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PHYTOTOXIC AND HERBICIDAL ACTIVITIES OF *Aspergillus* AND *Penicillium* SPECIES ISOLATED FROM RHIZOSPHERE AND SOIL

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ABSTRACT

Two fungi, one isolated from the rhizosphere of Mentha piperita and the other isolated from the top soil (20 cm) of the agricultural farm of the University of Agriculture Peshawar, Pakistan. The rhizospheric fungus was identified as Aspergillus and Penicillium as soil born. The dried crude extracts of Aspergillus and Penicillium species were screened for phytotoxic bioassay and herbicidal activities (against Lemna minor and the weed Silybum marianum L., respectively) in order to evaluate the agrochemical importance of the extracts. Different dose concentrations (10, 100 and 1000 μgmL^{-1}) of the ethyl acetate and n-Hexane fraction of both the species were tested against Lemna minor and seeds of Silybum marianum L. A dose concentration of 1000 μgmL^{-1} of ethyl acetate extract and n-Hexane fraction of Aspergillus species inhibited the growth of Lemna minor by 65 and 85%, respectively; while the same dose concentration of ethyl acetate extract and n-Hexane fraction of Penicillium species inhibited the growth of Lemna minor by 56.66 and 30%, respectively. In addition, the same dose concentrations of the crude extract from both the species completely inhibited the germination of the seeds of Silybum marianum L. The results showed that extract of both fungi has potential agrochemical constituents which might inhibit the growth of Silybum marianum L. and Lemna minor. The results showed potent herbicidal activity against the test weed and therefore is suggested for further isolation and purification work of the active component(s).

Key words: *Aspergillus*, herbicidal activities, *Lemna minor, Penicillium*, phytotoxic activities and *Silybum marianum.*

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INTRODUCTION

The kingdom fungi have more than 100,000 described species and about 1.5 million undescribed species (Kirk *et al.,* 2008) that can be found in wide range of environments including soil, rhizosphere, within and upon the organs of plants and in water (Santamarı´a *et al.,* 2005; Smithand Read*,* 2008; Clay*,* 1988; Kagami *et al.,* 2007). Fungi have a number of applications in pharmacology (Kayser *et al.,* 2003; Zjawiony*,* 2004) and fungi are the trusted source of structurally complex and pharmacologically important secondary metabolites (Wang *et al.,* 2011).

The genus *Penicillium* consists of more than 200 known species which mostly live in soil (Petit *et al.,* 2009) while the genus *Aspergillus* comprise of more than 180 species which live in diverse range of environments (Fei *et al.,* 2013). Most of the members of the genus *Aspergillus* are reported to be the producers of bioactive secondary metabolites e.g. polyketides, terpenoids and xanthones etc. which have been shown to have antibacterial, antifungal and cytotoxic properties (Sun *et al.,* 2012, He *et al.,* 2012). Since the discovery of penicillin in 1928 many species of *Penicillium* have been shown to be the producers of diverse range of secondary metabolites with antibacterial (Rancic *et al.,* 2006, Lucas *et al.,* 2007) and antifungal properties (Nicoletti *et al.,* 2007).

Both *Penicillium* and *Aspergillus* species are being screened in modern times and they continue to be the sources of new bioactive secondary metabolites (Ghisalberti *et al.,* 1990; Takahashi *et al.,* 2008; Rao *et al.,* 2000). We have screened soil and rhizosphere fungi for bioactive compounds with specific interest in secondary metabolites produced biotechnologically for agrochemical industry. The most active extracts were obtained from *Penicillium* and *Aspergillus* sp. which were subjected for phytotoxic and herbicidal activities. Phytotoxicity was tested against *Lemna minor* while herbicidal activities were performed against the seeds of *Silybum marianum*.

