African Journal of Biotechnology Vol. 9(47), pp. 8048-8057, 22 November, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Efficacy of Jatropha, Annona and Parthenium biowash on *Sclerotium rolfsii*, *Fusarium oxysporum* f. sp. *ciceri* and *Macrophomina phaseolina*, pathogens of chickpea and sorghum

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Accepted 28 October, 2010

The demand for products and technologies based on plants to control plant pathogens has increased in recent years due to concern about the use of hazardous pesticides. In the present investigation, washings of vermicompost (called biowash) prepared from foliage of Jatropha (*Jatropha curcas*), Annona (*Annona squamosa*) and Parthenium (*Parthenium hysterophorus*) were evaluated against fungal pathogens viz. *Fusarium oxysporum* f. sp. *ciceri* (FOC; causes wilt in chickpea), *Sclerotium rolfsii* (causes collar rot in chickpea) and *Macrophomina phaseolina* (causes charcoal rot in sorghum). Crude biowash of the botanicals were partitioned against ethyl acetate and the resultant organic and aqueous fractions were tested against the fungi. Similarly, crude biowash was also passed through C18 solid phase extraction cartridges and the resultant adsorbed and non-adsorbed fractions were tested against the fungi. Organic fractions of all the three biowash at 0.5% inhibited the growth of *S. rolfsii* between 78 and 87%, *M. phaseolina* between 62 and 65%, whereas only Parthenium was able to effectively inhibit FOC (91%), compared to control. Adsorbed fractions of all the three biowash at 0.5% inhibited the growth of *S. rolfsii* between 81 and 92%, *M. phaseolina* between 76 and 77% and FOC between 26 and 49%, compared to control. Both aqueous and non-adsorbed fractions of all the three biowash did not inhibit any of the fungi. Since Jatropha biowash showed consistently higher levels of inhibition (>80%) in both fractionation methods on *S. rolfsii*, this was selected for further purification of their secondary metabolites. When the organic fraction of Jatropha biowash was further fractionated by C18 open column chromatography with eluent 5, 10, 20, 40, 60, 80 and 100% MeOH fractions, only 80% methanol (MeOH) fraction was found to inhibit *S. rolfsii*. The active 80% MeOH fraction showed three clear bands when chromatographed on Silica Gel 60 F_{254} thin layer chromatography (TLC) plates with Rf values 0.95, 0.90 and 0.70. Hence, it was concluded that one of these three bands could be the active ingredients that inhibited *S. rolfsii* and can be further exploited as a bio-fungicide.

Key words: Botanicals, jatropha, annona, parthenium, biowash, *Sclerotium rolfsii*, *Fusarium oxysporum* f. sp. *ciceri*, *Macrophomina phaseolina*, secondary metabolites.

INTRODUCTION

Fusarium wilt and collar rot of chickpea, caused by *Fusarium oxysporum* f. sp. *ciceri* (FOC) and *Sclerotium rolfsii* Sacc., respectively and charcoal rot of sorghum caused by *Macrophomina phaseolina*, are the three major soil and seed borne diseases of chickpea and sorghum prevalent in most chickpea and sorghum growing countries (Gopalakrishnan et al., 2005; ICRISAT, 1978 and 1991). To some extent, it has been possible to manage these diseases by use of chemicals, however, with the increasing concern over environmental pollution by pesticides, major efforts are being made to develop environ-

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ment friendly methods of plant disease control. These include use of antagonist or competitor populations of a third organism and botanicals to suppress a pathogen population, making it less abundant and thus less damaging than it would be otherwise.

In recent years, much attention has been given to medicinal plants, for example, leaf extracts (20%) of Neem and chinaberry were reported to inhibit *Alternaria solani* and *F. oxysporum* f. sp. *lycopersici*, the pathogens of early blight and wilt diseases of tomato (Hassanein et al., 2008); rhizome extract (10%) of turmeric was found to effectively inhibit *Helminthosporium oryzae*, causal agent of rice brown spot (Harish et al., 2004) and plant extracts of *Syzibium aromaticum* (5 g/kg on rice grains) were shown to completely inhibit *Aspergillus flavus* growth and aflotoxin B1 (AFB1) production (Reddy et al., 2009). Plants produce a range of chemical compounds to protect themselves from various pathogens and insect pests that include alkaloids, terpenoids, flavonoids and acetogenins, for example, azadirachtin, isolated from the foliages of Neem. Secondary metabolites (compounds) from plants such as *Pyrethrum*, *Sabadilla* and *Carvone* were shown to have biological activity, protecting the plant from pathogens and at the same time non-toxic to mammals, fish and pollinators (Dubey et al., 2010). Compounds derived from such plants in general, possess no mammalian toxicity and hence should be exploited for controlling pathogens and insect pests of agriculturally important crops.

