INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
13–1199/2014/16–3–639–643
http://www.fspublishers.org

Full Length Article

Potential of Different Parts of Neem (*Azadirachta indica*) Extracts in Controlling *Rhizoctonia solani* Infestation

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Abstract

Black scurf is one of the oldest and common diseases of potato stems and stolons below the soil surface caused by *Rhizoctonia solani*. The present study was carried out to investigate the antifungal potential of *Azadirachta indica* L. against this soil-borne fungal pathogen. Different concentrations (1, 2, 3, 4, 5%) of aqueous, methanol, *n*-hexane and chloroform extracts of leaves, stems and fruits of *A. indica* were prepared and were evaluated for their *in vitro* antifungal activity. Data showed that all the extracts showed variable antifungal activity. In general, leaf and fruit extracts exhibited the highest inhibitory effect against growth of the fungal pathogen followed by stem-bark. Among the various extracts, leaf methanol extract, fruit and stem-bark chloroform extract showed the best antifungal activity resulting in 0–83% and 0-79% reduction in fungal biomass over corresponding control treatments, respectively. The implications of using the neem extracts in controlling *R. solani* are discussed.

Keywords: *Rhizoctonia solani*; *A. indica*; Antifungal activity; Fungal biomass

Introduction

Diseases related to *Rhizoctonia* are seed-borne or soil borne. Analysis of infected tubers in plant residue and infested depict that the pathogen overwinters as sclerotia and mycelium. Mostly the spring plantation of infected seed tubers results in the infection of root primordial, stolon primordial and leaf primordial as the fungus travels from the seed surface to the developing sprout(Wharton *et al*., 2007). Studies have revealed that seed inoculum is the primary source of the disease (Ritchie *et al*., 2006; Tsai *et al*., 2012). *Rhizoctonia solani* is among those soil-inhabiting fungi that infect numerous plant species including potato, tomato and wheat (Keiser *et al*., 2012; Bartz *et al*., 2013; Handiseni *et al*., 2013).

The biggest threat to potato crop is from *R. solani* (Wilson *et al*., 2008). Quality of the crop gets deteriorated through the development of black scurf on tubers but also in some instances deformed tubers and cracks (Grosch *et al*., 2005). Due to the stem and stolon infection at the beginning of the season, yield can be reduced and in some cases it can cause complete death of the stem. These losses can go up to 30% due to the disease (Lehtonen, 2009).

Phytopathogenic fungi are generally controlled by synthetic fungicides (Rauf, 2000). However, the use of these agro-chemicals is increasingly restricted due to their harmful effects on human health and the environment (Ma and Michailides, 2005; Myresiotis *et al*., 2007; Pande *et al*., 2012). Limitations on the use of agrochemicals due to development of resistance in the pathogens have led to imposition of strict regulations for their use, which have created a niche for new control strategies (Wedge *et al*., 2000; Dellavalle *et al*., 2011).

Phytochemicals have gained attention of microbiologists to use them against microbes as these products have been found to be degradable and safe for human health (Ghosh *et al*., 2008; Kumar *et al*., 2008; Behbahani *et al*., 2013). An important member of Meliaceae family *Azadirachta indica* (Neem) is well known for its unique characters of fast growth and resistance to the drought conditions (Dalziel, 1955). These unique characters make all parts of the tree a rich source of traditional drugs (Biswas *et al*., 2002; Ngure *et al*., 2009). Recently, neem has been of ecological importance and is effective as pesticide against about 200 insect species. Moreover, it has antiseptic, antifungal, antibacterial, antipyretic, anti-malaria, anti-diabetic and anti-fertility properties among several other uses (Nok *et al*., 1993, Natarajan *et al*., 2003; Fredros *et al*., 2007; Mbaya *et al*., 2010).

Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally variable with more than 140 compounds isolated from different parts of the tree (Subapriya and Nagini, 2005). Quercetin and ß-sitosterol, were the first polyphenolic flavonoids purified from neem fresh leaves and were known to have antibacterial and antifungal properties (Mahmoud *et al*., 2011). These authors purified the active fractions of neem in organic extracts using HPLC and found that their

To cite this paper: Bokhari, N.A., I. Siddiqui, K. Perveen, I. Siddique, M.S. Alwahibi, D.W.A. Soliman and M. Al-Subeie, 2014. Potential of different parts of neem (*Azadirachta indica*) extracts in controlling *Rhizoctonia solani* infestation. *Int. J. Agric. Biol*., 16: 639–643

content have major compounds such as 6-deacetylnimbin, azadiradione, nimbin, salannin and epoxy-azadiradione with appreciable bioactivity when bio-assayed on many pathogenic fungi. We hypothesize that various parts of nee plant and various solvents show differential inhibitory activities against *R. solani*. The objective of present study was to investigate the antifungal activity of aqueous as well as organic solvent extracts of leaf, stem bark and fruits of neem against *R. solani.*

Materials and Methods

Procurement of Fungal Species

Infected potato tuber with *R. solani* was cut into small pieces and surface sterilized with 1% sodium hypochlorite solution for 2 min, for the isolation of the pathogen. Sterilized water was used for thorough washing, and pieces were placed on potato dextrose agar (PDA) medium in 9-cm diameter petri plates and incubated at 25±2°C for one week. Pure culture was stored in the refrigerator at 4°C. The pathogen was identified by the First Culture Bank of Pakistan, Institute of Agricultural Sciences, University of the Punjab, Pakistan

Plant Material

The parts of neem (*Azadirachta indica* L.) tree, like leaves, stem-bark and fruits were collected. Plant parts were thoroughly washed in running tap water and surface sterilized with 1% sodium hypochlorite solution, thoroughly washed with sterilized water, dried at 40°C in an electric oven and grinded to form powder. This powder was then stored in polyethylene bags and used when needed to make extracts with water, methanol, n-hexane and chloroform.

Antifungal Activity

Powdered plant material of 20 g was soaked in 100 mL of sterilized distilled water, n-hexane, chloroform and methanol for 24 h and filtered to prepare 20% extract. The desired quantity of 2 mL was achieved by drying then in an oven at 45ºC. Final volume up to 100 mL was made by adding autoclaved sterilized distilled water. Potato dextrose broth was autoclaved and cooled to 40°C. Eighty mL of medium were poured in 250 mL flasks. Appropriate quantities of stock solutions and distilled water were added to make 1, 2, 3, 4, 5% (v/v) concentrations and to make a final volume of 100 mL in each flask. Control treatments were without plant extracts and only received distilled water or methanol, n-hexane or chloroform (2 mL in 98 mL water). Five mL diameter actively growing mycelial disks of *R. solani* were transferred to the flasks aseptically. Flasks were incubated at 25°C±2 for one week in an incubator. Each treatment was replicated three times. Fungal biomass in each flask was filtered, dried to constant weight and weighed after one week.

Statistical Analysis

Two factor Completely Randomized Design (CRD) was applied. All the data were analyzed by analysis of variance (ANOVA). The comparisons among means were worked out using Tukey HSD test at 5% level of significance.

Results

In the present study, solvent extracts with a concentration ranging from 1–5% of different parts of meem i.e., fruit, leaves and stem were studied against *R. solani*. Variability in the fungicidal effect of different parts and their extracts was observed in neem. Leaf and fruit extracts exhibited better antifungal activity than stem extract. Among the different concentration levels, 5% concentration for all the solvent extracts displayed the pronouncing decrease in the growth of the organism.

Among various concentrations of the plant extracts, the chloroform extract of stem produced the widest zone of inhibition as than the other solvents. The chloroform extract at 5% concentration produced the highest effect ($P<0.01$) with the mean reduction of 10. At the same concentration aqueous extract of stem produced the smallest diameter with mean reduction of 50±5.77. The inhibitory effect at 5% concentration was maximum with chloroform extract, 68% followed by *n*-hexane 63%, methanol 50% and least with aqueous extract of stem, 35%, respectively (Table 1).

The result showed that the fruit extract in different solvents had antifungal activity on the tested *R. solani*. The trend of the solvent fruit extract was similar to that of stem. The highest ($P<0.01$) antifungal activity was shown by the chloroform extract with a mean reduction of 32.67, whereas the least inhibitory effect was depicted by the aqueous extract of fruit with a mean reduction of 190. Concerning the efficacy of extract in percentages, more pronounced effect was with chloroform 79%, followed by with 68% *n*-hexane. However, water and methanol extracts showed a similar effect as both the extracts reduced the colony diameter up to 56 and 58%, respectively (Table 2).

