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*Full Length Article*

# *In vitro* Fungicidal Activity of Humic Acid Fraction from Oil Palm Compost

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## ABSTRACT

Fungicidal activity of humic acids isolated from empty fruit bunch of oil palm compost EFB-HA and commercial grade humic acid Co-HA were evaluated on the conidial germination and mycelial growth of *Choanephora cucurbitarum*. EFB-HA and Co-HA significantly ($P \leq 0.05$) inhibited the mycelial growth and conidial germination of *C. cucurbitarum in vitro* compared to control. The inhibition in mycelial growth increased with increase in humic acid concentrations tested, suggesting the presence of fungicidal activity. The maximum inhibition of conidial germination (71.96% after 24 h) was exerted by EFB-HA at the highest concentration (1000 mg $L^{-1}$). It was concluded that the efficiency of HA studied in controlling fungal growth was apparently related not only to their concentration and the pathogen examined, but also to HA origin and nature and in particular, HA structural and functional properties, especially COOH group content.

**Key Words:** Compost; Humic acid; *Choanephora cucurbitarum*; Mycelial growth; Conidia

## INTRODUCTION

Intensive agriculture tends to focus on production and often overlooks overexploitation of resources, reduced soil quality and poor soil protection. The repeated use of chemicals often leads to harmful environmental consequences including the development of resistance in pathogens and has negative effects on beneficial organisms (Lacomi-Vasilescu *et al*., 2004). For these reasons it is very clear that alternatives to these chemicals would be advantageous. Compost or compost extracts used as an organic fertilizer has beneficial effects on plant growth and considered as a valuable soil amendment (Gharib *et al*., 2008). Most of the people are aware that using composts is an effective way to increase healthy plant production, conserve natural resources, and reduce the cost of production and the use of chemical fertilizers. Despite these benefits, several composts have been limited in its application for agriculture use since, the product is weighty and bulky and is expensive to transport.

However, humic acids (HA), the major component of organic matter has been the subject of study in various areas of agriculture, such as soil chemistry, fertility, plant physiology as well as, environmental sciences because of the multiple roles played by these materials on plant growth (Tan, 1998). Humic acids introduced into soil by organic amendment can affect directly a number of physiological and biochemical processes occurring in plants and other soil-borne organisms, especially in the rhizosphere compartment (Vaughan & Malcolm, 1985; Chen & Aviad, 1990; Varanini & Pinton, 2001). Recent investigations have demonstrated that organic amendment is also able to control plant diseases caused by various soil-borne phytopathogenic microorganisms, such as fungi of various genera (Hoitink & Fahy, 1986; Zhang *et al*., 1996; Pascual *et al*., 2002). However, the relationships possibly existing between the chemical and functional properties of HA and the extent of the biological action that HA may exert have been scarcely explored and only in higher plants (Piccolo *et al*., 1992). Apparently, HA molecules or fractions rich in carboxylic groups and of small molecular size are the most biologically active in plants (Piccolo *et al*., 1992). There are few studies on pathogen suppression using humic acids however, they are suitable candidates for use as liquid biofertilizers and have been shown to have positive effect against plant pathogens (MckQuilken *et al*., 1994; Ayuso *et al*., 1996). At the best of our knowledge, no or very scanty information is available about the fungicidal activity of humic acid obtained from empty fruit bunch of oil palm compost. Therefore, the aim of this work was to study the "*in vitro*" effects of different concentrations of HA extracted from empty fruit bunch of oil palm (EFB) compost and commercially obtained humic acid on: (1) some chemical and functional properties of HA; (2) the conidial

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germination, mycelial growth inhibition and abnormalities of *Choanephora cucurbitarum* a commonly found air-borne phytopathogen in Malaysian climate.

## MATERIALS AND METHODS

Commercial grade soil humic acid (Co-HA) was obtained from Sigma-Aldrich (Ch-9471 Buchs, Switzerland). HA from EFB compost (EFB-HA) was extracted and purified according to the method described by Stevenson (1994). The fungal test pathogen was isolated from infected okra and chilli flowers according to Koch's postulates. The pure culture was maintained on Potato Dextrose Agar (PDA, Oxoid).

**Extraction and purification of humic acid.** Twenty gram of three-months-old EFB compost was shaken on a mechanical shaker for 24 h at room temperature (28 ± 2°C) with 180 mL of 0.1 M NaOH solution following separation of supernatant by centrifugation at 15,000 rpm for 20 min. The alkaline supernatant obtained was acidified with 6 N HCl to pH 1 and allowed to stand at room temperature for 24 h. All the steps were carried out under $N_2$ gas (fluxing the polyethylene bottles continuously with $N_2$ gas) so as to minimize chemical changes in the acidified extract. The precipitate (HA) was separated from supernatant (fulvic acids) by centrifugation. The humic acid obtained was purified by the HCl/HF method (5 mL conc. HCl + 5 mL 52% HF + 990 mL distilled water) (Schinitzer & Preston, 1986) until the ash content was less than 1%. Following this treatment, the humic acid pellet was washed repeatedly with distilled water until it gave a negative $Cl^-$ test with $AgNO_3$ and then freeze-dried.