The biological degradation and conversion of agricultural or herbal wastes by earthworms and microorganisms, called vermicomposting, is becoming the favoured method of recycling wastes (Edwards, 1998). Application of vermicompost prepared from herbs not only benefit crop plants as it contains beneficial microorganisms that help the plants to mobilize and acquire nutrients but also promote plant growth and inhibit many plant pathogenic microorganisms (Perner et al., 2006; Postma et al., 2003). Biowash or vermiwash is the watery extract of vermicomposts, extracted in the presence of rich population of earthworms and microbes. Biowash contains several enzymes, plant growth promoting hormones, vitamins along with micro and macronutrients (Shield, 1982) and beneficial microbes that increases the resistance of crops against various diseases and enhance the growth and productivity of crops (Nath and Singh, 2009; Suthar et al., 2005; Yadav et al., 2005). The objective of this study is to determine the efficacy of Jatropha, Annona and Parthenium biowash on some of the important pathogens of chickpea and sorghum viz. *S. rolfsii*, FOC and *M. phaseolina*.

MATERIALS AND METHODS

Experimental setup for vermicomposting

Foliages of *J. curcas*, *Annona squamosa* and *Parthenium hysterophorus* were collected from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) farm and air dried at room temperature. Container for vermicomposting was constructed by cutting a 200 L plastic barrel (collected from scrap) into two halves. A metal grill of about 10cm was placed at the bottom of the barrel and air dried foliages of Jatropha, Annona and Parthenium were composted on top with earthworms (*Eisenia foetida*). The plastic barrel was fixed with an outlet at the base to collect biowash (vermiwash). The whole setup was left for two months until all the foliages of herbals were digested.

Extraction of biowash

At the end of two months, when the foliages of Jatropha, Annona and Parthenium were completely composted, biowash was extracted. To the completely prepared compost, water was sprinkled slowly and uniformly. The quantity of water sprinkled was determined by the volume of compost in the tank. The watery extract of vermicompost drained out of the container was collected at the bottom of the drum and called biowash. This crude biowash was immediately transported to the lab and further processed for evaluation.

Solvent partitioning

The crude biowash collected from the vermicompost contained many solid particles as well as microorganisms. In order to remove these, the biowash were centrifuged at 9000 g for 20 min and the supernatants collected. After adjusting the pH to 3.0 the supernatants were partitioned three times against ethyl acetate (EtOAc). The EtOAc phases were combined, dried over anhydrous sodium sulphate and the EtOAc removed by film evaporation on a rotary evaporator (BUCHI V-850, Switzerland) at 35°C. The residues were dissolved in 10ml of methanol (MeOH) and stored in a freezer at –20°C. Aqueous samples were evaporated on a rotary evaporator at 35°C and dissolved in minimal volumes of MeOH (10 ml). The concentrated final samples (both organic and aqueous) were passed through a Millipore filter (MILLEX® filter unit, 0.22 µm, Millipore, Ireland) in order to remove any microbial contamination in it and placed in the freezer until assayed.

Solid phase extraction (SPE)

The crude biowash, after removing the solid particles and microorganisms as explained earlier, were introduced into a C18 SPE cartridge (Sep-Pak® C18 cartridge, Waters, Ireland) after solvation and equilibration of the cartridge with 30 ml each of MeOH and 5% MeOH in ultra purified water. After washing the cartridge with 5% MeOH (5ml) and drying in a current of air, the adsorbed metabolites were eluted in MeOH (10 ml; called as adsorbed fraction). The biowash that passed through the C18 cartridge was evaporated on a rotary evaporator at 35°C and dissolved in minimal volumes of MeOH (10 ml; called as non-adsorbed fraction). The concentrated final samples (both adsorbed and non-adsorbed) were passed through a Millipore filter and placed in the freezer until assayed.

Open column chromatography

The concentrated biowash (by solvent partitioning; organic phase as explained earlier) were dissolved in 5% MeOH before being loaded onto a reversed-phase open column (25 cm × 2.8 cm, Wakosil 40 C18, Wako, Japan). The column was eluted with 200 ml each of 5, 10, 20, 40, 60, 80 and 100% MeOH. All the above fractions were evaporated on a rotary evaporator at 35°C and the residues were collected in MeOH (10 ml). The concentrated final

