ABSTRACT

Chickpea is a major source of human food and the world’s third most important pulse crop after beans and peas. Similarly tomato is considered as ‘poor man’s orange’, a vitamin C rich vegetable and is eaten freely throughout the world. Chickpea production in India has declined considerably and tomato production has been limited due to the regular occurrence of wilt caused by *Fusarium oxysporum* f. sp. *ciceri* and *F. oxysporum* f. sp. *lycopersici*, respectively and root-knot caused by *Meloidogyne* spp. and the resulting fungus-nematode wilt disease complex. Annual yield losses to chickpea and tomato from the wilt vary from 10-15% in India. The disease under specific conditions may cause much greater losses or may destroy the entire crop in a field or area. Root-knot nematodes have been reported to reduce the yield of chickpea and tomato by 9-40% and 24-61% in India. The concomitant situation involving *Meloidogyne* spp. and *Fusarium* spp. leading to the development of wilt disease complex is, however, more damaging. The fungus-nematode wilt complex is one of the major constraints in the production of chickpea and tomato in India, and many growers have given up cultivation of these crops because of the disease complex. Management of wilt or root-knot is not an easy task, it becomes further difficult for wilt disease complex because of multi-pathogenic nature of the disease. When a pesticide is applied, it is targeted either against the wilt fungi or root-knot nematode, consequently the disease remains relatively unchecked. In view of lesser effectiveness of chemicals, high cost of application and adverse effects, biological control offers potential substitute for the management of wilt, root-knot and the wilt complex of chickpea and tomato.

Present study was undertaken with an objective to identify and characterize indigenous efficient isolates of *Aspergillus niger* for effectiveness against wilt (*F. oxysporum* f. sp. *ciceri/lycopersici*), root-knot (*M. incognita*) and the wilt disease complex (*F. oxysporum* f. sp. *ciceri/lycopersici + M. incognita*) and to develop their low cost formulations that could be adapted by poor and small farmers in India to control the target diseases of chickpea and tomato. The study was started with the isolation of *A. niger* aggregate from 32 different crop fields of 40 districts of the state of Uttar Pradesh, India. Initially 16 isolates out of 236 viz., AAn1, BAn4, BuAn3, BasAn5, BudAn3, GaAn1, JaAn2, LAn3, MeAn4, SkNAn3, SkNAn5, VAn4, ANAn1, ANAn4, AnC2 and AnR3 were short listed for further study for the reason that they showed faster growth rate, had more
ammonia and siderophore production and produced hydrogen cyanide and IAA than other isolates.

RAPD fingerprinting was conducted on the 16 efficient isolates of *A. niger* and three groups (Group I, II and III) were differentiated by RAPD markers and some amplicons were identified as isolate specific. Primer 02 (GCGACGCCTA) and Primer 06 (GATAGCCGAC) may be considered as *A. niger* specific. Primer OPA-16 can be treated as isolate specific for AnC2 and VAn4 (three amplicons produced by the primer as 2300 bp for AnC2 and VAn4, and 2800 bp for AnC2 only); Primer 04 (AGTGGGTCGCG) can be treated as SkNAn5 specific (as it produced only 1500 bp for isolate SkNAn5); OPA-12 can be treated as VAn4 specific (as it produced 700 pb in VAn4 only). The isolates SkNAn5, VAn4, AnC2, AnR3, ANAn4 and BuAn3 which were present in the Group I by RAPD profiling, solubilized more phosphorus, had negative production of ochratoxin A, had more compatible and adsorbed more toxic heavy metals and were found to possess relatively stronger ability to suppress the wilt fungi through antibiosis (production of volatile compounds) and mycoparasitism (dual culture test). Culture filtrates of these six isolates of *A. niger* inhibit the hatching of eggs and induced mortality to juveniles of *M. incognita*. These isolates were also found more compatible with common fungicides viz., carbendazim (Bavistin 50 WP), captan (Captaf 50 WP), mancozeb (Dithane M-45 75 WP), metalaxyl (Apron 35 SD), thiram (TMTD 75 WP), and two nematicides viz., carbofuran (Furadan 3G) and nemacur (Fenamiphos) than rest of the isolates.