**Elemental and spectroscopic characterization of humic acids.** Elemental composition (carbon, hydrogen, oxygen & sulphur) were determined by dry combustion method using a C/H/N Analyzer (VARIO-EL) (Table I). Acidic functional groups Carboxylic (-COOH) and phenolic (-OH) groups of HA were determined according to Inbar *et al*. (1990). Fourier Transform Infrared (FTIR) spectra were also recorded on KBr pellets (0.50 mg of HA + 200 mg of dry KBr) (Schinitzer & Preston, 1986) from 4000 to 600 $cm^{-1}$ on a Beckman Fourier transfer (FT) IR spectrometer.

**Fungicidal Activity of Humic Acids**

**Conidial germination.** The spores obtained from the 7-days-old *C. cucurbitarum* culture on PDA medium were collected, suspended in distilled sterile water and mixed with appropriate aliquots of stock aqueous suspensions/solutions of each HA to obtain a density of 5 × $10^4$ spores $mL^{-1}$ at a concentration of 0 (control), 250, 500, 750 and 1000 mg $L^{-1}$. About 50 µL of each medium were then placed on a microscopic cavity slide that was kept at 20°C in moist chambers consisting of Petri dishes lined with moist filter paper. After 24 h the germination percentage (40 conidia for each treatment) was measured using an Olympus CX 40 microscope. The conidia were considered germinated, when the germ-tube length was at least equal to the conidial diameter. The experiments were replicated six times.

**Mycelial growth.** The fungicidal activity of the purified humic acid from EFB compost and commercial grade soil humic acid was tested on *C. cucurbitarum* based on the poison food technique. A humic acid concentration (100%) was prepared in sterilized distilled water and filter sterilized by passing through 0.2 µm nylon membrane filter (low extractable membrane, Sartorius AG, Goettingen, Germany). The sterilized humic acid solution was amended with potato dextrose agar (PDA, Oxoid) at the rate of 0, 250, 500, 750 and 1000 mg $L^{-1}$. Each of the 15 mL of the amended PDA was poured into 90 mm petri plates and allowed to solidify. After 24 h the amended medium was inoculated centrally with a 5 mm diameter agar disc taken from the periphery of 5 - day-old culture of *C. cucurbitarum*. Plates with PDA medium alone served as control. All the plates were incubated at 28 ± 2°C for 5 days and percentage inhibition in radial growth (PIRG) was measured using the formula as described by Jinantana and Sariah (1998).

$$PIRG\ (\%) = \frac{R1-R2}{R1} \times 100\%$$

Where:

R1=Radius of *C. cucurbitarum* Colony in Control Plates.

R2=Radius of *C. cucurbitarum* Colony in Amended Culture Plates.

Attempts were also made to determine the survival of test pathogens from the inhibition zone in the amended plate by plating random mycelial disc on fresh PDA. The inhibited mycelial mat of test pathogens were also placed on a clean slide and mounted with a drop of lacto-phenol cotton blue (LCB) to observe morphological abnormalities using the light microscope (LM).

Experiment was conducted in Complete Randomized Design (CRD). Each treatment was replicated six times. Experiment was carried out at least three times unless otherwise stated. Data were subjected to analysis of variance (ANOVA) and tested for significance by Least Significant Difference (LSD) using PC-SAS.

## RESULTS AND DISCUSSION

The elemental composition and functional groups of the HA extracted from EFB compost and Co-HA were generally similar (Table I). The results of FTIR spectra for EFB-HA and Co-HA are presented in Table II. The interpretation of the FTIR spectra was based on Stevenson (1994). The results obtained were within the range of the functional group spectra of humic acid extracted elsewhere from different soils or compost sources (Schinitzer & Preston, 1986; Gracia *et al*., 1993; Stevenson, 1994; Ahmed, 2002).