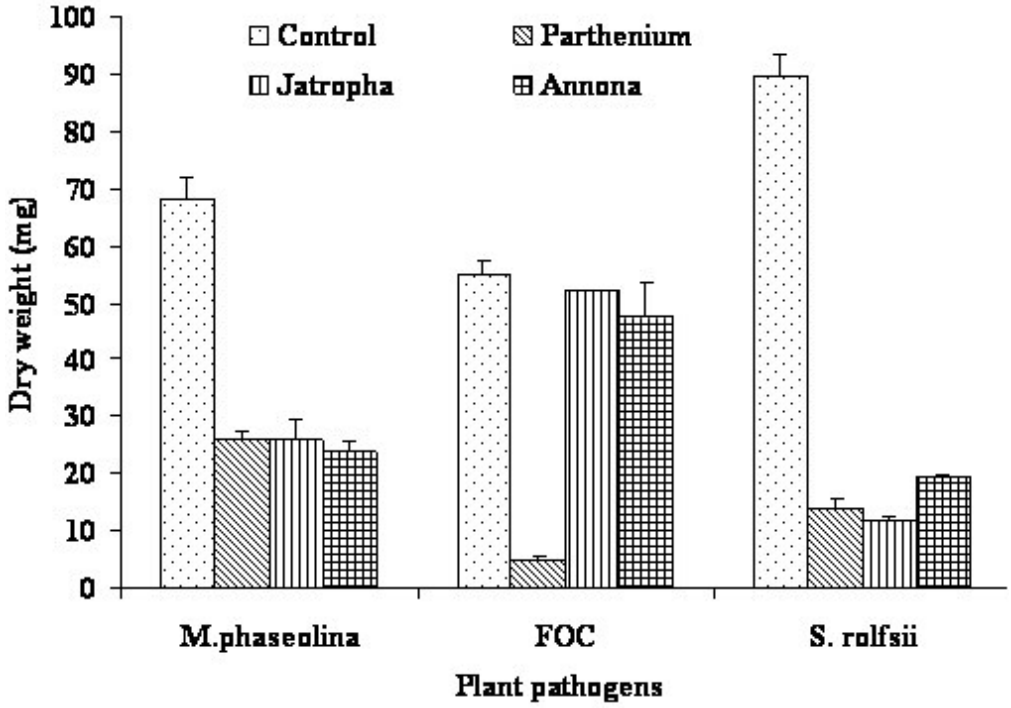

Figure 1. The effect of organic fraction (of solvent partitioning) of various biowash on the growth (dry weight) of plant pathogens.

fractions were passed through a Millipore filter and placed in the freezer until assayed.

Thin layer chromatography

Samples of the active open column fraction(s) in comparison with concentrated crude biowash (by solvent partitioning; organic phase) were spotted (20 µl) onto a silica gel TLC plate (9 X 7.5cm; Silica gel 60 F_{254}, Merck, Germany) as 1 cm bands alternating with 1 cm non-spotted areas. They were developed in a mobile phase consisting of dichloromethane: methanol (DCM: MeOH) at 2:1, 4:1, 8:1, 16:1 and 32:1 (v/v) and examined under normal light, short (254 nm) and long (365 nm) wave ultraviolet light. Conspicuous bands were marked with a pencil and Rf values were calculated.

Revival of the plant pathogens

All the three plant pathogenic fungi (*S. rolfsii*, *M. phaseolina* and FOC) were acquired from ICRISAT microbial gene bank. A loop full of the fungi were inoculated (individually on separate plates) on the center of quarter strength potato dextrose agar (PDA) plates and incubated at 28°C for 4 days.

Bioassay

Once the fungus was grown well on ¼ strength PDA plates, one disc (6 mm d) was bored with a borer from the growing mycelia (to get log phase culture) and inoculated into the flasks containing ¼ potato dextrose broth (PDB; 20 ml). In the treatment flasks, 100 µl of samples (organic, aqueous, adsorbed and non-adsorbed fractions) in MeOH was added whereas in the control, 100 µl of MeOH was added. The flasks were incubated at room temperature (26 to 29°C) on a shaker (G10 Gyrotary shaker, USA) at 160 rpm for 5 days. After incubation, fungal dry weight and count were done for the samples and compared with control.

The fungal dry weight was taken by dispensing the contents of each flask into a centrifuge tube (after taking the weight) followed by centrifugation at 9000 g for 20 min. Supernatant was carefully discarded and the tubes were kept in a oven (hot box oven; size 3; Gallenhamp, England) at 60°C for 2 days. The dry weight of the fungi was calculated as per the following formula:

Dry weight of fungi = dry weight of the tube with fungi – empty weight of the tube

For the fungal counts, the contents of the flask were homogenized with a tissuemizer (Teckmar type T 25, Japan), serially diluted up to 10^{-4} dilution and 0.1 ml was spread plated on ¼ strength PDA plates. After two days of incubation at 28°C, the fungal colonies in the treatment plates were compared with the control.

RESULTS

Effect of the organic fraction (of solvent partitioning) of Parthenium, Jatropha and Annona biowash on *M. phaseolina,* FOC and *S. rolfsii*

Organic fractions of Parthenium, Jatropha and Annona biowash at 0.5% inhibited the growth of *M. phaseolina* by 62, 62 and 65%, respectively and *S. rolfsii* by 85, 87 and 78%, respectively, compared to that of the control (Figures 1 and 3). Only organic fraction of Parthenium at 0.5% was able to inhibit FOC very effectively (91% compared to control) whereas Jatropha and Annona biowash were