The sixteen isolates were evaluated against target diseases on chickpea and tomato in 15 cm clay pots filled with 1 kg sterilized field soil and compost (3:1 ratio). The pathogens were inoculated in the form of 2 g sorghum seeds (SS) colonized by *F. oxysporum* f. sp. *ciceri/lycopersici* (22×10$^8$ CFUs/g) and/or nematode suspension containing 2000 freshly hatched juveniles (J2) of *M. incognita*/kg soil. The nematode suspension was prepared by incubating egg masses which were excised from the eggplants grown in a pure culture pots. The *A. niger* isolates were cultured on sorghum seeds or bagasse-soil mixture (BSM; 4:1). The *A. niger* isolates and pesticides were applied through seed treatment and soil application in chickpea, whereas nursery treatment and soil application were done in tomato. For seed treatment the dose was 4 g BSM or SS/kg seeds of chickpea that was applied to seeds along with the commercial *Rhizobium* of chickpea strain (25×10$^8$ CFUs/g formulation). In case of tomato, root-dip treatment with spore suspension of *A. niger* (10 g/100 seedlings) was given by grinding 10 g of colonized seeds
or the BSM in 100 ml distilled water. The roots of tomato seedlings were dipped in the suspension for 10 minutes. The pathogens were inoculated two days before the seed sowing or nursery transplanting. Five seeds of chickpea, *Cicer arietinum* cv. BGD-72 were sown in each pot. Whereas one seedling of tomato, *Lycopersicon esculentum* cv. Pusa Ruby was sown per pot. The pots were placed in an open space receiving uniform sunlight in a completely randomized block design. The plants were grown for four months (November to March). During this period they were regularly observed for symptom(s) attributable to a given treatment. The pots were watered uniformly with tap water whenever needed.

Inoculation with *F. oxysporum* f. sp. *ciceri/lycopersici* caused wilting which appeared on 7-8 weeks old plants and gradually intensified during vegetative growth. Some seedlings in a pot succumbed to the fungus infection. Chickpea cv. BGD-72 and tomato cv. Pusa Ruby were also found susceptible to the infection by *M. incognita* and developed characteristic root galls and egg masses. The galls were, however, smaller in size and lesser in number in chickpea than tomato. Concomitant inoculation with both the pathogens resulted to greater wilting but lesser galling (*P*≤ 0.01). The infected plants exhibited significantly reduced plant growth, yield and root nodulation. Phenol and salicylic acid contents of leaf and root of chickpea and tomato were considerably greater in the plants inoculated with *F. oxysporum* f. sp. *ciceri* or *F. oxysporum* f. sp. *lycopersici* and *M. incognita* singly or concomitantly. Infection with wilt fungi and root-knot nematode considerably decreased the leaf pigments. Application of *A. niger* isolates significantly checked the suppressive effect of the pathogens resulting to corresponding increase in the plant growth and yield variables; checked the loss of chlorophyll content of leaves, lycopene content of the tomato fruit and further increased the phenol content and salicylic acid of leaves and roots of chickpea and tomato. Seeds of chickpea and tomato obtained from *A. niger* applied plants showed considerably greater viability, germination and lower frequency of infection of *F. oxysporum* f. sp. *ciceri/lycopersici*. Among the treatments, *A. niger* SkNAn5 showed greatest effectiveness as it decreased the wilt severity by 60-68% and increase the yield by 84-88%, followed by VAn4 (decreased the wilt severity by 57-65% and increase the yield by 74-77%), AnC2 (decreased the wilt severity by 56-65% and increase the yield by 71-72%), carbendazim (decreased the wilt severity by 55-63% and increase the yield by 27-38%) / carbendazim + carbofuran (decreased the wilt severity by 56-57% and increase the yield by 38-50%). Other *A. niger* isolates also checked the disease and improved the yield of infected plants but less than AnC2. Soil population of pathogens
and *A. niger* isolates increased over time. In concomitantly inoculated pots, population of wilt fungi increased significantly (*P* ≤ 0.000001) but nematode population decreased (*P* ≤ 0.01). Application of *A. niger* isolates or pesticides resulted to significant decrease in the soil population of wilt fungi (*P* ≤ 0.000001) and root-knot nematode (*P* ≤ 0.0001) with the corresponding increase in the population of biocontrol agents.

