Bangladesh J. Bot. **47**(1): 141-146, 2018 (March)

BIOCHAR AND COMPOST ANTIFUNGAL ACTIVITY AGAINST *BOTRYTIS CINEREA* PERS. EX. FE.

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Keywords: Antifungal, Corn cob, Extracts, Grey mould, MIC

Abstract

The antifungal potential of biochar produced from corn cob and vegetable waste compost was evaluated against *Botrytis cinerea* Pers. Ex Fr. *In vitro* antifungal activities of biochar and compost were tested by preparing different concentrations (1, 2 and 3%) separately in methanol and distilled water. In this assay, methanolic biochar extract significantly suppressed the test fungus growth up to 50 - 79% as compared to control treatment. This effective methanolic extract of biochar was fractionated by using *n*-hexane, chloroform, ethyl acetate and *n*-butanol. These fractions were further serially diluted to check their minimum inhibitory concentration (MIC) along with synthetic fungicide metalaxyl + mancozeb as reference fungicide. After 24, 48 and 72 hrs incubation period, ethyl acetate fraction and fungicide were found to be the most effective showing MIC of 0.0097 mg/ml. The current study suggested that biochar has significant antifungal potential which can be exploited in future.

Introduction

Botrytis cinerea Pers. Ex. Fr. is a necrotrophic phytopathogenic "grey mold fungus" that is ubiquitous in existence (Williamson *et al.* 2007). A wide range of vegetables and plants in nursery, ornamental plants, agricultural products which are stored in warehouses, orchard and field crops are the potential host of *B. cinerea* (Elad *et al.* 2007). This fungus can persist on the necrotic tissue and produce spores and sclerotia, (a long lasting survival structures in host plant) (Holz *et al.* 2007).

To manage *B. cinerea* infection, there is a requirement of combination of traditional and chemical control approaches. Crop rotation is the most effective cultural control method to overcome Botrytis disease. Plantation of non-host crop plants provides a safe way to reduce grey mould outbreak (Gan *et al.* 2006, Elad *et al.* 2007). The practice of resilient cultivar is also an alternative useful and cost-effective solution (Mabrouk and Belhadi 2012).

Organic amendments, including composts and liquid preparations had been applied for centuries for the purpose of improved soil quality and crop nutrition. Compost is a solid particulate organic material that is the product of composting. During the process of composting, biodegradable materials are decomposed biologically under controlled aerobic conditions. As a result heat is produced biologically which permits the development of thermophilic temperatures, in order to attain compost that is sanitized and stable (Litterick and Wood 2009). Addition of compost in agricultural lands provides a good natural way to combat against plant pathogenic fungi (Mehta *et al.* 2013).

In Southern Europe and the Middle East at least 5500 years ago biochar was used as a fuel. Pre Columbian inhabitants of Amazon basin practiced charcoal as the soil additive together with pottery remains, fertilizers and bones (Elad *et al.* 2012). Biochar is produced through the process

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of pyrolysis i.e. (when biomass, such as manure, leaves or wood is heated in a sealed vessel in anaerobic conditions at < 700°C temperature) the thermal decomposition of organic material (Lehmann and Joseph 2012). In 2016, Zheng *et al.* reported that biochar reduced the fungal (zygomycetes, basidiomycetes) population in rice fields.

The literature survey showed that very little work available on the antifungal activity of corn comb biochar and compost particularly against *B. cinerea*. So, the present study was designed to evaluate antifungal properties of biochar and compost against the grey mold disease.

Materials and Methods

Biochar of corn cob was collected from Agro climatology, Department of Agronomy, University of Agriculture, Faisalabad, Pakistan. Food waste compost was collected from Compost Pvt. Ltd., Ring road Lahore. Biochar was ground thoroughly into fine powder through an electric blender. The *Botrytis* strain was isolated from diseased root hair of onion. The diseased root hair was isolated from the onion bulb and surface sterilized with 1% sodium hypochlorite solution. This sterilized onion bulb was placed on solidified 2% MEA (Malt Extract Agar) medium. It turned into a colony within 10 days of incubation at 20°C.

Powdered materials of biochar and compost were weighed (20 g) on digital weighing balance and placed in 250 ml beaker and then soaked in 100 ml of methanol and 100 ml of distilled water separately for 7 days at room temperature. Materials were filtered through an autoclaved muslin cloth. Filtrates were evaporated at room temperature to reduce the volume. Each filtrate (0.385 g) was separated in small beakers and then diluted by adding appropriate quantity of sterilized distilled water to make the final volume 1.9 ml. From each stock solution 3, 2 and 1% concentrations were prepared by adding 0.9, 0.6 and 0.3 ml of stock solution to 59.1, 59.4 and 59.7 ml of 2% ME medium, respectively. Malt extract (2%) was prepared by adding 1.2 g of malt extract in 60 ml of distilled water and sterilized through autoclaved at 121ºC, 15 lb $inch^2$ for 30 min. Similar procedure was followed for compost extracts as well. Control treatment was without any addition of the biochar and compost extracts. Chloromycetin 50 mg/l of the medium was added to avoid bacterial growth. Mycelial discs of *B. cinerea* were prepared using a sterilized 5 mm diameter cork borer from the actively growing margins of 7 days old test fungal culture and transferred to each flask. Each treatment was replicated three times. The flasks were incubated at room temperature for 7 days. The fungal biomass in each flask was filtered and dried to constant weight in an electric oven and weighed. Percentage growth inhibition of the fungal colonies was calculated by applying the following formula:

$$\text{Growth inhibition (\%)} = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100.$$