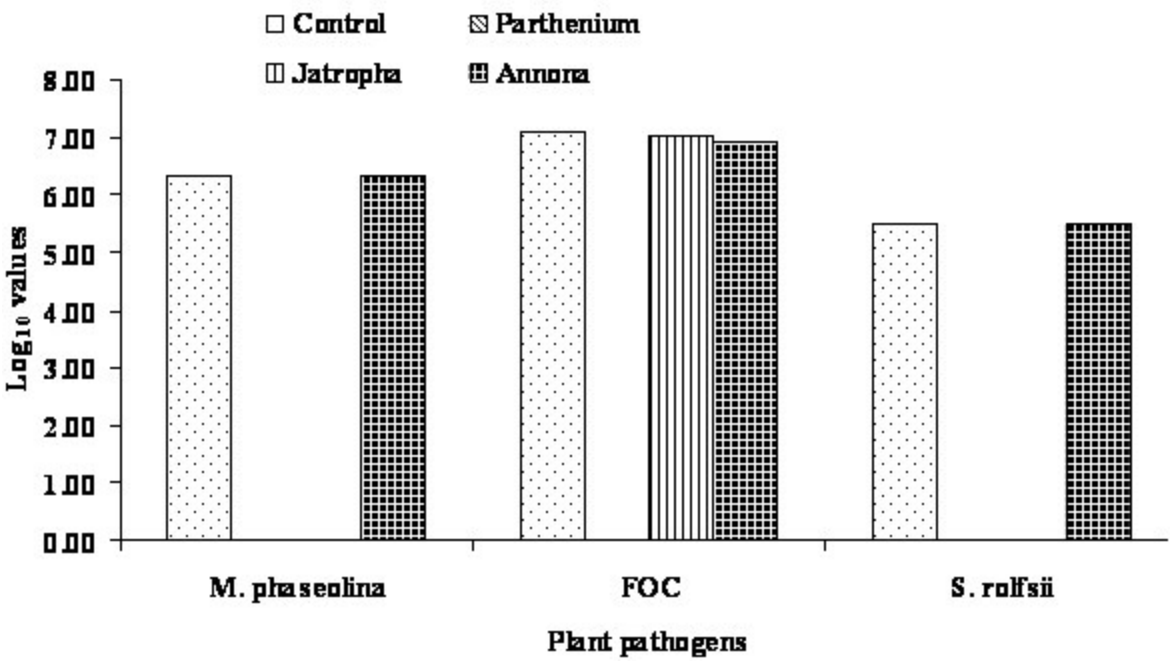

Figure 2. The effect of organic fraction (of solvent partitioning) of various biowash on the growth (counts) of plant pathogens.

ineffective (Figure 1 and 3). Similar results were found when the samples were plated on ¼ PDA as no colonies of *M. phaseolina* and *S. rolfsii* were found when treated with organic fractions of Parthenium and Jatropha whereas FOC was completely inhibited by only Parthenium but not Jatropha (Figure 2). Organic fractions of Annona were able to inhibit the growth (dry weight) of *M. phaseolina* and *S. rolfsii* to a greater extent (65 and 78%; Figure 1) but when the samples were plated on ¼ PDA no effect was found as it grew very well and results were comparable to the control (Figure 2), probably due to the presence of fungistatic compounds in the biowash rather than fungicidal. The aqueous fraction (of solvent partitioning) of all the three biowash was not able to inhibit any of the studied fungal pathogens.

Effect of the adsorbed fraction (of SPE) of Parthenium, Jatropha and Annona biowash on *M. phaseolina*, FOC and *S. rolfsii*

Adsorbed fractions of Parthenium, Jatropha and Annona biowash at 0.5% inhibited the growth of *M. phaseolina* by 36, 34 and 49%, respectively, FOC by 49, 26 and 31%, respectively and *S. rolfsii* by 89, 81 and 91%, respectively, compared to that of the control (Figures 4 and 6). When the samples were plated on ¼ PDA no colonies of *M. phaseolina*, it was found in all the three organic fraction treated biowash indicating its complete inhibition whereas, *S. rolfsii* was completely inhibited only by Parthenium and Jatropha but not by Annona (Figure 5). The counts of FOC were comparable to control in all the three biowash treated samples (Figure 5). The non-adsorbed fraction (of SPE) of all the three biowash was not able to inhibit any of the studied fungal pathogens.

Effect of open column fractions of jatropha biowash on *S. rolfsii*

Based on the results of the effect of organic and adsorbed fractions of Parthenium, Jatropha and Annona on *M. phaseolina*, *S. rolfsii* and FOC, it was concluded that Jatropha biowash relatively showed higher inhibition (more than 81%) on *S. rolfsii* in all the three types of observations, visual on flasks, dry wt of fungal growth and fungal count on ¼ strength PDA plates and hence Jatropha biowash was selected for further fractionation of their secondary metabolites against *S. rolfsii*. When the organic fraction of Jatropha biowash was further fractionated by open column chromatography, only 80% MeOH fraction was found to inhibit *S. rolfsii* (81%; Figure 7 and 8).

Thin layer chromatography of the active open column chromatography fraction

When the active 80% open column fraction was further fractionated on silica gel TLC plates using various mobile phase combinations of DCM and MeOH, three clear bands (dark bands) were found in DCM: MeOH (8:1) with

