



# Root Rot Disease Complex of Cotton: A Menace to Crop in Southern Punjab and its Mitigation through Antagonistic Fungi

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## ABSTRACT

Root rot is very drastic disease prevalent in major cotton producing areas of Pakistan. Complex nature of disease due to root rot fungi and root-knot nematode cause huge losses to cotton. Experiments were conducted to assess the role of fungi and root knot nematodes in root rot disease complex of cotton and to optimize their control. For this purpose fifteen cotton growing areas in Southern Punjab were surveyed in year 2013-14. Data were recorded on disease prevalence, incidence, frequency percent of root rot fungi and nematodes population. Maximum disease prevalence was recorded in Tibba Sultanpur (100%) and Duniyapur (88%) whereas disease incidence calculated was 5% and 4.4%, respectively, in these areas. *Rhizoctonia bataticola* (Taub.) was frequently isolated (78%) from roots as well as in soil samples (81.7%) collected from infected fields. Among nematodes, *Meloidogyne incognita* (Kofoid and White) chitwood showed the maximum frequency percentage (72.3%). Results confirmed the presence of fungi and nematodes interacting each other to enhance the disease. *Paecilomyces lilacinus* (Thom) and *Trichoderma harzianum* (Rifai) (alone and in combination) were used as bio-control agents against isolated pathogens at three different concentration viz., R, R/2 and R/4. Combination of bio agents (T+P) gave maximum results at R concentration against *R. bataticola* and *M. incognita*.

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## Authors' Contribution

MAK carried out the lab work. SAK and IH provided technical support and helped in writing the manuscript. RWK helped in statistical analysis.

## Key words

Root rot disease complex, *Gossypium hirsutum* L., *R. bataticola*, *M. incognita*, *Paecilomyces lilacinus* and *Trichoderma harzianum*.

## INTRODUCTION

Cotton (*Gossypium hirsutum* L.), a perennial shrub cultivated as an annual crop, is a major cash crop and has a greater share in the world's economy. About two third of cotton is cultivated in US, India, China and Pakistan. Pakistan ranks at fourth in cotton producing countries of the world while at 10<sup>th</sup> in term of yield. Cotton and its products contribute 10 percent to GDP and 55 percent to foreign exchange earning of Pakistan (Azam *et al.*, 2013). Nearly 60% of the Pakistan's total population lives in Punjab. Rice and cotton are the major crops of Punjab (Nia, 2000). It is a warm season crop and grows well in areas having 50 mm annual rainfall with heavy showers at the time of boll formation (Nazir, 2007). Now a days cotton production is on decline due to fungi, bacteria, nematodes and viruses that reduce the yield drastically. Among these, nematodes are considered major yield reducing pests (Agrios, 2005). Root rot is very severe and devastating disease of cotton prevailing in Punjab and Sindh (Iqbal *et al.*, 2012). Climatic conditions of Pakistan favor

root knot nematodes and root rot fungi and their further interaction results in crop failure (Anwar and McKerny, 2007). Disease outbreak causes severe losses to cotton crop (Summy, 1992). Plant disease complexes cause 16% of total crop reduction per annum in the world. In view of root rot disease complex, local and systemic modifications induced by root knot nematodes make plants vulnerable to attack by other soil borne fungi (Siddique *et al.*, 2004).

Chemicals are used to control nematodes but due to their high cost and hazardous effects, nematicides are not always attractive to farmers (Khan *et al.*, 2016a). Environment friendly and economical affordable products are now being used due to their advantage of less resistance against the targeted pathogens. As well as people becoming more conscious about environmental and health hazard that is why pesticides are removing from markets (Gerhardson, 2002; Khan *et al.*, 2016b). In the past few years use of chemical pesticides was strictly prohibited due to contamination of ground water, death of avian, toxemia of mammals and accumulation of hazardous chemicals in the food web that is why it is a need of the time to develop some biological management strategies (Bird and Kaloshian, 2003). A great number of efforts have been made for identification of the microorganisms capable of reducing the activity of soil borne pathogens.

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The majority of such strategies for biological control of such pathogens depend on a single microbial biological control agent for pathogen reduction (Larkin *et al.*, 1998). In diverse rhizosphere and soil conditions application of an individual biological control agent may not always perform against all the pathogens of the similar crop. A very little work has been done on the management of root rot disease complex of cotton in Pakistan. Present study was designed to check the prevalence and incidence of disease in different cotton growing regions of Southern Punjab and their control through bio-control agents.

## MATERIALS AND METHODS

### *Survey of cotton growing areas for root rot of cotton*

During 2013-14, an extensive survey of cotton fields was carried out in Southern Punjab, Pakistan to assess the root rot incidence. Different areas selected in these locations were Kabirwala, Tibba Sultanpur, Duniyapur, Dakota, Basti dharik, Fatehpur, Khanewal, Lodhran, D G Khan, Muzaffargarh, Grhamor, Bahawalpur, Multan, Vehari and Rajanpur of Punjab Province, Pakistan. Hundred samples of healthy and infected plants were collected from each locality. The diseased samples were first packed in paper bags and then in 15×20-cm polyethylene bags, labelled, brought to the lab and stored at 4°C until processed for identification. The disease prevalence was measured by following formula:

$$\text{Disease prevalence (\%)} = \frac{\text{No of fields infected}}{\text{Total No of fields inspected}} \times 100$$

The disease incidence was measured by the following formula (Rao *et al.*, 2016):

$$\text{Disease incidence (\%)} = \frac{\text{No of infected plants}}{\text{Total No of plants}} \times 100$$

Relative density of nematodes was calculated by using following formula (Anwar and McKerny, 2012):

$$\text{Relative density of nematode (\%)} = \frac{\text{No of samples with nematodes}}{\text{Total No of samples}} \times 100$$