Twenty grams powdered biochar was soaked in 100 ml methanol for seven days. After seven days the extract was evaporated at room temperature with a yield 0.541 g of gummy mass. This gummy methanolic mass was partitioned between *n*-hexane, chloroform, ethyl acetate and *n*-butanol (Jabeen *et al.* 2014). This partitioning by separating funnel gave gummy mass of *n*-hexane (0.01 g), chloroform (0.02 g), ethyl acetate (0.03 g), *n*-butanol (0.04 g).

The antifungal activity of four organic solvent fractions *viz. n*-hexane, chloroform, ethyl acetate and *n*-butanol of biochar was investigated against *B. cinerea* by MIC bioassays. The MIC values of these fractions along with a reference synthetic fungicide metalaxyl + mancozeb were tested in test tubes by the serial dilution assay (Jabeen *et al.* 2013). The highest concentration of 5 mg/ml was prepared by dissolving 5 mg of each extract in 0.5 ml of DMSO and 0.5 ml of distilled water. This concentration was further serially double diluted and minimum applied concentration

was 0.0097 mg/ml prepared. ME medium was added to seven days old fungal culture to reach the final concentration of 1×10^5. 100 µl of this suspension was added to each test tubes of 1.6 cm diameter and 15 cm length. Test tubes containing only DMSO and distilled water were used as control. These test tubes were left at room temperature. MIC was calculated after 24, 48 and 72 hrs. The fungal mycelial growth was visually determined by using hand lens and light (Shahbaz *et al.* 2015).

Data were analyzed statistically by applying ANOVA followed by DMRT to separate the treatment means (Steel and Torrie 1980).

Results and Discussion

Botrytis cinerea caused severe damage to a broad range of crop plants. This fungus damaged the host tissues by producing grey mold on flowers and fruits of grapes, tomatoes and strawberries (Baarlen *et al.* 2007). This is very essential to find out active ingredients from natural sources to control *B. cinerea* because use of chemical fungicide is not an environmental safe strategy (Singh *et al.* 2003, Murchie *et al.* 2005).

The aim of the current study was to discover antifungal potential of biochar and compost aqueous and methanol extracts against *B. cinerea*. From the results it is seen that corn cob biochar extracts showed significant antifungal activity against grey mold. All the applied concentrations (1, 2 and 3%) of methanolic extracts of biochar suppressed the test fungal growth. Although 3% conc. showed high antifungal activity by causing 79% reduction in the biomass of *B. cinerea.* Aqueous extract of biochar was also found to be effectual in retarding the growth of test fungus. All the concentrations of this extract significantly reduced the colony growth of *B. cinerea* by 36 - 64% (Fig. 1).These results correspond to the earlier results reported by (Elad *et al.* 2010) as biochar produced systemic resistance to the gray mold fungal pathogens of leaves, broad mite pest and powdery mildew of pepper and tomato.

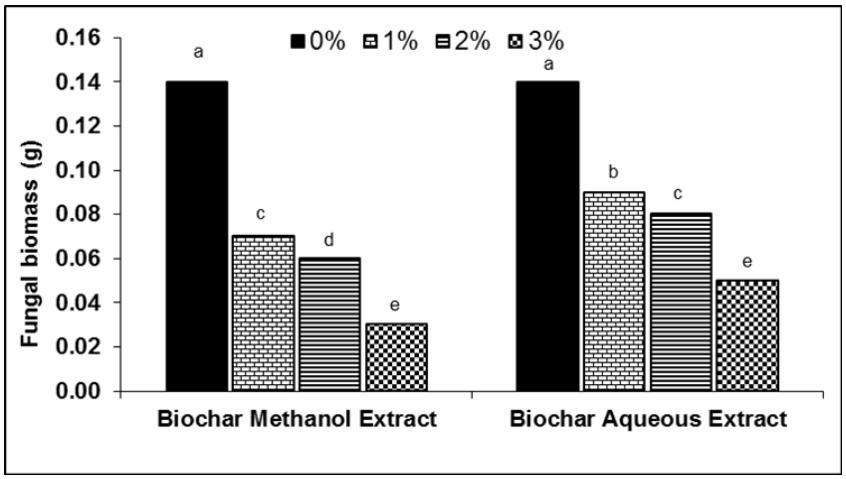

Fig. 1. Effects of methanolic and aqueous extracts of biochar on *in vitro* growth of *B. cinerea*. Values with different letters show significant differences as determined by DMRT.