MATERIALS AND METHODS

Isolation of fungal strains

Samples were taken from rhizosphere of *Mentha piperita*and from the top soil of agricultural farm of the University of Agriculture Peshawar. Potato Dextrose Agar (PDA) medium Acumedia® prepared according to the manufacturer's instructions was used for isolation of

desired fungal strains. After preparation of the medium and sterility checking for 24 hours, samples collected from soil and rhizosphere were inoculated on PDA medium and incubated for ten (10) days at 28°C (Onyegeme *et al.,* 2009). After ten days growth of different fungal strains was observed which were sub-cultured on fresh medium and further purification was performed.

Fungal strains identification

Purified fungal strains were studied under light microscope (100-200 magnification) for identification. Different characteristics like structure of colony and hyphae, arrangement of spores and pigmentation of the colonies were studied by growing the isolates on both PDA and CzapicDox Agar (CDA). We acknowledge Department of Plant Pathology, University of Agriculture Peshawar for proper identification and confirmation of the fungal strains.

Culturing fungal strains for secondary metabolites production

Czapic yeast broth (CYB) medium, containing 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 1% Peptone, 1% Glucose and 0.001% $FeSO_4 \cdot 7H_2O$; pH 7.3±0.2, supplemented with additional 2% glucose and 3% starch was prepared and autoclaved at 128°C. After autoclaving fungal strains were inoculated in separate flasks containing the media and the flasks were then transferred to shaking incubator for 14 days at 28 °C and 150 rpm.

Extraction of fungal metabolites from broth medium

After completion of incubation period 250 µl of 40 % HCl was added each flask which helps separating the media components. Using electrical blender fugal mycelia were grinded and equal volume of ethyl acetate was added to each flask. After mixing for 40 minute the mycelia were filtered using cheese cloth. The mixture was transferred to separating funnel to recover the organic layer containing metabolites. In order to remove the impurities the mixture was washed with 2M brine solution. Anhydrous sodium sulphate (Na_2SO_4) was used to dehydrate the organic layer which was again filtered. The recovered organic layer containing the crude metabolites were subjected to rotary evaporator at 45 °C in order to concentrate them.

Phytotoxic activities

Ethyl acetate extract and *n*-Hexane fractions of *Aspergillus* and *Penicillium* sp. were subjected for phytotoxic activity against Lemna plant. E- Medium was prepared by adding different inorganic salts in deionized water (1L) and final pH of the medium was adjusted between 5.5- 6.5 by adding sodium hydroxide. For preparation of E. Medium, 0.86 gm of KH_2PO_4, 1.515 gm of KNO_3, 1.18 gm of Ca $(NO_3)_2.4H_2O$, 0.492 gm of $MgSO_4.7H_2O$, 0.00286 gm of H_3BO_3, 0.00362 gm of $MnCl_2.4H_2O$, 0.00540 gm of $FeCl_3.6H_2O$, 0.00022g of $ZnSO_4.5H_2O$, 0.00022 gm $CuSO_4.5H_2O$ and 0.00012 gm of

$Na_2MO_4.2H_2O$ in 1000 ml (qsp) de-ionized water, then autoclaved at 121°C temperature for 15 minutes on 15 psi pressure. Sterile di-methyl sulfoxide (DMSO) was used to prepare a stock solution of 30 mg/ 1.5mL. From this stock solution different dose concentrations (i.e. 10, 100 and 1000 µg/mL) were then prepared.

20 mL medium was added to each sterilized flask and after proper examination ten healthy plants of Lemna were transferred to flasks containing the medium. It was ensured that each plant have a rosette of three healthy fronds. The flasks were marked as 10, 100 and 1000 and the desired dose concentration was added to each flask. One flask was taken as control in which only the medium was added. The flasks were tightly plugged with sterile cotton and flasks were kept for seven days in growth chamber by setting the temperature at 30 °C, light intensity was adjusted to 9000 lux, and humidity was maintained at 60 by placing a beaker of autoclaved water in the growth chamber and a photoperiod of 12 hours. Methanol was used as negative control and parquet as positive control. Three replicas of the experiment were performed and on eighth day the fronds were visually examined and percent growth inhibition was calculated using the following formula (Bashir *et al.,* 2013, McLaughlin, 1988).