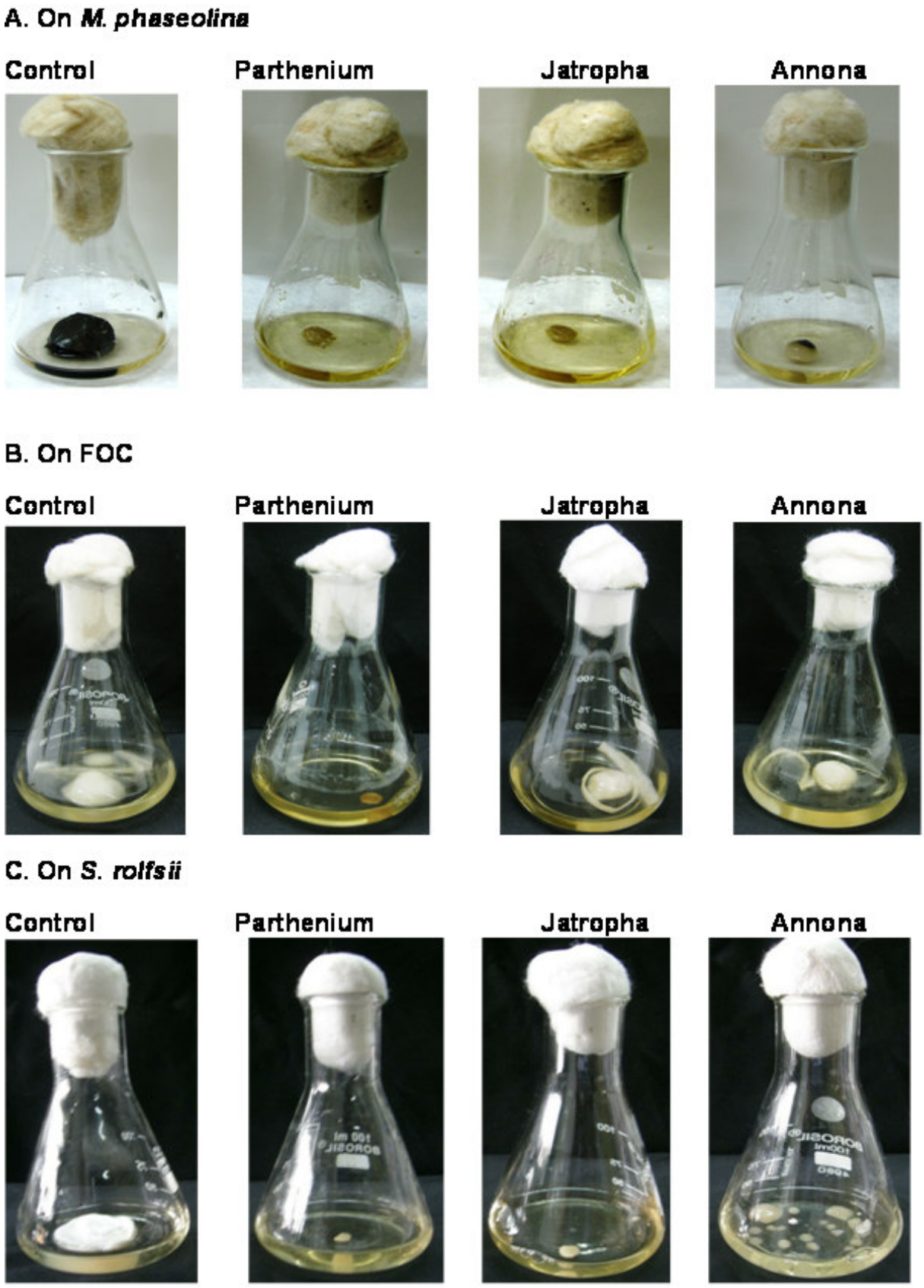

Figure 3. Efficacy of the organic fraction (of solvent partitioning) of Parthenium, Jatropha and Annona biowash on *M. phaseolina*, FOC and *S. rolfsii*.

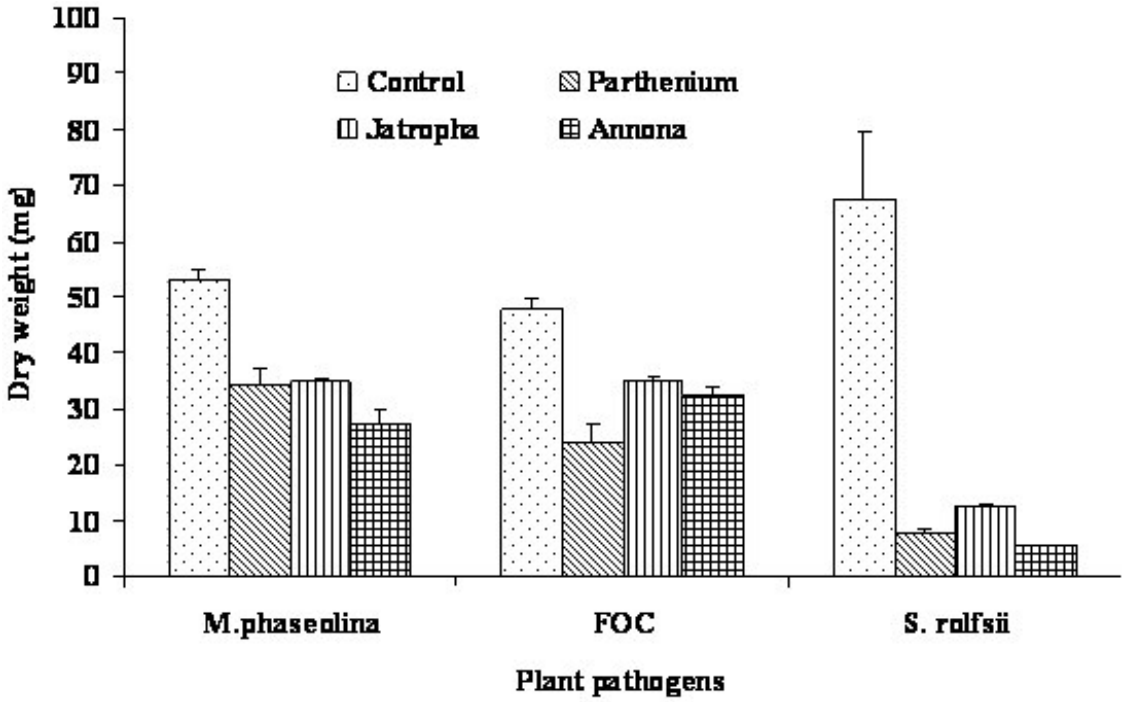

Figure 4. The effect of adsorbed fraction (of solid phase extraction) of various biowash on the growth (dry weight) of plant pathogens.