All the applied concentrations (1 - 3%) of both aqueous and methanol compost extracts showed efficient antifungal activity against the test fungus *B. cinerea.* However, 57% reduction in test fungal biomass was showed by 3% concentration of methanol extract of compost (Fig. 2). It is

likely that different compounds were dissolved in variable quantity of the extracts of solvents. Previous researches on antifungal properties of compost supported this present study. Pane *et al.* (2012) reported the biological control ability of compost tea, both *in vivo* and *in vitro*, against tomato pathogens. Kone *et al.* (2010) also studied five types of non-aerated compost tea for their capability to prevent the development of *Alternaria solani* and *Phytophthora infestans in vitro.*

Table. 1. MIC values of different organic fractions of methanolic extract of biochar and synthetic fungicide metalaxyl + mancozeb against *B. cinerea* after 24, 48 and 72 hrs incubation periods.

Fractions	mg/ml									
	5	2.5	1.25	0.625	0.3125	0.156	0.078	0.039	0.019	0.0097
24 hrs after incubation										
Control (H_2O)	-	-	-	-	-	-	-	-	-	-
Control (DMSO)	-	+	-	-	-	-	-	-	-	-
n-hexane	-	-	-	-	-	-	-	-	-	-
Chloroform	-	-	-	-	-	-	-	-	-	-
Ethyl acetate	-	-	-	-	-	-	-	-	-	-
n-butanol	-	-	-	-	-	-	-	-	-	-
Fungicide	-	-	-	-	-	-	-	-	-	-
48 hrs after incubation										
Control (H_2O)	+	+	+	+	+	+	+	+	+	+
Control (DMSO)	-	+	+	+	+	+	+	+	+	+
n-hexane	-	+	+	+	+	+	+	+	+	+
Chloroform	-	-	-	-	-	-	+	+	+	+
Ethyl acetate	-	-	-	-	-	-	-	-	-	-
n-butanol	-	-	-	-	-	-	-	-	-	-
Fungicide	-	-	-	-	-	-	-	-	-	-
72 hrs after incubation										
Control (H_2O)	+	+	+	+	+	+	+	+	+	+
Control (DMSO)	+	+	+	+	+	+	+	+	+	+
n-hexane	+	+	+	+	+	+	+	+	+	+
Chloroform	+	+	+	+	+	+	+	+	+	+
Ethyl acetate	-	-	-	-	-	-	-	-	-	-
n-butanol	+	+	+	+	+	+	+	+	+	+
Fungicide	-	-	-	-	-	-	-	-	-	-

Mycelium present (+), mycelium absent (-).

The four organic fractions *viz. n*-hexane, chloroform, ethyl acetate and *n*-butanol were isolated from methanolic extract of biochar. The MIC (5 mg - 0.0097 mg/ml) of these fractions of biochar was studied against *B. cinerea* and compared with a known fungicide metalaxyl + Mancozeb (Table 1). Among the four fractions ethyl acetate along with fungicide fractions were effectively suppressed the mycelial growth of *B. cinerea* with 0.0097 mg/ml. However, other fractions were found to be least effective. It seems that compound with antifungal property might be soluble in ethyl acetate fraction and can be further isolated for the production of environment friendly fungicides. Earlier findings suggested that biochar has the ability to suppress phtopathogenic fungi. Elmer and Pignetello (2011) reported that hardwood dust biochar possess

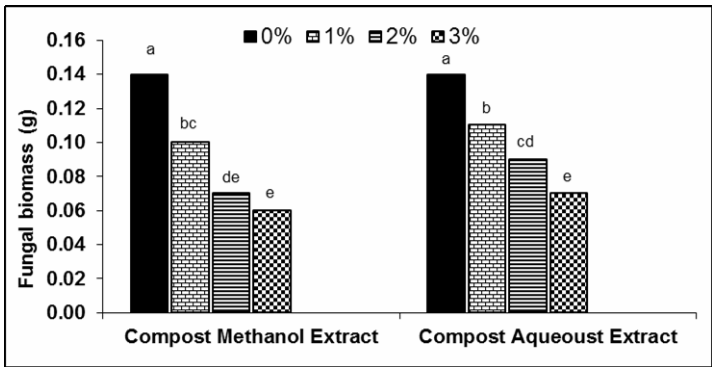

Fig. 2. Effects of methanolic and aqueous extracts of compost on *in vitro* growth of *B. cinerea*. Values with different letters show significant differences as determined by DMRT.

allelopathic potential against *Fusarium* crown and root rot disease of *Asparagus*. Graber *et al.* (2010) described that biochar has some chemical compounds like propylene glycol, benzoic acid, quinones and butyric acid which may suppress the growth of harmful pathogenic microbes and stimulate defense pathways in host plant against pathogen attack. So, from the present study it may be concluded that methanolic extract of corn cob biochar showed highly pronounced antifungal activity against *B. cinerea*, so further investigation to isolate effective antifungal compounds is needed.

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(Manuscript received on 2 February, 2017; revised on 7 March, 2017)