### *Isolation of plant parasitic fungi from infected samples*

The infected roots were cut in to 5-6 cm pieces, washed with tap water and surface disinfected by 2 percent sodium hypochlorite for two minutes. The pieces were given two washings in sterilized water and were blotted on sterilized filter paper sheet for drying. The segments were then plated on potato dextrose agar (potato starch, 20 g; agar agar, 20 g; dextrose, 20 g; distilled water, 1 L) for the isolation of suspected fungus in petri plates for each isolate

collected from different locations. All plates were placed at 28±2°C in an incubator, exposed to 12 h photoperiod under white light of wavelength between 350 and 750 nm for 5-7 days for recovery of pathogens (Sharma *et al.*, 2012). The plates were examined under the microscope and identified on the basis of their morphological characters and the growth of fungal hyphae from the segments of plant roots was assessed regularly (Ellis, 1971). It was determined whether more than one fungal species are growing. Purification was done at approximately 5 mm of hyphal growth. A small block of medium from the margin of each colony was transferred to another Petri plate containing PDA and incubated for 5 days at 28±2°C. The frequency of each isolated fungus from each part in the cultured pieces was calculated by using the formula (Saxena and Singh, 1987):

$$\text{Relative density of fungus (\%)} = \frac{\text{No of pieces colonized by a pathogen}}{\text{Total No of pieces}} \times 100$$

### *Isolation and identification of fungi from soil*

The soil sample were air-dried and grinded it lightly with a mortar and pestle (a marble kitchen mortar and pestle is appropriate) and mixed thoroughly. Then transferred a 10 g sub sample to 100 mL of sterile 0.01% WA (water agar) in a bottle, to give a dilution of 1:10. After that 10 mL was transferred to a second bottle containing 90 mL of 0.01% WA to give a dilution of 1:100 and mixed well to ensure an even spread of the soil in the solution. Repeated this step to give a 1:1000 dilution, which is usually satisfactory for the isolation of Fungus from vegetable or field crop soils. One mL of soil suspension was Dispersed (spread) across the medium in a 9 cm diameter Petri dish. Plates were prepared and let them dry for a few days to eliminate water from the surface of the plate. One mL of soil suspension was carefully pipetted onto the edge of the medium to one side of the plate. Plate was held on a slight slope away from the suspension and gently shaken at right angles to the slope, spreading the suspension in a uniform wetting front across the plate. Isolated plates were incubated under lights for 5-7 days. The colonies were sub-cultured and purified using the single spore technique on PDA. The number of these colony-forming units (CFU) per gram of soil were then be calculated for each species using the following formula (Warcup, 1955):

$$\text{Dilution} \times \text{Mean No. colonies of fungal species on isolation plates} = \text{CFU/g soil}$$

Pure cultures were identified by observing the following features: micro-morphological features (*e.g.* asexual and sexual fruiting bodies), spore morphology or morphology of sclerotia, mode of formation of spores (*e.g.*

nature of the conidiogenous cell and the presence or absence of chains of conidia). Some important plant pathogens were distinguished on the presence and morphology of sclerotia (e.g. *Rhizoctonia*).

#### *Collection of diseased samples for nematode isolation and identification*

For nematode isolations soil, and plant roots were taken at 4-6 inches depth. Samples were collected and mixed thoroughly and a composite sample of soil was taken in paper bags and then in 15×20-cm polyethylene bags. Polythene bags having samples were tightly sealed to maintain humidity and brought immediately to Laboratory of Nematology, Department of Plant Pathology, University of Agriculture, Faisalabad. The roots were separated from the soil, washed and weighed. The entire root system was chopped and incubated in a mist chamber for 5 days to hatch the eggs (McKerny and Roberts, 1985). Soil samples were thoroughly mixed and processed by Baermann funnel technique for 3 days to collect nematodes (Thistlethwayte, 1970). The isolation of nematodes from soil and root samples was carried out by Whitehead and Hemming tray method (Whitehead and Hemming, 1965) and Baermann funnel method (McKerny and Roberts, 1985), respectively. Relative density of nematodes was calculated by using following formula (Anwar and McKerny, 2012):

$$\text{Relative density of nematode (\%)} = \frac{\text{No of samples with RKN}}{\text{Total No of samples}} \times 100$$

#### *Staining methods*

Two staining methods were used. Acid fuchsin was used for staining nematodes within the root systems whereas phloxine B was used to stain *Meloidogyne* spp. egg masses. Identification of root knot nematodes was done on Perineal patterns of mature females (Eisenback et al., 1981).

#### *Preparation of concentrations*

Cultures of antagonistic fungi obtained from Department of Plant pathology, University of Agriculture Faisalabad at different concentrations of culture filtrates of *P. lilacinus* and *T. harzianum* R ( $10^6$  spores/ml), R/2 ( $10^4$  spores/ml) and R/4 ( $10^2$  spores/ml) were prepared by adding requisite amount of distilled water.

For mortality test, freshly hatched second stage  $J_{2s}$  within 48 h of *M. incognita* were used. Juveniles of *M. incognita* were extracted from the eggs and 50  $\mu$ l of the suspension containing 50  $J_{2s}$  was placed in each petri dish. Petriplate without extract of microbial antagonist was served as control. Four treatments including control of each concentration (*P. lilacinus*, *T. harzianum*, *P. lilacinus*+*T. harzianum*) with five replications were tested. Data were

recorded after 24, 48, 72 h of incubation.

Juvenile mortality was calculated and corrected by Abbot's formula (Abbott, 1925).

$$\text{Mortality (\%)} = \frac{t - c}{100 - c} \times 100$$

Where t is percent mortality in the chemical (bio/synthetic) and c is percent mortality in the control. Juveniles were considered dead if they did not move when probed with a fine needle (Abbasi et al., 2008) and were considered alive if they moved or appeared as a winding shape (El-Rokiek and El-Nagdi, 2011).

For hatching test, population of *M. incognita* maintained on the roots of eggplant from single egg mass culture was used, eggs of *M. incognita* were isolated by the method of Hussey and Barker (1973). A drop of distilled water containing approximately 50 eggs was placed in each petri dish. Three treatments of antagonistic fungi were added in the petri dishes and a control containing only eggs was used. Each treatment was replicated five times and incubated at  $28^\circ\text{C} \pm 2$  in a completely randomized design. Data were recorded after at 24, 48 and 72 h intervals.

Percent egg hatching was calculated and corrected by Abbot's formula (Abbott, 1925).

$$\text{Hatching inhibition (\%)} = \frac{t - c}{100 - c} \times 100$$

Where t is percent hatching inhibition in the chemical (bio/synthetic) and c is percent hatching inhibition in the control.