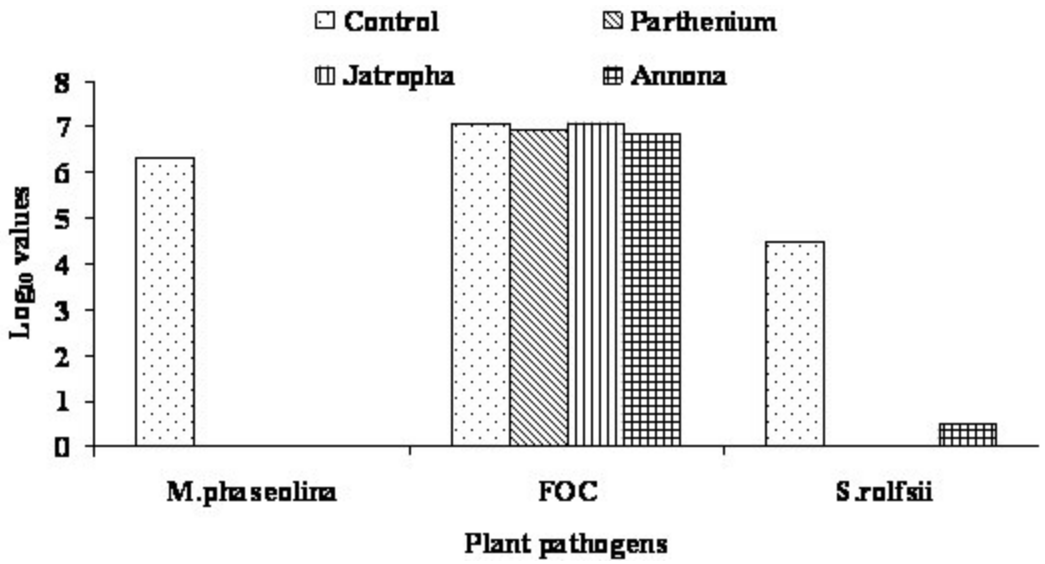

Figure 5. The effect of adsorbed fraction (of solid phase extraction) of various biowash on the growth (counts) of plant pathogens.

Rf values 0.95, 0.90 and 0.70 when examined under short wave ultraviolet light (254 nm).

DISCUSSION

Natural products with pesticidal activity have been and are being explored in order to make pesticides which are easily biodegradable, selective and can be locally produced, especially for farmers who can not afford expensive synthetic pesticides. At present, serious attention is drawn to extracts from vermicomposts of higher plants that contain antifungal substances in the form of alkaloids or prohibitins, which help in resisting the pathogens. One

