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Organic control of phytonematodes with *Pleurotus* species

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Abstract

The nematicidal ability of *Pleurotus ostreatus*, *P. florida* and *P. citronopileatus* against phytonematodes of vegetables was determined in this study. Crude extract from *Pleurotus* spp., were tested against *Pratylenchus*, *Xiphinema*, *Tylenchorhynchus*, *Tylenchus*, *Helicotylenchus*, *Ditylenchus*, *Psilenchus*, *Aphelenchus*, *Hoplolaimus*, *Longidorus*, *Aphelenchoides* and *Paralongidorus* spp. Extracts from the fruiting bodies, mushroom waste and broth culture of *Pleurotus* species at four different concentrations and three time intervals were tested against the nematodes. Nematodes killed by the application of crude extracts of *Pleurotus* species never recovered to life after placing in simple water. *P. citronopileatus* was found more effective than other species and it killed 100% nematodes after 24 hrs followed by fruiting body extracts of *P. florida* 99% and waste of *P. ostreatus* 77%.

Keywords: Nematicidal ability, *Pleurotus* spp., phytonematodes, crude extract, fruiting bodies, mushroom waste

Different vegetables are grown in Khyber Pakhtunkhwa province of Pakistan due to the diverse climatic conditions. Frost free pockets in Malakand division, give an opportunity to the farmers to grow even off season vegetables like tomatoes, chilies, potatoes and many others. Potato, onion, tomato, chili, gourds, bitter gourd, brinjal and okra have been cultivated in Malakand division in both seasons. However, despite environmental suitability, yield/unit area recorded quite low in Pakistan i.e. 10.12 tones/hectares than other vegetables growing countries (Anonymous, 2010). Reasons are viral, bacterial, fungal and nematode pathogens which cause economic losses in vegetables. Plant parasitic nematodes are one of the major pathogens responsible for such losses. Worldwide economic losses caused by plant parasitic nematodes have been reported in a broad range of agricultural crops (Sasser & Freckman, 1987). Root-knot nematodes, reniform nematodes, cyst nematodes and several ectoparasitic nematodes are known to attack vegetable crops in different parts of the world. Moreover, these nematodes not only infect the vegetables directly but also as vectors and transmit other important pathogens. Pea early-browning virus and tobacco rattle virus (tobra viruses) are generally ingested by nematodes through feeding upon infected root cells (Baujard, 1995).

Different nematicides have been used for the management of nematodes. However, chemical treatments are not reliable, practicable and economically justified. Nematode management with chemicals also becomes impractical as nematode penetrates deep into the soil layers when conditions are unfavorable (Prot, 1980). Consequently, many chemicals have been withdrawn from the market due to environmental concerns and pesticide residues in food products (Thomason, 1987).

Strategies other than chemicals have been developed to control nematodes. Increasing soil temperature through soil solarization is a good method in the management of nematodes but it needs sunshine for long periods of time (FAO,

1991). Studies have confirmed that heat reaches only up to 10 cm and not beyond this level (Gaur & Perry, 1991). Further studies have confirmed that nematodes change their metabolic activities during stress and survive by anhydrobiotic means (Womersly, 1987). The control of nematodes by flooding has been advocated in certain areas (Gowen & Queneherve, 1990). However, all soil pore spaces are filled and oxygen supply for soil microflora and fauna becomes limiting. Further, this method is not applicable on large scale due to shortage of water.

A series of nematicidal substances have been obtained from several edible mushrooms (Anke & Sterner, 1997). For example several species in the genus *Pleurotus* have been reported to capture and consume nematodes (Thorn & Barron, 1984; Barron & Thorn, 1987). The ability of *Pleurotus ostreatus*, *P. pulmonarius* and *P. eryngii* to prey on the pinewood nematode has previously been reported. A nematicidal toxin trans-2-decenedioic acid was isolated from *P. ostreatus* (Kwok *et al*., 1992). It was found that 11 species of gilled fungi, including *P. ostreatus* had the ability to kill nematodes.

Keeping in view the importance of nematodes in vegetables and the environment, this research was initiated with the objective to test *in vitro* bio-control efficacy of *Pleurotus* species against nematodes.

Materials and methods

Collection and processing of soil samples: Soil samples were collected in polyethylene bags from the root zone of vegetables along with roots from vegetable growing areas in Malakand division and stored at 4 °C. The soil sample was put in a large bucket containing water and the suspension was vigorously stirred for 15 minutes. The heavy soil particles settled down in two minutes while nematodes remained suspended in water. The supernatant was filtered through a coarse sieve (100 mesh). The nematode suspension was poured over a piece of tissue paper attached to a perforated plastic sheet placed in a funnel fitted with a rubber tube and clamped at the lower end. Nematodes were collected after 48 hrs in a beaker.