#### *Effect of antagonistic potential of T. harzianum and P. lilacinus against R. bataticola*

Culture of *R. bataticola* was maintained on PDA (potato starch, 20 g; agar agar, 20 g; dextrose, 20 g; distilled water, 1 L) in a 9 cm petri plate. Culture *T. harzianum* and *P. lilacinus* was maintained on PDA. Dual culture plate technique was used to check antagonistic potential of *T. harzianum* and *P. lilacinus* in inhibiting growth of the *R. bataticola* as described by Morton and Strouble (1955). A mycelial A mycelial disc of 0.3 cm from margin of the 10 days old culture of *T. harzianum* and *P. lilacinus* and the pathogen were taken with the help of sterilized needle and placed on the opposite edges of the plate at equal distance from the periphery. Five replications for each treatment alone and in combination of antagonistic fungi were maintained under completely randomized design. Petri plates containing antagonistic fungi alone and in combination and a petri plate containing pathogen were used as control for the comparison of the mycelial growth. All the inoculated plates were incubated at  $28 \pm 2^\circ\text{C}$  until the end of inoculation period (10 days after inoculation).

Data was recorded 2, 4, 6, 8 and 10 days interval after inoculation by measuring radial growth of the pathogen and percent inhibition of average radial growth was calculated in relation to growth of controls as follows:

$$L = [(C-T)/C] \times 100$$

Where L represents the inhibition of radial mycelial growth, C is radial growth measurement of the pathogen in control and T is radial growth of the pathogen in the presence of antagonistic isolates (Singh *et al.*, 2002).

#### Data analysis

Data were analyzed statistically using Statistics 8.1 software and means were compared by LSD test at 5 % significant level (Steel *et al.*, 1997).

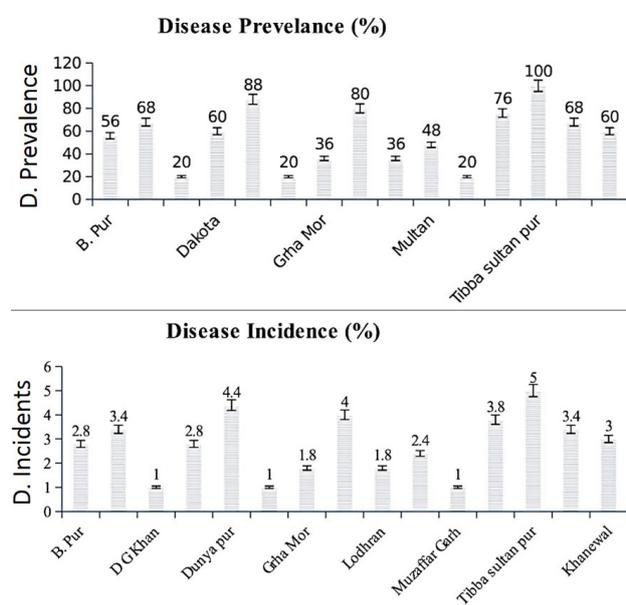


Fig. 1. Disease prevalence and indices of root rot of cotton at different locations during survey.

## RESULTS

#### Disease prevalence of root rot of cotton in different cotton growing areas of Southern Punjab

Data on disease prevalence was 100% in Tibba Sultanpur which revealed that all the fields visited were infected with root rot. Dunyapur showed 88% while Kabirwala showed 80% prevalence that means 4 out of 5 fields were infected, whereas fifth field was healthy. Rajanpur showed 76% and both Dakota and Khanewal showed 60% prevalence. Bahawalpur represented 56% disease prevalence followed by Multan 48%, Lodhran 36% and Grhamor 36%. Minimum disease prevalence was observed in D G Khan, Fatehpur and Muzaffargarh

that was 26%. Results showed that root rot of cotton was most prevalent disease in cotton fields. The results were significantly different from each other at  $P=0.05$  (Fig. 1).

#### Disease incidence of root rot of cotton in different cotton growing areas of Southern Punjab

During visit the total number of plants from each field were counted and recorded. The total number of plants showing root rot symptoms was recorded. Disease incidence was maximum at Tibba Sultanpur (5%) and Dunyapur (4.4%) while there was less incidence in Kabirwala (4%) followed by Rajanpur (3.8%), Basti dharik (3.4%) and Vehari (3.4%). Khanewal showed 3% while Bahawalpur and Dakota showed 2.8%. Incidence observed in Garhamor and Lodhran was 1.8%. Minimum incidence occurred in D. G. Khan, Fatehpur and Muzaffargarh (1%) (Fig. 1). The results were significantly different from each other at  $P=0.05$ .

#### Frequency % of fungi isolated (root samples) from different locations during survey

Root samples taken from different locations expressed frequency percentage of different fungi. *R. bataticola* was most frequently isolated fungi. Frequency % of *Rhizoctonia bataticola* was found to be highest (78%) in root samples collected from Tibba Sultanpur followed by *B. theobrome* (33.4%), *A. alternate* (22.8%) and *F. oxysporum* (17.8%). Frequency % of *Rhizoctonia bataticola* in Rajanpur was 71.2 % followed by Vehari (71%), Dunyapur (70%), Bahawalpur (66%), Dakota (56.2%) and Lodhran (56%). Basti Dharik and D G Khan showed 55% frequency of *R. bataticola*. Frequency percentage of *R. bataticola* calculated in Kabirwala was (52.2%) while Grhamor showed (49.6%), Fatehpur showed (49%) and Khanewal (46%). Multan and Muzaffargarh showed 36.2% and 30.6% *R. bataticola* frequency, respectively. *Botryodiplodia theobrome* was second frequent fungus (67%) isolated from cotton root samples in Dunyapur followed by Rajanpur (55.2%), Vehari (48%), Bahawalpur (46%), Dakota (43.8%), Fatehpur (39.6%), Tibba Sultanpur (33.4%), D G Khan (30.4%), Lodhran (28.8%), and Kabirwala (28.4%). Grhamor and Khanewal showed 25.4% and 25%. Basti dharik (23.4%) showed more frequency percentage of *B. theobrome* than Multan (22.8%) while minimum percentage was observed in Muzaffargarh (19.2%). Maximum frequency percentage of *Alternaria alternata* (51.4%) was observed in root samples collected from Rajanpur followed by Vehari (32.6%), Fatehpur (32.4%), Dakota (27.6%), Bahawalpur (26%), Kabirwala (25%) and Basti dharik (24%). Lodhran, D G Khan and Muzaffargarh showed 23.8%, 23.6% and 23.2%, respectively. Grhamor (18.6%) showed more

percentage than Duniyapur (17.8%) while Multan (17.4%) showed more percentage than Khanewal (13.2%) and Tibba Sultanpur (12.8%). *Fusarium oxysporum* was less frequently fungi isolated from all samples collected at different locations. Maximum frequency percentage was observed in Vehari (26.4%) while minimum percentage was observed in Khanewal (10.4%). The results were significantly different from each other at  $P=0.05$  as shown in Figure 2A.