**Table I. Elemental composition (on a moisture-and ash - free basis) of humic acids examined**

| Sample | C | H | N | O | S | COOH | Phenolic OH |
|---|---|---|---|---|---|---|---|
| EFB-HA | 56.28 | 5.69 | 4.42 | 32.95 | 1.24 | 445 | 250 |
| Co-HA | 55.62 | 5.52 | 4.45 | 34.42 | 1.15 | 433 | 241 |
| Literature | 53.8-58.7* | 3.2-6.2* | 0.8-4.3* | 32.8-38.3* | 0.1-1.5* | 380-450** | 220-300** |

* Steelink (1985)
* Schnitzer (1977)

**Table II. Absorption bands in the FTIR spectra of humic acids derived from EFB compost using KBr pellets, the commercial grade humic acid Co-HA was used as standard**

| EFB $cm^{-1}$ | Co-HA $cm^{-1}$ | *Frequency $cm^{-1}$ | Proposed Grouping |
|---|---|---|---|
| 3304 | 3372 | 3400-3300 | OH stretching |
| 2920 | 2935 | 2940-2900 | Aliphatic C-H streching |
| 1715 | 1724 | 1725-1720 | C=O stretching of COOH; C=O ketonic carbonyl |
| 1633 | 1650 | 1660-1630 | C=O stretching of amide groups, quinone; C=O and/or C=O conjugated ketones |
| 1608 | 1617 | 1620-1600 | Aromatic ring stretching |
| 1451 | 1459 | 1460-1450 | Aliphatic C- H |
| 1259 | 1270 | 1280-1200 | C-O stretching and OH deformation of COOH, C-O stretching of aryl esters |
| 1100 | 1115 | 1170-950 | C-O stretching of polysaccharides |

Stevenson (1994)

**Table III. Effects of HAs at different concentrations on the germination (percent) of *C. cucurbitarum* conidia at different sampling times with respect to control (100%)**

| Treatment | Sampling time (h) | | |
|---|---|---|---|
| | 12 | 24 | 48 |
| Control | 100.00±4.25 | 100.00±4.20 | 100.00±3.12 |
| EFB-HA 250 $mgL^{-1}$ | 64.12±5.02 | 62.02±3.01 | 62.19±2.32 |
| EFB-HA 500 $mgL^{-1}$ | 47.11±6.17 | 46.05±4.02 | 46.12±2.58 |
| EFB-HA 1000 $mgL^{-1}$ | 29.01±3.26 | 28.04±2.56 | 28.00±2.29 |
| Co-HA 250$mgL^{1}$ | 82.25±5.66 | 81.22±3.79 | 81.23±4.28 |
| Co-HA 500 $mgL^{1}$ | 68.25±2.37 | 68.22±4.28 | 67.12±2.33 |
| Co-HA 1000 $mgL^{1}$ | 57.25±6.21 | 56.12±3.24 | 56.08±3.32 |

Each value is the mean of six replicates (n=6) ± Standard Error according to Least Significant Difference (LSD) at $P \leq 0.05$

The humic acids from EFB displayed main absorption bands in regions of 3004 $cm^{-1}$ (hydrogen bonded OH), 2920 $cm^{-1}$ (Aliphatic C-H stretch), 1715 $cm^{-1}$ (C=O of $C_2H$, C=O of ketonic carbonyl), 1633 $cm^{-1}$ (C=O stretch of quinones, COO, hydrogen-bonded C=O), 1616-1608 $cm^{-1}$ (C=C of aromatic rings), 1451 $cm^{-1}$ (aliphatic C-H ). Absorption bands in the 1259 and 1100 $cm^{-1}$ region were assigned to symmetrical bonding of aliphatic CH2, OH or C-O stretch of various groups, suggesting that humic acids are engaged in pronounced hydrogen bonding. Thus, the humic acid extracts can be used successfully to evaluate for its biological activity on *C. cucurbitarum*. The precise properties of a given humic acid may also depend on the particular substrate chosen and the specific extraction conditions. Nevertheless, there is a remarkable uniformity in the average properties (Schinitzer, 1977; Rice & MacCarthy, 1991).

**Fungicidal activity of humic acid.** In general, EFB-HA and Co-HA depressed the mycelial growth and conidial germination of the *C. cucurbitarum* during the five day experimental time, with variations statistically significant with respect to the control, at concentrations of 250 to 1000 mg $L^{-1}$.

**Conidial germination.** Statistical analysis showed a significant or highly significant reduction in the germination percentage of *C. cucurbitarum,* conidia in the presence of HA at higher concentrations at any time, with respect to the control. The strongest inhibition of conidia germination (71.96% after 24 h) was exerted by EFB-HA at the higher concentration. Further, the inhibition effect of HA on the germination of conidia remained un-changed with increasing the treatment time (Table III).

**Mycelial growth.** The morphology of *C. cucrbitarum* colonies showed marked variation as observed under light microscope in the presence of both HAs amended with PDA. These results are in line with those of Moliszewska and Pisarek (1996) that a soil HA could inhibit the mycelial growth of *F. culmorum* in PDA medium and especially, in water-agar medium. In particular, EFB-HA significantly inhibited the radial mycelial growth of C. *cucurbitarum* (Fig. 1). The inhibition in mycelial growth increased with increase in humic acid concentrations tested, suggesting the presence of fungicidal effect. Light microscopic observations showed severe malformation of hyphal tips and thickening of hyphal walls of *C. cucurbitarum* grown on EFB-HA amended media at higher concentration, when compared to control (Fig. 2). Normal hyphal walls and germ tubes were smooth and with no swellings or vacuolations. No lysis of hyphae was observed in the water control.