$$\text{Growth Inhibition (\%)} = 100 - \left(\frac{\text{No. of fronds in sample}}{\text{No. of fronds in control}}\right) \times 100$$

Herbicidal activities

The experiment was conducted in Petri-dish using Completely Randomized Design (CRD) having three replications. A tissue paper was placed in Petri dish having ten seed of *Silybum marianum* L. keeping appropriate distance. Each petri dish was replicated three times and a control treatment was kept with each concentration. Different dose concentrations (10, 100, 1000 µ g/mL) of the fungal extracts were applied to petri dish through dropper and then water was provided as per requirement each day. The seed germination data was recorded on daily basis. The whole experimental setup continued for 14 days. The data was recorded on the parameters i.e. percentage of seed germination, length of shoot (cm), root length (cm), shoot weight (g) and root weight (g) (Hassan *et al.,* 2004; Saeed *et al.,* 2013).

RESULTS AND DISCUSSION

Phytotoxic activities

Crude metabolites of *Penicillium* and *Aspergillus* sp. were tested against *Lemna* for phytotoxicity. The results are shown in Fig. 1 and Fig. 2. Different concentrations of Ethyl acetate and n-hexane fractions of both fungal strains showed different degrees of phytotoxicity on the leaves of *Lemna* plant and different types of necrotic lesions were

observed. The lesions varied from reddish brown to dark brown in color. To determine the phytotoxic ability of the crude metabolites, three dose concentrations (10, 100 and 1000 µg/mL) were used.

A dose of 10, 100, and 1000 µ g/mL of ethyl acetate fraction of *Aspergillus* sp. showed 0, 30 and 65% mortality respectively while the same dose concentration of n-hexane fraction of this fungus showed 6.66, 78.33 and 85% mortality against the *Lemna* plant.

Ethyl acetate extract of *Penicillium* sp. showed more phytotoxicity against *Lemna* plant in comparison to its n-hexane fraction. Dose concentration of 10,100 and 1000 µ g/mL of crude ethyl acetate fraction of *Penicillium* sp. showed mortality percentage of 0, 25 and 56.66 % respectively whereas the same dose concentrations of its n-hexane fraction showed 0, 11.66 and 30 % mortality respectively against *Lemna.*

Herbicidal Activities

Results showed that both fungal species with their respective concentrations ((10, 100, 1000 µ g/mL)) significantly controlled growth of *Silybum marianum* L. The seed germination data in percentage showed that maximum germination was recorded for control plots i.e. (90 %) while no germination were found in other extract applied treatments. Furthermore, the mean data showed that highest shoot length (7.8 cm) and root length (6.3 cm) were observed for control plots while no data was recorded from other extract applied treatments with their respective concentration. In addition, the mean data showed that maximum shoot weight (0.39 g) and root weight (0.32) were noticed for control treatments while no data was recorded from other extract applied treatments with their respective concentration. Herbicidal activities of both fungal strains and their respective concentrations are given in Table-1.

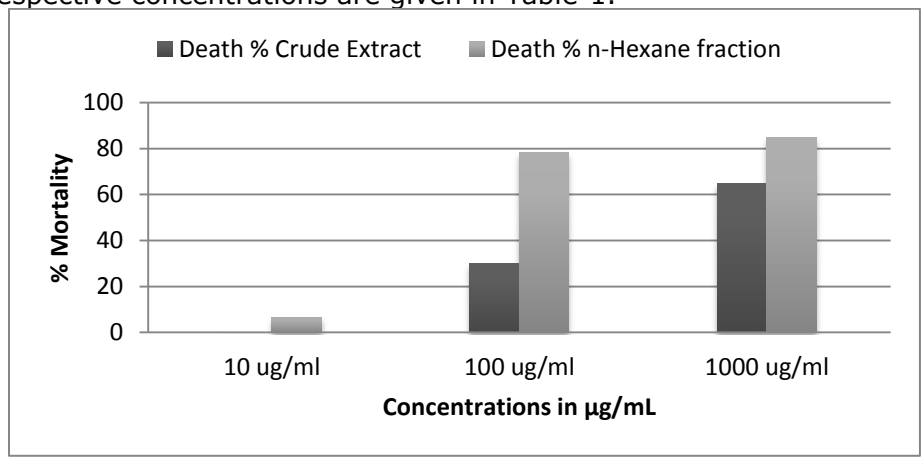

Figure 1. Phytotoxic activities of crude extract of *Aspergillus* sp.