*Frequency % of fungi isolated (soil samples) from different locations during survey*

By using a soil dilution technique, the mycological analysis of soil samples was studied. Different fungi including *R. bataticola*, *B. theobrome*, *F. oxysporum*, *A. alternata*, *Aspergillus*, *Penicillium* and *Mucor* were isolated from samples collected at different locations. Maximum frequency percentage (81.7%) of *R. bataticola* was calculated in Tibba Sultanpur followed by Duniyapur (66.2%), Basti dharik (66.2%), Kabirwala (61.7%), Vehari (52.5%), Bahawalpur (51.2%) and Khanewal (43.7%). *R. bataticola* isolated from Grhamor (41.7%) soil samples showed higher frequency percentage than Lodhran (40%) while minimum frequency percentage was observed in Multan (25.2%). Maximum frequent fungus was *R. bataticola* at Tibba Sultanpur followed by *A. alternata* (34.2%), *B. theobrome* (33%), *F. oxysporum* (31%), *Aspergillus* (25.2%), *Penicillium* (17.2%) and *Mucor* (7%). Frequency percentage of fungi isolated from Kabirwala was *R. bataticola* (61.7%), *A. alternata* (29.2%), *B. theobrome* (40%), *F. oxysporum* (20.7%), *Aspergillus* (17.2%) and *Mucor* (4.5%). Frequency percentage of fungi isolated from Multan was *R. bataticola* (25.2%), *A. alternata* (22.2%), *B. theobrome* (29.7%), *F. oxysporum* (16.2%), *Aspergillus* (14.5%), *Penicillium* (5.5%) and *Mucor* (4%). Frequency percentage of fungi isolated from Dakota was *R. bataticola* (27.5%), by *A. alternata* (42.5%), *B. theobrome* (50%), *F. oxysporum* (22%), *Aspergillus* (2.5%), *Penicillium* (14.5%) and *Mucor* (11.7%). Frequency percentage of fungi isolated from Khanewal was *R. bataticola* (43.7%), *A. alternata* (27.5%), *B. theobrome* (44.2%), *F. oxysporum* (23.7%), *Aspergillus* (8.5%), *Penicillium* (0.0%) and *Mucor* (5.5%). Frequency percentage of fungi isolated from Basti Dharik was *R. bataticola* (66.2%), *A. alternata* (42.5%), *B. theobrome* (54.7%), *F. oxysporum* (29.2%), *Aspergillus* (33%), *Penicillium* (0.0%) and *Mucor* (3.5%). Frequency percentage of fungi isolated from Lodhran was *R. bataticola* (40%), *A. alternata* (51%), *B. theobrome* (30.5%), *F. oxysporum* (20.2%), *Aspergillus* (6.5%), *Penicillium* (7.2%) and *Mucor* (5%). Frequency percentage of fungi isolated from Fatehpur was *R. bataticola* (35%), *A. alternata* (57.5%), *B. theobrome* (83.7%), *F. oxysporum* (57.5%), *Aspergillus* (15.7%), *Penicillium* (24%) and

*Mucor* (19.5%). Frequency percentage of fungi isolated from D G khan was *R. bataticola* (26.5%), *A. alternata* (24.2%), *B. theobrome* (32.5%), *F. oxysporum* (11.2%), *Aspergillus* (4.7%), *Penicillium* (10.2%) and *Mucor* (4.7%). Frequency percentage of fungi isolated from Rajanpur was *R. bataticola* (38.2%), *A. alternata* (26.5%), *B. theobrome* (47%), *F. oxysporum* (27.5%), *Aspergillus* (2%), *Penicillium* (30%) and *Mucor* (9.5%). Frequency percentage of fungi isolated from Muzaffargarh was *R. bataticola* (26.5%), *A. alternata* (29.5%), *B. theobrome* (29.7%), *F. oxysporum* (11.2%), *Aspergillus* (10%), *Penicillium* (5%) and *Mucor* (2.2%). Frequency percentage of fungi isolated from Grhamor was *R. bataticola* (41.7%), *A. alternata* (13.7%), *B. theobrome* (24.5%), *F. oxysporum* (32.5%), *Aspergillus* (2.2%), *Penicillium* (0.0%) and *Mucor* (6.2%). Frequency percentage of fungi isolated from Bahawalpur was *R. bataticola* (51.2%), *A. alternata* (24.5%), *B. theobrome* (34.5%), *F. oxysporum* (23.5%), *Aspergillus* (24%), *Penicillium* (11%) and *Mucor* (3.7%). Frequency percentage of fungi isolated from Duniyapur was *R. bataticola* (66.2%), *A. alternata* (42.5%), *B. theobrome* (61.2%), *F. oxysporum* (28.2%), *Aspergillus* (26.7%), *Penicillium* (7%) and *Mucor* (13.5%). Frequency percentage of fungi isolated from Vehari was *R. bataticola* (52.2%), *A. alternata* (35.5%), *B. theobrome* (66.2%), *F. oxysporum* (31.2%), *Aspergillus* (23.2%), *Penicillium* (10.5%) and *Mucor* (15.2%). The results were significantly different from each other at  $P=0.05$  (Fig. 2B).