The substantial inhibition shown on *C. curbitarum* during the entire experimental period by the HA fraction isolated from the composted empty fruit bunch of oil palm may be attributed to the presence of OH, quinonoid C=O and carboxylic groups in humic acids as revealed by FTIR spectra (Table II). A recent study (Pascual *et al*., 2002) showed that soil amendment with municipal waste compost or its HA-like fraction significantly decreased the population of *Pythium ultimum* causing damage to pea plants. The free radicals of humic acids consist mainly of semiquinone (Senesi *et al.*, 1991), which are known to

**Fig. 1. Effect of different concentration of EFB-HA and Co-HA on Inhibition in mycelial growth (percent) of *C. cucurbitarum* after five days of incubation at 28 ± 2°C, each value is the mean of six replicates (n=6). Vertical bars indicate standard error**

EFB-HA=Humic acid obtained from empty fruit bunch of oil palm compost

Co-HA=Commercial grade humic acid

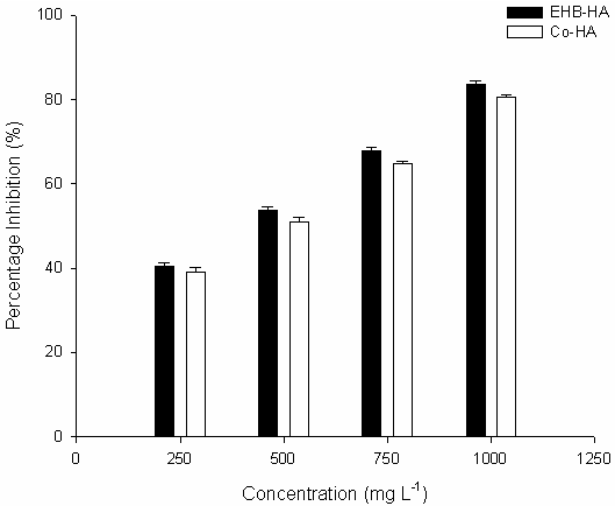
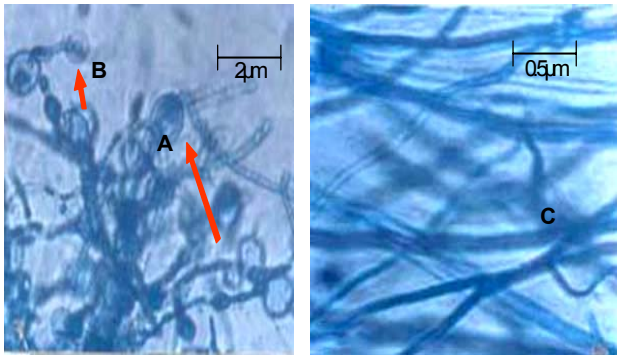



**Fig. 2. Effect of EFB-HA on mycelial morphology of *C. cucurbitarum* as observed under light microscope at 40X magnification**

A- Thickening in hyphal walls
B- Swollen hyphal tips
C- Healthy mycelium




produce super oxide ion ($O^-_2$) by auto oxidation under aerobic conditions. $O^-_2$ changes to $H_2O_2$ in the absence of superoxide dismutase and then to hydroxy radical (OH) (Saito *et al*., 1980). Catalase is a preventive antioxidant for the detoxification of $H_2O_2$ (Niki, 1990). The study carried out by Fujimura and Katayam (1994) showed that the inhibitory effect of humic acids on bacterial growth was attenuated by the addition of catalase. The denatured catalase did not prevent the growth inhibition by bacteria (B116), suggesting that a hydroxy radical was involved in the inhibitory action of humic acids. These findings are consistent with the biocidal action of free radicals from synthetic hydroquinone humic acid and catechol humic acid as reported by Hassett *et al*. (1987). It is considered that the humic acids contains 4, 9-dihydroxyperylene-3, 10-quinone as chromophore (Kumad, 1987). The inhibitory action of humic acids might also be attributed to the direct attachment of pathogen to humic acids. They react with the cell wall constituents, causing alterations in cytoarchitecture and cell functions, which lead to cell death (Pflug & Ziechman, 1982; Hassett *et al*., 1987). On the other hand involvement of other mechanisms such as deficiency of essential microelements trapped by humic acids can also be suggested.

In conclusion, the efficiency of HA studied in controlling fungal growth was apparently related not only to their concentration and the pathogen examined, but also to HA origin and nature and in particular, HA structural and functional properties, especially COOH group content.

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