Based on the relative performance in pot experiment, SkNAn5, VAn4, AnC2, AnR3, ANAn4 and BuAn3 were found more effective against the target diseases, and hence were tested further under field condition against wilt, root-knot and wilt disease complex of chickpea and tomato during November-March 2007-08. Three microplots each of 2 × 4 m were prepared for each treatment, and were randomly distributed in the field. Inoculation of *F. oxysporum* f. sp. *ciceri/lycopersici* (sorghum colonized seeds) and *M. incognita* (second stage juvenile suspension) was applied in soil @ 2 g colonized sorghum seeds or 2000 juveniles/kg soil two days before seed sowing/nursery transplanting. The efficient isolates of *A. niger* were mass cultured on bagasse-soil mixture (4:1). The soil treatment @ 40 g/microplot were applied at the time of seed sowing/nursery planting. Seed treatment with the *A. niger* isolates @ 4 g/kg chickpea seed was given along with *Rhizobium* application. root-dip treatment with *A. niger* isolates was done whereas seed treatment was not done. Roots of tomato nursery were dipped in the suspension of *A. niger* isolates (10 g/100 seedlings) for 10 minutes. Suspension of *A. niger* was made by grinding 10 g of colonized seeds in 100 ml distilled water. Carbendazim and carbofuran were applied @ 2.5 kg a.i./h (2 g a.i./microplot) and @ 2 kg a.i./h (1.6 g a.i./microplot), respectively, as soil application, and 2 g a.i./kg seed as seed treatment. In root-dip treatment, two weeks old tomato seedlings were dipped in 200 ppm solution of carbendazim/carbofuran for 15 minutes before transplanting. For combined treatment of fungicide and nematicide half dose of carbendazim was mixed with the half dose of carbofuran to get the equal dose to other treatments. Thereafter seeds/nursery were sown/transplanted in four rows in a microplot. Adequate moisture was maintained in the soil at the time of chickpea seeds sowing and irrigated just after transplanting of tomato nurseries. Two irrigations (as per requirement) in case of chickpea and bi-weekly irrigation (as per requirement) in case of tomato were given without over flooding of water from microplots to avoid contamination and plants were grown for 4 months. Soil population of the wilt fungi and *A. niger* isolates were estimated monthly by dilution plate method on *Fusarium* specific medium, and *A. niger* specific
medium supplemented with 50 mg/l nystatin fungicide in Petri dishes. Background population of the *Fusarium* spp. and *A. niger* were also determined.

Chickpea and tomato plants grown in the wilt fungus and/or root-knot nematode infested plots exhibited the characteristic symptoms of wilt and root-knot on above ground parts and roots, respectively. Severity of wilt was greater in plots inoculated concomitantly with *F. oxysporum* f. sp. *ciceri/lycopersici* and *M. incognita*. Seed/nursery treatment or soil application with the *A. niger* isolates provided the disease control that varied with pathogen and isolate. Greatest decrease in the wilt incidence and corresponding increase in the yield of chickpea and tomato occurred with SkNAn5 (75-76% lower incidence and 59-65% greater yield) followed by VAn4 (69-70% lower incidence and 52-57% greater yield), AnC2 (65-66% lower incidence and 50-53% greater yield) and carbendazim (56-58% lower incidence and 19-20 % greater yield) compared to the control. A suppression of 47-51% was recorded in the gall formation and 50-58% in egg mass production of root-knot nematode with SkNAn5 followed by VAn4 (39-45% galls and 40-47% egg masses), AnC2 (39-45% galls and 33-43% egg masses) and carbofuran (39-44% galls and 29-43% egg masses) over respective controls. In concomitantly inoculated plots, application of SkNAn5 suppressed the wilt and root-knot disease by 55-61% and 36-43%, respectively, and increased the yield of chickpea and tomato by 41% and 44%, respectively in comparison to the control followed by VAn4, AnC2 and joint application of carbendazim and carbofuran. Joint application of carbendazim and carbofuran decreased the wilt incidence by 54-58% and increase the yield by 27-28% compared to the control. Soil population of wilt fungi increased over time in plots without a treatment. The nematode population, however, decreased in the presence of wilt fungus. Various treatments also caused substantial decrease in the soil population of pathogens, being greatest with SkNAn5 followed by VAn4 and AnC2. In such plots population of the *A. niger* isolates increased correspondingly. Seed/nursery treatment and soil application of the *A. niger* isolates against the target diseases were more or less equally effective.

In view of effectiveness of *A. niger* SkNAn5, VAn4 and AnC2 demonstrated under field condition, their commercial formulations (biopesticides) were prepared on sawdust-soil-molasses mixture (stock culture) and fly ash-soil-molasses (immobilizing agent) in the ratio of 1:20. Shelf life test of the formulations revealed that the fly ash based carrier supported the survival as well as multiplication of the *A. niger* isolates during storage. At ambient temperature, the CFU count of *A. niger* isolates/g formulation increased
significantly in comparison to other temperatures. The temperature next in supporting
the greater CFU count was 25°C. Greatest CFU load/g formulation (10^{10}) was recorded during
March to August. From August onwards, the CFU count gradually declined, but even in
September it was greater than the control at 25°C or ambient temperature. The biocontrol
genus was, however, detected in the formulation upto 12 months. Among the three isolates
of \textit{A. niger}, greatest CFUs/g formulation (mean of 12 months population) was recorded for
SkNAn5 (14.5 \times 10^9), followed by VAn4 (13.2 \times 10^9) and AnC2 (12.6 \times 10^9). The
significant differences between the CFU counts of SkNAn5 and VAn4 (P \leq 0.05); SkNAn5
and AnC2 (P \leq 0.01); and VAn4 and AnC2 (P \leq 0.05) were recorded during different storage
periods.