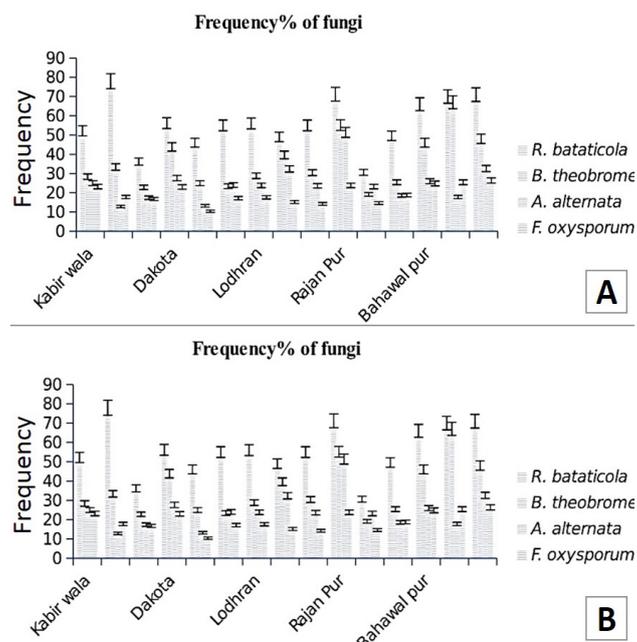


Fig. 2. Frequency (%) of isolated fungi from root samples (A) and soil samples (B) at different locations.

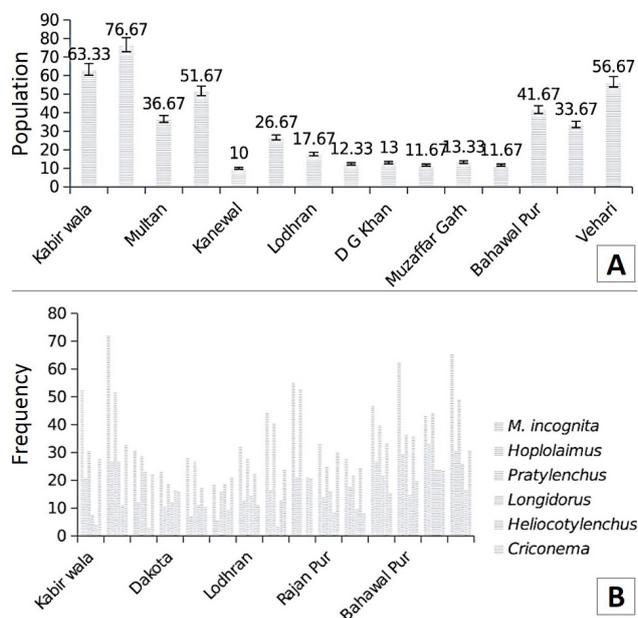


Fig. 3. Population (A) and frequency (%) (B) of nematodes at different locations.

#### Population of nematodes at different locations during survey

The results of survey at different locations indicated that maximum nematode population was recorded in Tibba Sultanpur (76.67%) followed by Kabirwala (63.33%). The nematode infestation recorded in Vehari was 56%, Dakota 51%, Bahawalpur 41% and Multan 36%. Infestation recorded in Dunyapur was 33% whereas in Basti Dharik, Lodhran, Muzaffargarh and D G Khan was 26.6%, 17.6%, 13.3% and 13% respectively. Minimum nematode infestation was observed in Fatehpur 12.3% followed by Grhamor 11.6% and Khanewal 10%. The results were significantly different from each other ( $P=0.05$ ) as shown in Figure 3A.

#### Frequency % of nematodes at different locations during survey

*M. incognita* showed maximum frequency percentage (72.3%) in Tibba Sultanpur followed by Vehari (65.6%) and Bahawalpur (62.6%). Second most frequent nematode was *Pratylenchus* (53%) in D G Khan followed by *Hoplolaimus* (33%) in Dunyapur, *Heliocotylenchus* (33.6%) in Grhamor, *Longidorus* (26%) in Vehari and *Criconema* (23.6%) in Dunyapur. Frequency percent of *M. incognita* in D G Khan was 55.33% and Kabirwala showed 52.6%. Grhamor showed 47% followed by Fatehpur (44.6%), Dunyapur (43.3%), Rajanpur (33.3%), Lodhran (32.3%) and Multan (31%). Khanewal showed 28.4% whereas Muzaffargarh showed 28%. Minimum frequency percent

of *M. incognita* was observed in Dakota (23.5%) and Basti Dharik (18.6%). Frequency percent of *Pratylenchus* in Tibba Sultanpur was 52%, Vehari was 49.3%, in Dunyapur was 44.3%, in Fatehpur was 40.6% and in Grhamor was 40%. Bahawalpur showed 36.6% frequency followed by Kabirwala (30.6%), Multan (29%), Lodhran (28%), Khanewal (27%), Rajanpur (25%), Muzaffargarh (22%), Dakota (19%) and Basti Dharik (16%). Frequency percent of *Hoplolaimus* in Vehari was 30.6% followed by Bahawalpur (29.6%), Tibba Sultanpur (27%), Grhamor (27%), D G Khan (21.3%), Muzaffargarh (18%), Fatehpur (16.6%), Rajanpur (14.3%), Lodhran (13%), Multan (12.3%), Dakota (10.6%), Khanewal (7.4%) and Basti Dharik (5.7%). Frequency percent of *Heliocotylenchus* in Bahawalpur was 36% followed by Muzaffargarh (24.6%), Lodhran (22.7%), D G Khan (21.3%), Khanewal (17.4%), Dakota (16.8%), Vehari (16.7%), Fatehpur (13%), Tibba Sultanpur (11.4%), Basti Dharik (9.6%), Rajanpur (8.6%) and Multan (3.3%). Frequency percent of *Longidorus* in Tibba Sultanpur was 27% followed by Multan (23.3%), Basti Dharik (19%), Rajanpur (16.3%), Bahawalpur (15%), Lodhran (14.6%) and Dakota (12.4%) (Fig. 3B).