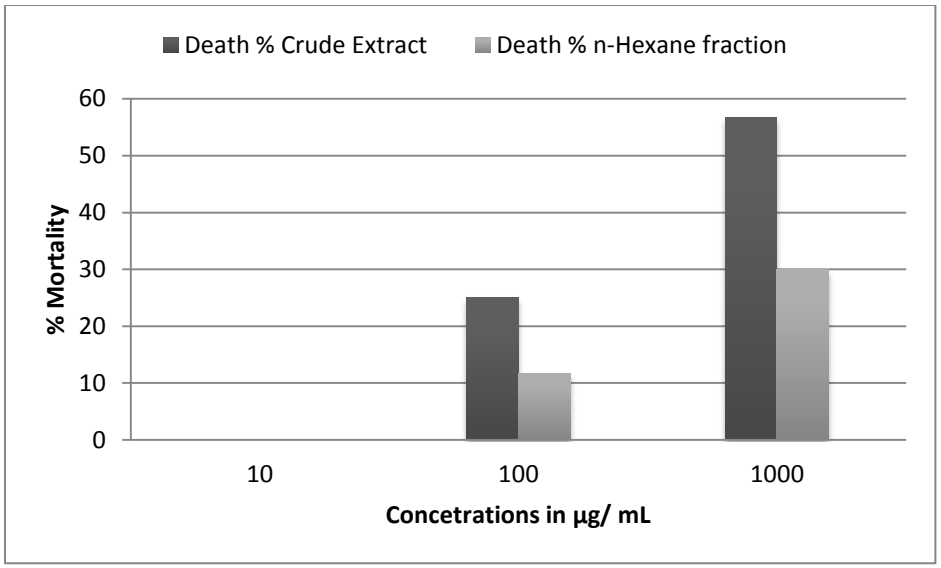

Figure 2. Phytotoxic activities of *Penicillium* sp.

Table-1. Herbicidal activities of n-Hexane and ethyl acetate fractions of the crude extracts of *Aspergillus* and *Penicillium* species

Treatments	Germin-ation (%)	Shoot length (cm)	Root length (cm)	Shoot weight (gm)	Root weight (gm)
*Penicillium*sp.(10, 100, 1000 µ g/mL of both ethyl acetate and n-hexane fractions)	X	X	X	X	X
*Aspergillus*sp. (10, 100, 1000 µ g/mL of both ethyl acetate and n-hexane fractions)	X	X	X	X	X
Control	90	7.8	6.3	0.39	0.32

X = no or zero growth

Plants have been used traditionally since ancient times for treatment of various chronic diseases because they have the potential of allelopathy and that's why plants have attracted the consideration of

the biochemical researchers to use plants for primary health care purposes. Despite of their medicinal importance plants produce some toxic metabolites, exhibited phytotoxicity against other plants, for example Flavonoids extracted from a variety of plants have been reported as allelochemicals (Samanta *et al.,* 2011). Therefore, alternative to plants fungi have been recently utilized as allelochemicals for application in agriculture (Martha *et al.,* 2008). Fungi are easy to isolate and culture comparative to plants, attention of researchers have focused on the isolation of bioactive compounds from fungi. Antonio *et al.* (2000) tested the crude extract of *Ascochyta caulina* a mycoherbicidal fungus against different plants of monocot and dicot families and some weeds. The dry crude extract was further subjected for the isolation of pure compound and therefore isolated a novel compound with phytotoxic activities. The metabolite was characterized as trans-4-aminoprolineusing both 1D and 2D NMR techniques. Hyun-Ju Kim *et al* 1999 studied *Helminthosporium* specie isolated from the grasses which upon exploitation in the laboratory condition produced six compounds (Ophiobolin A, 6-Epiophiobolin A, 3-Anhydroophiobolin A, 3-Anhydro-6-epiophiobolin A, Ophiobolin B, and Ophiobolin I) of biological interest. All the compounds inhibited the growth of bacteria and fungi with different extent; however, Ophiobolin A has also shown stronger phytotoxic properties by inhibiting the growth of eight plants in leaf wounding assays.