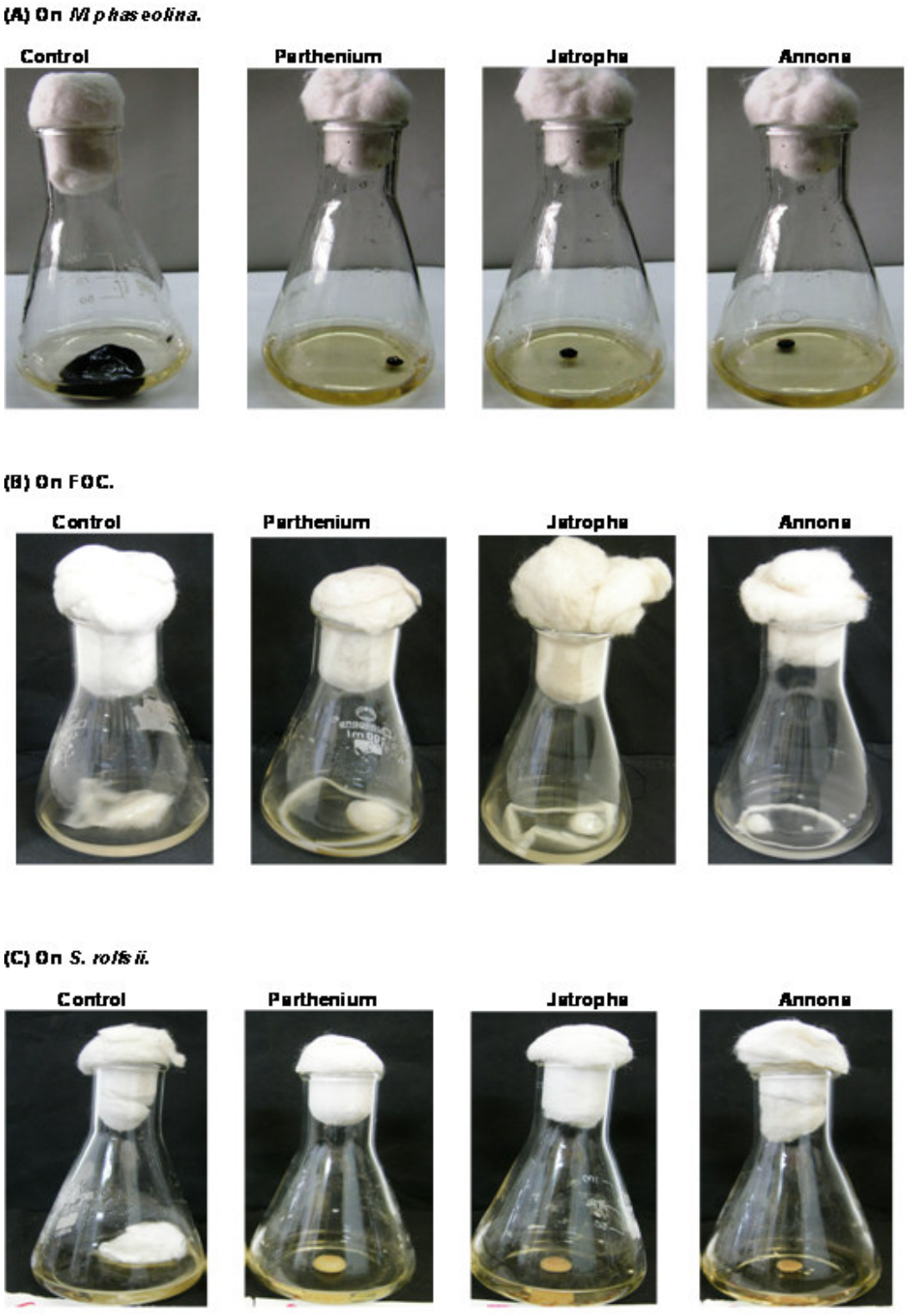

Figure 6. Efficacy of the adsorbed fraction (of solid phase extraction) of Parthenium, Jatropha and Annona biowash on *M. phaseolina*, FOC and *S. rolfsii*.

of the advantages of using earthworms in composting is that it creates aerobic conditions thus inhibiting the action of anaerobic microorganisms, which causes foul odour and release coelomic fluids in the decaying biomass,

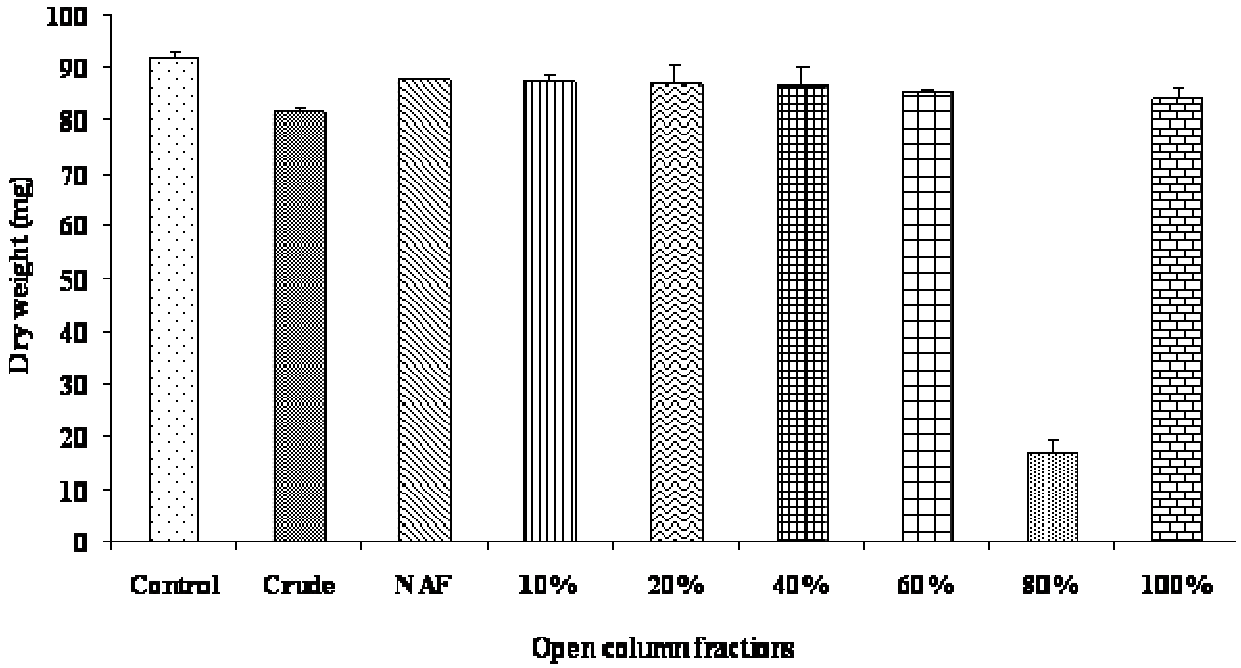

Figure 7. The effect of open column fractions of Jatropha biowash on the growth of *S. rolfsii* – dry weight. NAF- Non adsorbed fraction.