#### Egg hatching inhibition of *M. incognita* at R, R/2, R/4 concentration of *P. lilacinus* and *T. harzianum*

Hatching percent of *M. incognita* varied significantly at different concentrations of *P. lilacinus* and *T. harzianum*. Antagonistic fungi were applied alone and in combination at R concentration of spores. Concentration of spores was maintained using haemocytometer. At R ( $1 \times 10^6$  spore/ml), R/2 ( $1 \times 10^4$  spore/ml) and R/4 ( $1 \times 10^2$  spore/ml) concentrations *P. lilacinus* significantly inhibited egg hatching of *M. incognita* as compared to *T. harzianum*, whereas combined application showed more significant results after 24, 48 and 72 h. After 24 h, *P. lilacinus* showed 3.6% egg hatching as compared to *T. harzianum* (7%) at R concentration, whereas their combined application proved more effective (1.6% egg hatching) against nematode. At R/2 after 24 h, 12.6% eggs were hatched in *P. lilacinus* treatment and *T. harzianum* showed 22% hatching inhibition, whereas combine application of both showed significant results. At R/4 after 24 h, 18.6% eggs were hatched in *P. lilacinus* treatment and *T. harzianum* showed 22.4% hatching inhibition, whereas combined application of both showed significant results (13.2% egg hatching). After 48 h, *P. lilacinus* showed maximum inhibition (5.6%) compared to *T. harzianum* (13.8%) at R concentration, whereas their combine application proved more effective (2.8% hatching inhibition) against nematode. At R/2 after 48 h, 18.4% inhibition was recorded in *P. lilacinus* treatment and *T. harzianum* showed 28.6% egg hatching, whereas combined application of both showed significant

results (12.4%) hatching inhibition. At R/4 after 48 h, 25% hatching was recorded in *P. lilacinus* treatment and *T. harzianum* showed 30.6% hatching, whereas combined application of both showed significant results (20.6%). After 72 h, *P. lilacinus* showed minimum percentage of hatched egg (9.4%) compared to *T. harzianum* (18.2%) at R concentration, whereas their combined application proved more effective (4.4% egg hatching inhibition) against nematode. At R/2 after 72 h, 24% eggs were hatched in *P. lilacinus* treatment and *T. harzianum* showed 32.4% hatching, whereas combined application of both showed significant results (17%) hatching. At R/4 after 72 h, 29.4% eggs were inhibited in *P. lilacinus* treatment and *T. harzianum* showed 32.4% hatching, whereas combine application of both showed significant results (24.6%). The results were significantly different from each other ( $P=0.05$ ) (Fig. 4).

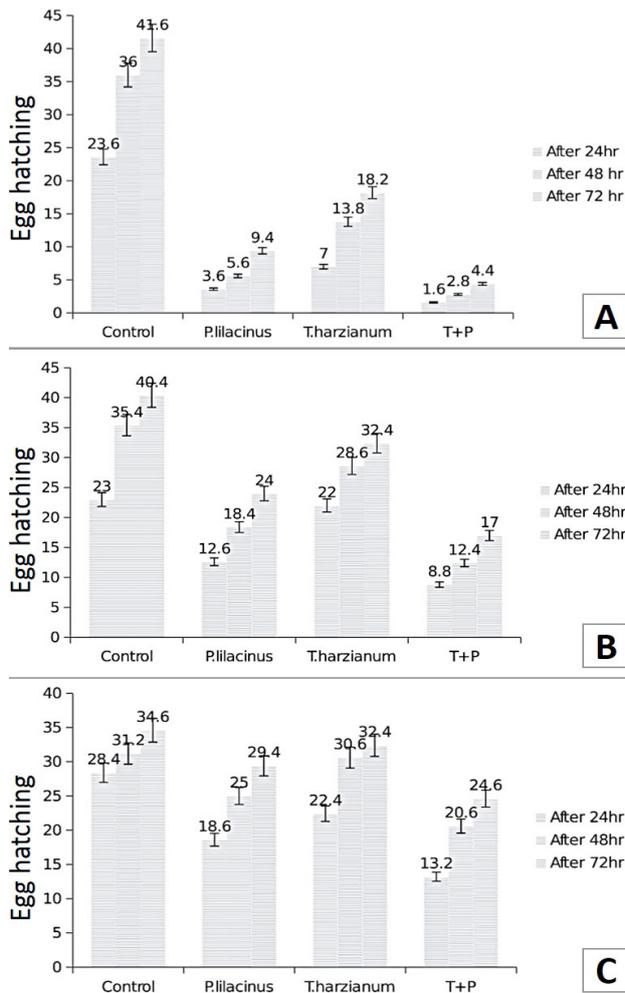


Fig. 4. Egg hatching inhibition of *M. incognita* at R (1/10<sup>2</sup> spores/ml) (A), R/2 (1/10<sup>2</sup> spores/ml) (B) and R/4 (1/10<sup>2</sup> spores/ml) (C) concentrations.

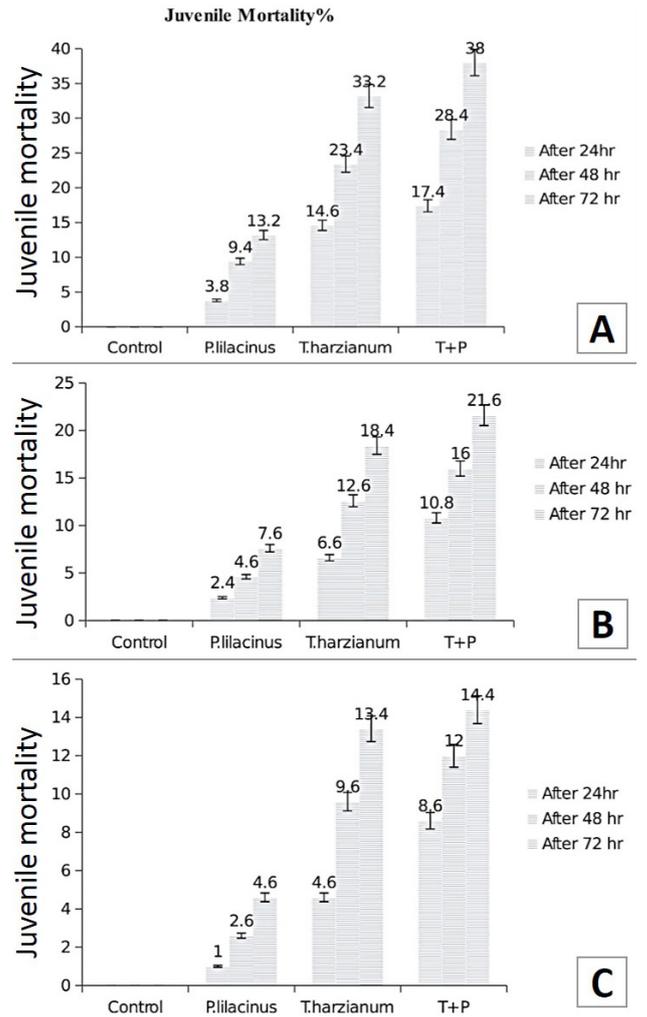


Fig. 5. Percent mortality of *M. incognita* at R (1/10<sup>2</sup> spores/ml) (A), R/2 (1/10<sup>2</sup> spores/ml) (B) and R/4 (1/10<sup>2</sup> spores/ml) (C) concentrations.