Nematodes were killed by pouring hot water (90 °C) into the beaker containing nematodes. Specimens were immediately fixed and preserved in a solution of 3% formaldehyde and 2% glycerin. They were later placed in a TAF (Triethanolamine 2%, 7 ml formaldehyde 38%, 91 ml distilled water) for 24 hours. Fixed nematodes were processed in glycerin by a slow dehydration method. The specimens were placed in a cavity block containing 2 ml of 1.25% glycerin and traces of picric acid. The cavity block was placed in an incubator at 55 ^{o}C for 5-6 days. Nematodes were placed in the centre of the slide in a small drop of glycerin. Paraffin wax was placed as four small lumps around the drop (19 mm diameter) cover slip on the wax lumps. The slide was gently heated on a hot plate (62-65 ^{o}C) to melt the wax (Siddiqi, 1986). Fixed nematodes were processed for light microscopy (LM) using methods of Hooper (1970) and Golden (1978). Photomicrograph of the eelworms, cyst, females, males and juveniles were taken with the help of an automatic digital camera attached to a compound microscope equipped with an interference contrast system in the Pathology Laboratory of Animal Nutritional Sciences Department, The University of Agriculture, Peshawar, Pakistan.

Collection of *Pleurotus* species and extraction of natural products: Fruiting bodies and wastes of *Pleurotus* (*P. ostreatus*, *P. florida* and *P. citronopileatus)* were collected from mushroom house of The University of Agriculture, Peshawar. The fruiting bodies were carefully removed from the wheat straw substrate, weighed and ground and later weighed separately and subjected to extraction with ethyl acetate. The solvent was removed by evaporation at 45 ^{o}C for 24 hrs in a rotator evaporator.

For the production of secondary metabolites, the *Pleurotus* species were inoculated on broth malt extract media. For this purpose 100 ml medium

was poured separately in 500 ml flask and a block from pure culture of *Pleurotus* spp., dropped in each flask with a sterilized spatula. The flasks were covered with aluminum foil to avoid contamination and kept in incubator at 25 °C for 14 days. The growth of *Pleurotus* spp., on media was observed daily to check any kind of contamination up to two weeks and the observations were recorded.

Bioactive compounds were extracted from *Pleurotus* spp., with organic solvent (ethyl acetate). Separation of aqueous solution and organic solvent were carried out with the help of separately funnel, till separations achieved by observing two distinct layers. Aqueous solution was poured into the flask and the organic solvents were placed in separate sample flask and switched on rotary evaporator. During this process, the temperature was kept at 45 °C. After required time, the ethyl acetate was collected in receiving flask and crude in sample flask. These were left for few days till ethyl acetate in the crude was evaporated. This crude was then used for the preparation of stock solutions and further concentrations according to requirement.

Four different concentrations (C_1 =100 ppm, C_2 = 200 ppm, C_3 = 300 ppm and C_4 = 400 ppm) of the organic solvent were applied to the Petri plates which contained 100 plant parasitic nematodes for three different intervals of times (T_1 = data recorded after one hour, T_2= data after four hour and T_3 = data after twenty four hour). Data on the mortality of nematodes were recorded under the stereo microscope in laboratory.

Statistical analysis: Logistic regression model was used for the statistical analysis. The variables used in the experimental analysis were: Time: It is an independent variable and was used at three different levels of time intervals like: T1, data recorded after one hour, T2 data recorded after 4 hours and T3 the data recorded after 24 hrs, Concentration: It is an independent variable and is the extracts from *Pleurotus* species. These extracts were applied against nematodes at 4 different levels of concentrations. The concentration levels were C1 at 100 ppm, C2 at 200 ppm, C3 at 300ppm and C4 at 400 ppm, different parts of the same mushroom: Extracts of the three parts (fruiting body, mushroom waste extracts, broth culture extracts) of same the mushroom were used in the experiment against nematodes, four random replications with one control was applied and for nematodes mortality: This is a dependent variable as it dependent upon concentrations, time intervals and extracts of different parts of mushroom.