While in our study the n-Hexane and ethyl acetate fractions of both the fungal species inhibited growth of *Lemna* plant and stopped the germination of the seeds of *Silybum marrianum*L. has confirmed the presence of an active component in the extract. N-Hexane fraction of *Aspergillus* sp. inhibited the growth of *Lemna*by 85% and Ethyl acetate fraction inhibited the growth by 65%, which clearly shows that the fatty components of the dry crude extract of *Aspergillus* sp. are more toxic as compared to the ethyl acetate because the n-Hexane fraction contain maximum of the fatty and/or oily compounds. Whereas the n-Hexane fraction of *Penicillium* sp. inhibited the growth of *Lemna* by 30% and ethyl acetate fraction inhibited the growth by 56.6 % which reflect the same story as of *Aspergillus*. However, still we have to investigate which specific metabolites either in the n-Hexane or in ethyl acetate fraction is responsible for the phytotoxic activities because both the fractions has already inhibited the growth of bacteria and fungi as well. Now the question is that either this inhibition is due to some biologically potent metabolites or due to some toxins. If there are separate metabolites which are involved in the inhibition of *Lemna*as well as in the in inhibition of bacteria and fungi then it is desirable to isolate them. Otherwise if these are toxins

then we need to stop the production of theses toxin by gene knockout studies.

Based on bioassay guided as mentioned earlier, Nakajima *et al.* (1991) isolated a novel compound from the crude extract of a newly reported fungus *Paecilomyces variotii* SANK 21086 with herbicidal properties against young annual and perennial plants of both monocot and dicot families with limited effect on corn plants. After the physiochemical analysis this compound was identified as Cornexistin belonging to nonaride group. Similar studies were conducted by Akbar and Javaid (2012). They tested the fungal filtrate of four *Drechslera* sp. on two species of weeds i.e. *Chenopodium album* L. (dicot) and *Avenafatua L.* of the wheat crop using petriplate method. According to their results the fungus filtrate effected the germination of *C. album* by 28 -50%, shoot length was reduced by 54 to 91% and shoot biomass decreased by 58 to 81%. Root length and biomass also showed significant decrease as it was reduced by 66 to 88%. For *A. fatua* germination decreased by 28-54% because of different filtrate concentrations. Roots of *A. fatua* proved more susceptible as compared to shoots. The fungal filtrate reduced the shoot length of *A. fatua* by 27 to 67% , shoot biomass was reduced by 27 to 57%, root length and root biomass was reduced by 55 to 86% and 47 to 77% respectively as different dose concentrations were applied (Akber *et al.,* 2012). These findings are quite similar to our study because the extracts of both the fungal species have shown promising inhibition of the germination of seed, while the extract from *Aspergillus* and *Penicillium* have shown more toxicity against *Silybum marrianum* L. by inhibiting its growth of roots and shoots initiation.

CONCLUSION

From the present study it is concluded that the dry crude extracts of both the fungal species have the potential of inhibiting the growth of *Lemna,* can retard the germination of the seeds of *Silybum marianum* L. and have shown inhibitory effects on pathogenic bacteria and fungi. These results have developed the interest of our group to further investigate the appropriate metabolite responsible for the actual inhibition and hence we are working to investigate these interesting metabolites. However, it is also suggested for other researchers that the dry crude extract of both *Aspergillus* and *Penicillium* sp. may contain effective biologically interesting metabolites which upon purification can lead to the discovery of novel potent metabolite.

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