*Juvenile mortality of M. incognita at R, R/2, R/4 concentration of P. lilacinus and T. harzianum*

The effect of all concentrations varied significantly on juvenile mortality of *M. incognita*. Results showed that *T. harzianum* was found very effective against *M. incognita* juveniles as compared to *P. lilacinus*, whereas combined application of both showed significant results at all concentration after 24, 48 and 72 h. At R concentration after 24 h, *T. harzianum* showed 14.6% J<sub>2s</sub> mortality compared to *P. lilacinus* (3.8%), whereas combined application of both showed 17.4% J<sub>2s</sub> mortality. At R/2 concentration after 24 h, *T. harzianum* showed 10.8% J<sub>2s</sub> mortality compared to *P. lilacinus* (2.4%), whereas combined application of both showed 10.8% J<sub>2s</sub> mortality. At R/4 concentration after 24 h, *T. harzianum* showed 4.6% J<sub>2s</sub> mortality compared to

*P. lilacinus* (1%), whereas combined application of both showed 8.6%  $J_{25}$  mortality. At R concentration after 48 h, *T. harzianum* showed 23.4%  $J_{25}$  mortality compared to *P. lilacinus* (9.4%), whereas combined application of both showed 28.4%  $J_{25}$  mortality. At R/2 concentration after 48 h, *T. harzianum* showed 12.6%  $J_{25}$  mortality compared to *P. lilacinus* (4.6%), whereas combined application of both showed 16%  $J_{25}$  mortality. At R/4 concentration after 48 h, *T. harzianum* showed 9.6%  $J_{25}$  mortality compared to *P. lilacinus* (2.6%), whereas combined application of both showed 12%  $J_{25}$  mortality. At R concentration after 72 h, *T. harzianum* showed 33.2%  $J_{25}$  mortality compared to *P. lilacinus* (13.2%), whereas combined application of both showed 38%  $J_{25}$  mortality. At R/2 concentration after 72 h, *T. harzianum* showed 18.4%  $J_{25}$  mortality compared to *P. lilacinus* (7.6%), whereas combined application of both showed 21.6%  $J_{25}$  mortality. At R/4 concentration after 48 h, *T. harzianum* showed 13.4%  $J_{25}$  mortality compared to *P. lilacinus* (4.6%), whereas combined application of both showed 14.4%  $J_{25}$  mortality. The results were significantly different from each other ( $P=0.05$ ) (Fig. 5).

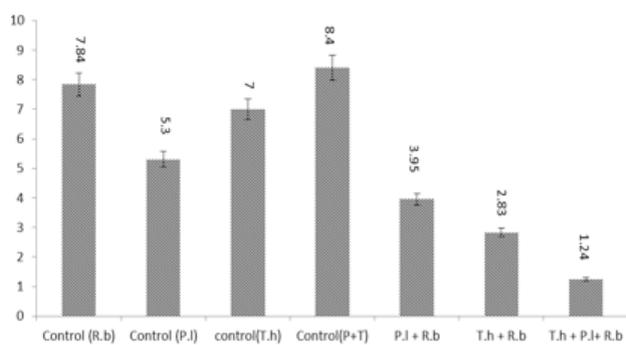


Fig. 6. Radial growth (cm) of *R. bataticola* (root rot fungus) in dual culture plate technique.

#### Evaluation of antagonistic fungi (*T. harzianum* and *P. lilacinus*) against *R. bataticola* (root rot fungus)

*T. harzianum* and *P. lilacinus* were tested against *R. bataticola* in dual culture plate technique. *R. bataticola*, *T. harzianum*, *P. lilacinus* and both (*T. harzianum* and *P. lilacinus*) were cultured separately to check their radial growth and then combined and alone antagonistic effect of *T. harzianum* and *P. lilacinus* was studied against *R. bataticola*. *R. bataticola* showed 7.84 cm radial growth in control. Radial growth of *P. lilacinus* in control was 5.3 cm. *T. harzianum* showed 7 cm radial growth in control whereas control with both *P. lilacinus* and *T. harzianum* showed 8.4 cm. Results showed that in separate treatments *T. harzianum* showed better results as compared to *P. lilacinus*. Radial growth of *R. bataticola* measured in

case of *T. harzianum* was 2.83 cm, whereas in the case of *P. lilacinus* radial growth of *R. bataticola* was 3.95 cm. *R. bataticola* growth was significantly suppressed by *T. harzianum* than *P. lilacinus*. Maximum growth of *R. bataticola* was suppressed in combination treatment. *R. bataticola* showed 1.24 cm radial growth when both *T. harzianum* and *P. lilacinus* were applied (Fig. 6).

## DISCUSSION

Root knot nematodes are most devastating and wide spread group of plant parasitic nematodes, prevalent in almost all cultivated areas of the world. Root knot nematodes are most common in areas having sandy soil with comparatively warm climate. Root knot nematodes take all the nutrients from the diseased plant and reduce the market value of the crop. Higher reproduction potential of root knot nematodes make them difficult to control. They parasitize roots by making injuries and predispose plants to the other soil borne fungal pathogens such as *Rhizoctonia solani*, *Rhizoctonia bataticola*, *Fusarium oxysporum* and *Botryodiplodia theobromae*.

Root rot caused by the *Rhizoctonia bataticola* (Taub.) and *Rhizoctonia solani* (Kuhn) is one of the most damaging diseases of cotton and considerable losses caused by them have been reported. Various reports on the variability of the pathogens of root rot of cotton *i.e.* *R. solani* and *R. bataticola* are available in literature on the basis of physiology, morphology and pathogenicity of different isolates (Monga *et al.*, 2004). Among the four provinces of Pakistan, Punjab's share in cotton production is estimated to be approximately 81% (Nia, 2000). Disease incidence of root rot, potential severity and population of nematodes during survey have been calculated by many scientists (Kiran *et al.*, 2006; Wheeler *et al.*, 2000; Haq *et al.*, 2012). In present study, survey of different cotton growing areas in Southern Punjab were conducted to calculate the disease prevalence, incidence and the frequency of pathogens present in roots and rhizosphere of cotton plants. *R. bataticola* was the most frequently isolated fungi from the samples taken. In current study, results revealed that the higher density of the fungus causing root rot of cotton was isolated from the roots having higher population of nematodes.