Effectiveness of the biopesticides against wilt (\textit{F. oxysporum} f. sp. \textit{ciceri/lycopersici}),
root-knot (\textit{M. incognita}) and the wilt disease complex (\textit{F. oxysporum} f. sp. \textit{ciceri/lycopersici} + \textit{M. incognita}) of chickpea and tomato was tested in microplots (4 \times 2 m^2) in different fields. The biopesticides were applied in soil (40 g/microplot) or on
chickpea seeds (4 g/kg seed) or on tomato nursery roots (10 g/100 seedlings; root dipped for
10 minutes in 10\% \textit{A. niger} biopesticide suspension). Treatments with carbendazim and
carbofuran were maintained to compare effectiveness of the biopesticides applied \@ 2.5 kg
a.i./h (2 g a.i./microplot) and \@ 2 kg a.i./h (1.6 g a.i./microplot), respectively, as soil
application, and 2 g a.i./kg seed as seed treatment. In root-dip treatment, two weeks old
tomato seedlings were dipped in 200 ppm solution of carbendazim/carbofuran for 15
minutes before transplanting. For combined treatment of fungicide and nematicide half dose
of carbendazim was mixed with the half dose of carbofuran. Chickpea cv. BGD-72 and
tomato cv. Pusa Ruby grown in the plots infested with pathogens singly or concomitantly
developed characteristic wilt and root-knot symptoms, and exhibited significant yield
decline being significantly greater with the concomitant infestation (P \leq 0.001). Application
of the biopesticides checked the severity of the disease and associated yield declines.
Application of biopesticide SkNAn5 decreased the wilt incidence by 75\% and 79\% and
promoted the yield by 66\% and 71\% of chickpea and tomato, respectively grown in \textit{F.
oxysporum} f. sp. \textit{ciceri/lycopersici} infested plots. Its application was also found effective
against root-knot disease and suppressed the galling by 63 and 72\% and promoted the yield
by 27 and 28\% of chickpea and tomato, respectively in comparison to the control.
Application of biopesticide SkNAn5 was found highly effective against the fungus-
nematode wilt disease complex of chickpea and tomato, and its seed/nursery treatment
substantially controlled the wilt and root-knot, and increased the yield of chickpea and tomato by 46 and 44%, respectively. The biopesticide SkNAn5 also acted as a bio-fertilizer and improved the yield of chickpea and tomato in the plots not infested with either pathogens by 39% and 41%, respectively. Soil population of the wilt fungi and root-knot nematode decreased in the plot applied with biopesticides/pesticides. Population of the *A. niger* isolates applied through biopesticides, however, increased in the presence as well as absence of pathogens, being greatest in the former.

The present study has demonstrated that biological management of wilt, root-knot and wilt disease complex of chickpea and tomato can be satisfactorily achieved with the application of the biopesticides prepared with indigenous soil isolate of *A. niger* SkNAn5. Hence, it can be used in all three disease situations i.e., wilt, root-knot and fungus-nematode wilt disease complex. The isolate *A. niger* SkNAn5 was found ochratoxin A negative, highly tolerant to the fungicides such as carbendazim (Bavistin 50 WP), captan (Captaf 50 WP), mancozeb (Dithane M-45 75 WP), metalaxyl (Apron 35 SD), thiram (TMTD 75 WP), and two nematicides viz., carbofuran (Furadan 3G) and nemacur (Fenamiphos) commonly used by farmers. It was also found highly tolerant to toxic heavy metals such as Ni, Cr and Cd, and also showed great potential to adsorb the metals. Hence, pesticide contamination in soil will not influence effectiveness of the formulation. The pesticide tolerance ability has broadened the use of present biopesticide as this formulation in conjugation with pesticides can be applied under integrated disease management. However, a further fine tuning of the formulation and extensive field trials in different agro-climatic conditions and cropping patterns are needed before the commercial production and application.