Out of four solvent extracts of the leaves methanol extract reduced the diameter of the fungus to a greater extent. However, this was contrary to the results of stem and fruit extracts where chloroform extract was more effective in decreasing the colony diameter of the fungus (Table 3). Methanol extract of leaves produced the inhibition zone with a mean reduction of 10But aqueous extract repeated the trend in the leaves. The minimum inhibitory effect (60%) of the aqueous extract was on an average 190. The zone of inhibition for the fungus was more($P<0.01$) with methanol (83%), followed by *n*-hexane (70%) and chloroform (71%), respectively. The results clearly depicted that n-hexane and chloroform produced a similar effect.

Overall a non-significant affect $P>0.05$ was observed for 1% concentration with all the extracts and parts of the plant.

Table 1: Biomass of *Rhizoctonia solani* affected by the aqueous and organic extracts of *A. indica* stem

Extract concentration	Treatment				Mean
(%)	Water	Methanol	n-hexane	Chloroform	
0	120.00 ± 5.77a	88.67 ± 0.88cd	89.00 ± 0.58cd	48.67 ± 0.88hij	86.58 ± 7.74A
1	116.67 ± 3.33ab	80.00 ± 5.77de	69.00 ± 0.58ef	43.00 ± 3.06ijk	77.17 ± 8.13B
2	103.33 ± 3.33bc	65.00 ± 2.89efg	55.33 ± 3.18f-i	34.67 ± 2.19jk	64.58 ± 7.61C
3	89.00 ± 0.58cd	62.67 ± 3.18fgh	47.33 ± 0.88hij	28.67 ± 0.33kl	56.92 ± 6.70D
4	70.00 ± 5.77ef	49.00 ± 0.58hij	40.00 ± 0.58ijk	17.00 ± 0.58lm	44.00 ± 5.87E
5	50.00 ± 5.77g-j	39.00 ± 0.58jk	28.67 ± 0.33kl	10.00 ± 0.00m	31.92 ± 4.61F
Mean	91.50 ± 6.28A	64.06 ± 4.23B	54.89 ± 4.80C	30.33 ± 3.34D	

Table 2: Biomass of *Rhizoctonia solani* affected by the aqueous and organic extracts of *A. indica* fruit

Extract concentration	Treatment				Mean
(%)	Water	Methanol	n-hexane	Chloroform	
0	683.3 ± 1.67a	59.00 ± 0.58b	58.00 ± 0.58b	88.7 ± 0.09gh	48.55 ± 7.03A
1	590.0 ± 0.58b	59.00 ± 0.58b	48.00 ± 0.58c	87.3 ± 0.09gh	43.68 ± 6.24B
2	490.0 ± 0.58c	46.00 ± 3.51c	38.00 ± 0.58d	78.7 ± 0.03gh	35.22 ± 4.97C
3	390.0 ± 0.58d	36.33 ± 3.67d	28.00 ± 0.58e	76.3 ± 0.09gh	27.74 ± 3.79D
4	280.0 ± 1.53e	21.67 ± 1.67ef	18.33 ± 0.67f	66.3 ± 0.09gh	18.66 ± 2.39E
5	190 ± 0.58f	10.00 ± 0.00g	17.33 ± 0.33f	26.0 ± 0.65h	12.23 ± 1.97F
Mean	43.72 ± 4.15A	38.67 ± 4.49B	34.61 ± 3.64C	7.06 ± 0.52D	

Table 3: Biomass of *Rhizoctonia solani* affected by the aqueous and organic extracts of *A. indica* leaf