Results and Discussion

Nematodes belonging to twelve genera i.e., *Pratylenchus*, *Xiphinema*, *Tylenchorhynchus*, *Tylenchus*, *Helicotylenchus*, *Ditylenchus*, *Psilenchus*, *Aphelenchus*, *Hoplolaimus*, *Longidorus*, *Aphelenchoides* and *Paralongidorus* were identified in the surveyed area. *Helicotylenchus* spp., was the most frequently recorded from each locality of vegetables growing areas of Dargi Malakand, followed by *Xiphinema*, *Tylenchorhynchus*, *Tylenchus*, *Hoplolaimus*, *Ditylenchus*, *Psilenchus*, *Aphelenchus*, *Pratylenchus*, *Longidorus*, *Aphelenchoides* and *Paralongidorus*. Results showed that *Pleurotus* species contained nematicidial components lethal to nematodes. *P. citronopileatus* extracts application was found more effective than other *Pleurotus* spp. Culture filtrates of *P. citronopileatus* at 400 ppm killed all nematodes. Results showed that the extracts application of broth culture of *P. citronopileatus* at all dose applications had lethal effect on nematodes. This was followed by fruity bodies and mushroom waste application (Table 1 a).

The fruiting body extracts of *P. florida* found lethal to nematodes and killed 99% followed by broth cultural and mushroom waste extracts application (Table 2 a). Mushroom waste extract of *P. ostreatus* was also effective and killed 77% nematodes at 400 ppm (Table 3 a).

Similarly, statistical analysis of *P. citronopileatus* (Table 1 b), *P. florida* (Table 2 b) and *P. ostreatus* (Table 3 b) shows that extracts of

different parts of *Pleurotus* spp., different concentrations and time had a significant effect on percent nematodes survival rate. Result showed that *P. citronopileatus* extracts contained nematicidial chemical in larger amount.

The organic approach used in this study has been applied for the first time in Pakistan for nematodes control and is applicable for the management of many other soilborne plant diseases and nematodes. Results confirmed that *Pleurotus* species contain nematicidal components. Previously, Stadler *et al*., (1994) reported that mycelia in Petri plates physically captured and killed nematodes. *Pleurotus citronopileatus* can be used for the organic control of nematodes.

Table 1 a. Effect of extracts derived from *Pleurotus citronopileatus* on nematodes mortality.

Concentration	*Pleurotus citronopileatus* extracts			Control
	Fruiting body	Mushroom waste	Broth	
100 ppm				
1 Hour (T_1)	18	13	16	100 ± 5
4 Hour (T_2)	31	30	32	100 ± 5
24 Hour (T_3)	34	40	38	100 ± 5
Average	28	28	29	
200 ppm				
1 Hour (T_1)	34	28	34	100 ± 5
4 Hour (T_2)	60	59	66	100 ± 5
24 Hour (T_3)	65	67	83	100 ± 5
Average	53	51	61	
300 ppm				
1 Hour (T_1)	40	30	63	100 ± 5
4 Hour (T_2)	63	63	77	100 ± 5
24 Hour (T_3)	73	83	90	100 ± 5
Average	59	59	77	
400 ppm				
1 Hour (T_1)	60	57	76	100 ± 5
4 Hour (T_2)	90	85	99	100 ± 5
24 Hour (T_3)	93	96	100	100 ± 5
Average	81	79	92	

Table 1 b. Statistical analysis of *P. citronopileatus* extracts application against nematodes mortality.

Factors	B	Standard error	Wald test	Degree of freedom	Significance	Odds of survival
P. citronopileatus			182.952	2	0.00	
Fruiting body	0.572	0.049	137.929	1	0.00	1.772
Mycelia	0.578	0.049	140.670	1	0.00	1.782
Time			1.287E3	2	0.00	
T_1	1.750	0.051	1.197E3	1	0.00	5.753
T_2	0.517	0.049	110.470	1	0.00	1.677
Concentrations			2.266E3	4	0.00	
C_0	23.255	644.049	0.001	1	0.97	1.257E10
C_1	2.943	0.064	2.118E3	1	0.00	18.969
C_2	1.654	0.060	749.067	1	0.00	5.228
C_3	1.137	0.061	348.594	1	0.00	3.118
Replications			1.369	3	0.71	
R_1	0.059	0.056	1.117	1	0.29	0.943
R_2	0.045	0.056	0.650	1	0.42	0.956
R_3	0.017	0.056	0.093	1	0.76	0.983
Constant	2.973	0.077	1.501E3	1	0.00	0.051

Table 2 a. Effect of extracts derived from *Pleurotus florida* on nematodes mortality.