Figure 8. The effect of 80% methanol open column fraction (right flask) on the growth of *S. rolfsii* compared to control (left flask).

which may have antibacterial properties that kills pathogens example, *Salmonella*, *Serratia marcescens* and *Escherichia coli* (Jeyaraaj and Jayaraaj, 2005; Prabha, 2009). Earthworms also promote microbial activity and diversity in organic wastes to levels even greater than those in thermophilic composts (Edwards, 1998). Hence, there seems to be an even greater potential for suppression of plant disease by vermicomposts than by composts, probably due to stimulatory effects of soil microbial activity (Perner et al., 2006; Postma et al., 2003). Number of literatures are available in support of this hypothesis e.g. vermicompost at 25% provided good control of damping off in Patience-plant (*Impatiens wallerana*) caused by *Rhizoctonia solani* (Asciutto et al., 2006).

In the available literatures, among the three botanicals studied in this investigation, only Annona was proven to have biopesticide potential. For instance, crude ethanolic/ acetonic seed extracts of *Annona squamosa* (5 to 7.5%) was found to have suppression on polyphagous lepidopteran *Spodoptera litura* and diamondback moth, *Plutella xylostella* (Leatemia and Isman, 2004a; 2004b), cabbage head caterpillar *Crocidolomia binotalis* (Prijono et al., 1997) and inhibitory effect on conidial germination of *Colletotrichum gloeosporioides* causing anthracnose in papaya (Albiter et al., 2007). The insecticidal activity of the seed extracts of *A. squamosa* is attributed to annonins (Sahai et al., 1994).

In the present investigation, biowash (washings of vermicompost) prepared from the foliage of Jatropha, Annona and Parthenium were shown to have high level of inhibition against *S. rolfsii* (78 to 92%), FOC (26 to 91%) and *M. phaseolina* (62 to 77%), the causal organisms of collar rot of chickpea, Fusarium wilt of chickpea

and charcoal rot of sorghum, respectively. Only biowash of Parthenium was able to inhibit FOC more effectively (91%) whereas biowash of the other two botanicals, Jatropha and Annona, did not show much inhibition. Based on these results, it can be concluded that biowash prepared from Jatropha contains secondary metabolites that are capable of inhibiting *S. rolfsii,* very efficiently. Biowash of vermicompost is well known for its plant growth promoting traits for example, application of biowash of vermicompost not only enriches nutrients and plant growth promoting microorganisms required for the crop but also increases soil organic carbon (20 to 25%) and yield (22 to 33%) in okra (Gopal et al., 2010), paddy (Thangavel et al., 2003) radish, lobia and okra (Nath and Singh, 2009) and marigold (Sivasubramanian and Ganeshumar, 2004). Biowash of herbal vermicompost is also known for suppressing various insect pests including cucumber beetles (*Acalymna vittatum*) on cucumber and tobacco hornworm (*Manduca sexta*) on tomatoes (Edwards et al., 2010) and nematodes, root-knot nematode, *Meloidogyne incognita*, (Gulsar and Rohini, 2006). However, not much information is available on the effect of biowash of herbal vermicompost on inhibiting disease causing plant pathogens with one exception. Sathianarayanan and Khan (2008) reported that the biowash of vermicomposted coffee husk, coir pith and cow manure completely suppressed the growth of *Rhizoctoniae solani*, the causal organism of damping off of cabbage, cauliflower and cucumber.

In the present investigation, when the biowash of Jatropha was further fractionated on an open column chromatography, only one fraction (80% MeOH) was found to inhibit *S. rolfsii*. The active 80% MeOH fraction showed three clear bands when chromatographed on Silica Gel 60 F_{254} TLC plates with Rf values 0.95, 0.90 and 0.70; one of these three bands could be the active ingredients that inhibited *S. rolfsii*. Hence it can be firmly concluded that there are some secondary metabolites present in the biowash of Jatropha that are capable of inhibiting *S. rolfsii*. Suppression of *S. rolfsii* might be due to soluble compounds (such as soluble nutrients, free enzymes, soluble phenolic compounds and a wide range of microorganisms) passing from the solid Jatropha vermicompost into the biowash. Suppression by soluble nutrients and microorganisms can be ruled out since the biowash was passed through various chromatographies including solvent partitioning, SPE and open column chromatography and finally passed though Millipore filters which filters microorganisms. It is possible that some free enzymes could influence the pathogen (*S. rolfsii*) suppression, but not on the scale and consistency demonstrated in these experiments. Hence, the most likely reason for pathogen suppression could be the soluble phenolic substances taken up into biowash from vermicomposts of Jatropha. Exploitation of such an allelochemicals needs to be done in the integrated management of plant pest and pathogens. Further research in this area has the potential to extend the usefulness of vermicomposts prepared from Jatropha, Annona and Parthenium as biopesticide.

ACKNOWLEDGEMENTS

We thank National Bureau of Agriculturally Important Microorganisms for providing financial support. We also thank all the staff of biocontrol unit of ICRISAT including M/s PVS Prasad, P. Manohar, B. Nagappa, M. Satyam, D. Barath, A. Jabbar and D. Nagabushnam for their significant inputs in the sample preparation and lab studies.

Abbreviations

TLC, Thin layer chromatography; **FOC,** *Fusarium oxysporum* f. sp. *ciceri*; **EtOAc,** ethyl acetate; **PDB,** potato dextrose broth.

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