Extract concentration	Treatment				Mean
(%)	Water	Methanol	n-hexane	Chloroform	
0	600.00 ± 57.74a	96.67 ± 3.33f	96.67 ± 3.33f	88.33 ± 1.20f	220.42 ± 67.23A
1	613.33 ± 18.56a	96.67 ± 3.33f	88.67 ± 0.88f	88.67 ± 0.88f	221.83 ± 68.28A
2	490.00 ± 05.77b	88.33 ± 0.67f	69.00 ± 0.58f	78.00 ± 0.58f	181.33 ± 53.79B
3	390.00 ± 05.77c	89.33 ± 0.67f	69.00 ± 0.58f	67.33 ± 0.88f	153.92 ± 41.20C
4	290.00 ± 05.77d	68.00 ± 1.53f	48.00 ± 1.53f	49.00 ± 5.77f	113.75 ± 30.83D
5	190.00 ± 05.77e	66.67 ± 3.33f	48.00 ± 0.58f	32.67 ± 3.18f	84.33 ± 18.82E
Mean	428.89 ± 38.67A	84.28 ± 3.13B	69.89 ± 4.49B	67.33 ± 5.09B	

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05). Small letters represent comparison among interaction means and capital letters are used for overall mean

Discussion

The present study revealed that leaves of *A. indica* possessed more antifungal properties than fruit and stem-bark (Table 3). Abubakar *et al.* (2010) showed that leaves of *Tamarindus indica* extract possess better antifungal properties when compared to fruit and stem extracts. Leaf is one of the major accumulators of bioactive compounds, and has therefore preferred for therapeutic purposes (Maji *et al.*, 2010). Mahmoud et *al.* (2011) reported that leaf extracts of neem had a characteristic effect on human pathogenic fungi. Shivpuri *et al.* (1997) noticed that the extracts in ethanol of *A. indica* had fungitoxic properties against five pathogenic fungi when tested under laboratory conditions at concentrations ranging between 500 and 1000 µg mL^{-1}. Verma *et al.* (1998) found that a purified fraction (ethyl acetate: chloroform, 3:1) of extracts in methanol from neem seed coat showed strong antifungal activity against *A. niger* and *Curvularia lunata* with MIC of 250 ppm. They found also that the extracts in petroleum ether from the neem leaves were highly active at a lower MIC (100 ppm) against the same pathogens. Kishore *et al.* (2001) reported that ethanolic leaf extracts of *A. indica* inhibited the conidial germination of *Phaeoisariopsis personata* by > 90% to control late leaf spot of groundnut.

More than 135 compounds have been isolated from different parts of neem. The compounds have been divided into two major classes: isoprenoids [like diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type of compounds and C-secomeliacins such as nimbin, salanin and azadirachtin] and non-isoprenoids, which are proteins/amino acids and carbohydrates [polysaccharides], sulphurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds, etc. Most of the compounds have the fungistatic ability (Asif, 2012). Overall, methanolic and chloroform extracts showed considerable antifungal activity over *n*-hexane in this study. This suggested that some compounds are more active in methanolic extracts than they were present in the chloroform extracts in the present study. Similarly, Rizwana *et al.* (2012) reported that alcoholic extracts possessed more antibacterial activity than chloroform. This showed that

some compounds are more effective and extraction is more efficient in polar solvents rather than nonpolar (Zaker and Mosallanejad, 2010; Mahmoud et *al.*, 2011; Bassey *et al*., 2013).

Aqueous extracts of the plants also show considerable antifungal effects by reducing the fungal biomass (Abubacker *et al*., 2008). Ashraf and Javaid (2007) screened aqueous extracts of three plants of Meliaceae and found that both *A. indica* and *M. azedarach* significantly reduced biomass of *M. phaseolina* by 34–85% and 43–78%, respectively. In our experiments, the highest concentration of the extracts showed maximum inhibition of *R. solani.* It has been also reported by earlier workers that as the concentration increases antifungal activity also increases (Aslam *et al.,* 2010). Recently, Javaid *et al.* (2012) evaluated methanolic extracts of three parts of *Sorghum halepense* against *M. phaseolina* and found that the highest concentration possessed the highest antifungal property.

In conclusion, neem leaf, stem and fruit displayed reduction in the growth of the fungus. Simultaneously all the concentration of the extracts affected the fungal colony; however, the highest concentrations of the extracts had more pronounced effect in decreasing the growth of *R. solani*. The compounds in the extract of the plant could be commercially exploited for the reduction of the fungi causing fatal diseases of plants and animals.

Acknowledgement

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding the work through the research group project No. RGP-VPP-066.

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(Received 01 October 2013; Accepted 12 November 2013)