Concentration	*Pleurotus florida* extracts			Control
	Fruiting body	Mushroom waste	Broth	
100 ppm				
1 Hour (T_1)	14	26	28	100 ± 5
4 Hour (T_2)	28	31	27	100 ± 5
24 Hour (T_3)	65	34	39	100 ± 5
Average	**36**	**30**	**31**	
200 ppm				
1 Hour (T_1)	22	50	41	100 ± 5
4 Hour (T_2)	55	57	55	100 ± 5
24 Hour (T_3)	67	57	70	100 ± 5
Average	**48**	**55**	**56**	
300 ppm				
1 Hour (T_1)	58	58	51	100 ± 5
4 Hour (T_2)	77	69	73	100 ± 5
24 Hour (T_3)	95	69	86	100 ± 5
Average	53	65	70	
400 ppm				
1 Hour (T_1)	88	66	76	100 ± 5
4 Hour (T_2)	91	79	93	100 ± 5
24 Hour (T_3)	99	79	97	100 ± 5
Average	93	74	88	

Table 2 b. Statistical analyses of *Pleurotus florida* extracts application against nematodes mortality.

Factors	B	Standard error	Wald test	Degree of freedom	Significance	Odds of Survival
Pleurotus florida			180.052	2	0.00	
Fruiting body	-0.242	0.047	26.169	1	0.00	0.785
Mycelia	0.382	0.046	67.876	1	0.00	1.465
Time			664.877	2	0.00	
T_1	1.232	0.048	657.333	1	0.00	3.428
T_2	0.555	0.048	134.306	1	0.00	1.742
Concentrations			1.998E3	4	0.00	
C_0	23.093	654.32	.001	1	0.97	1.069E10
C_1	2.553	0.06	1.786E3	1	0.00	12.846
C_2	1.565	0.059	713.953	1	0.00	4.783
C_3	0.911	0.060	231.532	1	0.00	2.486
Replications			21.312	3	0.00	
R_1	0.189	0.054	12.147	1	0.00	1.208
R_2	0.078	0.054	2.070	1	0.15	1.081
R_3	0.222	0.054	16.817	1	0.00	1.248
Constant	-2.545	0.073	1.226E3	1	0.00	0.078

Table 3 a. Effect of extracts derived from *Pleurotus ostreatus* on nematodes mortality.

Concentration	*Pleurotus ostreatus* extracts			Control
	Fruiting body	Mushroom waste	Broth	
100 ppm				
1 Hour (T_1)	0.25	14	0	100 ± 5
4 Hour (T_2)	6	28	4	100 ± 5
24 Hour (T_3)	11	35	8	100 ± 5
Average	**6**	**26**	**4**	
200 ppm				
1 Hour (T_1)	2	25	1	100 ± 5
4 Hour (T_2)	14	37	18	100 ± 5
24 Hour (T_3)	29	52	21	100 ± 5
Average	**15**	**38**	**13**	
300 ppm				
1 Hour (T_1)	6	43	3	100 ± 5
4 Hour (T_2)	33	58	26	100 ± 5
24 Hour (T_3)	43	60	30	100 ± 5
Average	**27**	**54**	**20**	
400 ppm				
1 Hour (T_1)	9	63	9	100 ± 5
4 Hour (T_2)	46	74	31	100 ± 5
24 Hour (T_3)	48	77	31	100 ± 5
Average	**34**	**71**	**24**	

Table 3 b. Statistical analyses of *Pleurotus ostreatus* extracts application against nematodes mortality.

Factors	B	Standard error	Wald test	Degree of freedom	Significance	Odds of Survival
P. ostreatus			1.580E3	2	0.00	
Fruiting body	-0.414	0.058	50.629	1	0.00	0.661
Mycelia	-1.980	0.055	1.279E3	1	0.00	0.138
Time			755.155	2	0.00	
T_1	1.557	0.058	730.915	1	0.00	4.742
T_2	0.334	0.049	46.788	1	0.00	1.397
Concentrations			927.558	4	0.00	
C_0	21.19	624.41	0.001	1	0.97	1.594E9
C_1	2.000	0.069	850.660	1	0.00	7.392
C_2	1.027	0.058	310.077	1	0.00	2.794
C_3	0.493	0.055	79.705	1	0.00	1.638
Replications			6.766	3	0.08	
R_1	-0.037	0.061	0.368	1	0.54	0.964
R_2	-0.118	0.060	3.842	1	0.05	0.888
R_3	0.03	0.061	0.238	1	0.62	1.030
Constant	0.642	.069	87.294	1	0.00	1.900

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