Environment friendly and economically affordable products are now being used due to their advantage of less resistance against the targeted pathogens. Moreover, people are becoming more conscious about environmental and health hazard, as a result of which pesticides are being removed from markets (Gerhardson, 2002). In the past few years use of chemical pesticides was strictly prohibited due to contamination of ground water, death of avian, toxemia

of mammals and accumulation of hazardous chemicals in the food web that is why it is a need of the time to develop some biological management strategies (Bird and Kaloshian, 2003). A great number of efforts have been made for identification of the microorganisms capable of reducing the activity of soil borne pathogens. The majority of such strategies for biological control of such pathogens depend on a single microbial biological control agent for pathogen reduction (Larkin *et al.*, 1998). In diverse rhizosphere and soil conditions application of an individual biological control agent may not always perform against all the pathogens of the similar crop.

A combination of biological control agents in a single preparation can overcome this inconsistent behavior of the antagonistic agent. A greater variety of traits responsible for reduction of one or more pathogen can be found in a combination of biological control agents. There is a possibility of expression of these traits over a wide range of environmental conditions (Pierson and Weller, 1994; Crump, 1998). Present study pointed that culture of *T. harzianum* and *P. lilacinus* have nematocidal activity. Nematocidal effect of culture filtrates of different fungi on hatching and mortality of root knot nematodes have been studied by many scientists (Zaki and Maqbool, 1991). In our study it was observed that culture filtrates of *T. harzianum* and *P. lilacinus* has capability to parasitize eggs and juveniles of *M. incognita* on different concentrations and this phenomenon increase with increase in time of exposure. Maximum egg parasitism and juvenile mortality was observed at higher spore concentration of *T. harzianum* and *P. lilacinus* after 72 h. Both antagonistic fungi possess larvicidal and as well as ovipocidal activities to compel *M. incognita*. Biocontrol agents used in the management of *M. incognita* are ecofriendly and do not have any human health hazards (Ashoub *et al.*, 2009).

These findings are in synchronized with the results obtained by Bonants *et al.* (1995). Present study is also similar with the results obtained by Montealegre *et al.* (2005). It was concluded that *T. harzianum* releases enzymes such as chitinase, glycanase and protease in culture filtrates. Similar results were obtained by Getha *et al.* (2005). Our results were in conformity with Lumsden *et al.* (1992) and Ashoub *et al.* (2009).

Combined application of both *P. lilacinus* and *T. harzianum* have strong ovipocidal activity. In combine application after 72 h at higher concentration of antagonists spore, very few eggs were hatched as compared to control while in *P. lilacinus* alone showed maximum egg inhibition as compared to *T. harzianum*. Similar results were obtained by Jatala (1986), Dos Santos *et al.* (1992) and Carnerio and Gomes (1993). Our results are synchronized with that of Sun *et al.* (2006) during their in vitro experiments

they reported a significant parasitism rate of *Meloidogyne* spp. egg masses by *P. lilacinus*. Al Kader (2008) enlisted 30 strains of *P. lilacinus* which have ability to parasitize *Meloidogyne* spp. eggs. In the present study, maximum egg inhibition was observed during first few hours this indicate that immature eggs are more prone to parasitism as compared to older ones. These results are harmony with Eapen *et al.* (2005), who reported that *P. lilacinus* provide significant parasitism during first 24 h.

Present study revealed that combine application of both antagonistic fungi were responsible for the maximum mortality of juveniles of *M. incognita* as compared to alone treatment. In combined application after 72 h at higher concentration of antagonist spore, maximum juvenile mortality was observed compared to control while in *T. harzianum* alone showed maximum juvenile mortality as compared to *P. lilacinus*. Similar findings were reported by Dennis and Webster (1971), who reported that *T. harzianum* has the ability to produce various enzymes like chitinase, glucanase and protease. These enzymes digest cuticle of *M. incognita* juvenile and thus destroy cell wall integrity and kills juveniles. Similar results were obtained by Huang *et al.* (2004). To invade juvenile fungus need to parasitize cuticle that is composed of collagen, keratin and fiber. When cuticle is invaded by fungal hyphae nematode become paralyzed and digested by fungus (Tunlid and Jansson, 1991).

Biological control potential of antagonistic fungi against *R. bataticola* has been indicated in number of reports (Vasudeva and Sikka, 1941; Jakkar *et al.*, 1997). Utilization of antagonistic microbes is an attractive, environment friendly and economical strategy alternative to chemical management (Bramawy and Sarag, 2012). Keeping in view the above mentioned facts the present study was designed to find out a management strategy for *Rhizoctonia* root rot of cotton. *In vitro* evaluation of antagonistic potential of *T. harzianum* and *P. lilacinus* was carried out against *Rhizoctonia bataticola*. It was found that *T. harzianum* had higher inhibitory potential than *P. lilacinus* against the pathogen. The results were supported by Devi *et al.* (2012) that inhibition of the growth of pathogen by *T. harzianum* isolates is due to its fast growing potential and competition for space and nutrition. The combined application of *T. harzianum* and *P. lilacinus* showed best result by inhibiting the growth of pathogen than alone. The results were supported by Nagesh *et al.* (2006), who reported that the combined application of *T. harzianum* and *P. lilacinus* significantly inhibited mycelia growth of *F. oxysporum* f.sp. *lycopersici*.

## CONCLUSION

The results revealed that root rot disease complex

is very prevalent disease in the cotton growing areas of the Southern Punjab. Root rot of cotton is caused by *Rhizoctonia bataticola* and its interaction with root knot nematode cause severe disease complex and is very difficult to manage. Root rot disease complex is becoming a problem in Punjab as no proper measures are taken to manage the disease. Present research is highlighting the disease prevalent areas. This will help the farmers to make an integrated disease management plan and biological control will also help to minimize environmental pollution, preserve the agro-ecosystem and bio diversity and keep management process more economical.

#### Statement of conflict of interest

Authors have declared no conflict of interest.

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