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Molecular and biochemical characterization of soil isolates of *Aspergillus niger* aggregate and an assessment of their antagonism against *Rhizoctonia solani*

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Summary. Sixteen *Aspergillus niger* aggregate isolates collected from different crop fields were subjected to RAPD-PCR using 20 Operon primers and 8 synthetic primers. Twenty-two primers led to the amplification of 727 fragments ranging from 3500 bp (OPA 11) to 200 bp (primer 06). Two bands were monomorphic, while the rest were polymorphic. Three amplicons produced by OPA 16 were recorded as isolate-specific as 2300 bp by AnC2 and AnR3, and as 2800 bp by AnC2 only. The highest genetic similarity (0.79) was measured between AnC2 and AnR3, and the lowest (0.17) between AnC2 and AnR2. Multivariate analysis of genetic similarity revealed three major clusters, named group I, group II and group III. All isolates were ochratoxin A negative (<1 ng g^{-1}). Isolates AnC2 and AnR3, which produced HCN and solubilized the greatest amount of phosphorus, belonged to group I. These isolates also significantly increased eggplant yield and caused the greatest inhibition of colonization by *R. solani* in dual culture. They also suppressed the root rot on eggplant and the soil population of *R. solani* in pot soil.

Key words: biological control, root rot, eggplant, RAPD-PCR, ochratoxin A.

Introduction

In an environmentally conscious world, an eco-friendly management of a disease, especially biological control, are a potential substitute for chemical pesticides. A great number of micro-organisms have shown some capacity to antagonize plant-pathogenic fungi (Papavizas, 1985; Mukerjii, 1991; Mukhopadhyay *et al.,* 1992; Khan and Khan, 1995; Khan *et al.,* 2004). *Trichoderma* spp. have been widely explored for biological control (Papavizas, 1985), and *Trichoderma* formulations to control plant diseases are commercially available (Khan *et al.,* 2004). *Trichoderma* spp. suppress disease by antagonizing the pathogen. Some biocontrol agents, such as the plant growth promoting organisms (PGPOs), control a disease by dual action, not only suppressing a pathogen but also promoting plant growth and the defense mechanism of plants. The commercial application of PGPOs may provide a better and more reliable means to counter a disease.

Aspergillus niger is a versatile phosphate solubilizer that is abundant in various soil types (Gaur, 1990). This fungus also has a fair capacity to suppress plant pathogens (Fujimoto *et al.,* 1993; Khan *et al.,* 2006) and it increases the yield of the plants it colonizes. The effectiveness of the fungus, howe-

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ver, varies with the isolate (Sen *et al.,* 1993; Khan *et al.,* 2006). The accurate characterization of an isolate is an important aspect of the exploration and search for efficient biocontrol agents. Quite often isolates that are genetically similar are treated as different on account of a lack of proper characterization. Moreover, isolates cannot be differentiated by morphological or other conventional methods. Determination of genomic DNA by RAPD-PCR is one of the common tests for the initial assessment of genomic variation within and between fungal populations, and closely related organisms (Jacobson *et al.*,1993; McDonald and McDermott, 1993; Dalgleish and Jacobson, 2005). The RAPD has been extensively used to differentiate isolates of *A. niger* (Megnegneau *et al.,* 1993; Pekarek *et al*., 2006), *A. fumigatus* (Lin *et al*., 1995; Brandt *et al*., 1998), *A. parasiticus* (Yuan *et al*., 1995) and *A. flavus* (Bayman and Cotty, 1991; Tran-Dinh *et al*., 1999).

The aim of the present paper was to collect soil isolates of *A. niger* aggregate from different crop fields and to identify those isolates that are effective in controlling root rot of eggplant caused by *Rhizoctonia solani*. Root rot is a devastating disease of various vegetable and legume crops throughout India causing significant yield losses (Mehrotra, 1993). In order to detect variation in antagonism and phosphate solubilization between different isolates of *A. niger* aggregate, RAPD-PCR and some biochemical tests were performed and the difference in the yield of eggplants in pots treated with various *A. niger* aggregate isolates was measured. The antagonism of *A. niger* soil isolates against *R. solani* was investigated *in vitro* and *in vivo* on eggplant (*Solanum melongena*) cv. Pusa Kranti in clay pots.

Materials and methods

Isolation, identification and pure culture of *A. niger* aggregate isolates

Soil was collected from a number of crop fields in the Aligarh district, India, placed in sterilized polythene bags and brought to the laboratory. The soil samples were processed using a standard serial dilution technique (Wakman, 1927). The procedure was repeated three times to obtain a dilution of 1: 10,000, which was pipetted over PDA in a Petri dish (0.3 ml dish^{-1}) under a laminar flow. Three dishes were maintained for each treatment. Inoculated dishes were incubated at 25±2ºC for 5 days in a BOD incubator. After incubation, the dishes were inspected and isolates belonging to *A. niger* aggregate were identified on the basis of cultural and morphological characters as described by Raper and Fennel (1965) and Gilman (2001). A pure fungal culture was prepared by inoculating spores from each identified colony to a culture tube containing PDA and incubating at 25±2°C in a BOD incubator for 5 days. Details on the coding of the isolated cultures were as follows: AnPM1, AnPM2, AnPM3, AnPM4 from cultivated fields of pearl millet; AnC1, AnC2, AnC3 from cauliflower; AnR1, AnR2, AnR3, AnR4 from rice; AnL1, AnL2, AnL3 from lentil, and AnM1, AnM4 from mustard.

Isolation of genomic DNA

Genomic DNA from each soil isolate of *A. niger* aggregate was isolated as follows. Vacuum dried mycelium (3 g fresh or frozen) was crushed in 15 ml of grinding buffer (10 ml 3 M sodium acetate; 37.5 ml 4 M NaCl; 30 ml 0.5 M EDTA, pH 8.0; 15 ml 1 M Tris Cl; 6 g PVP; 4.2 g SDS and double distilled water to bring the volume up to 300 ml) using a mortar and pestle. The resulting paste was transferred to sterile centrifuge tubes using a sterile spatula. The tubes were incubated in a water bath at 60–65°C for one hour, with a gentle inversion of the tubes about every 15 min. Then 3 ml of 10 M ammonium acetate was added to each tube and kept for a further 30 min at 65°C, after which the tubes were centrifuged at 10,000 rpm for 10 min. The supernatant of each tube was then transferred to another tube to which an equal volume of chilled isopropanol had been added and kept at -20°C for 60 min, or at 0°C overnight. DNA was pelleted out by centrifugation (6,000 rpm for 25 min or 10,000 rpm for 15 min at 4˚C), washed twice with 70% ethanol and dissolved in $T_{10}E_1$ buffer (1 ml 1 M Tris; 200 μl 0.5 M EDTA, pH 8.0; double distilled water to bring the volume up to 100 ml). The dissolved DNA solution was extracted with chloroform: iso-amyl alcohol (24:1), and RNA was removed by RNAse (enzyme) treatment (4 μl ml^{-1} of supernatant from a stock of 10 mg ml^{-1} of RNAse) at 37°C for one hour. RNAse-treated DNA was further extracted twice with chloroform:iso-amyl alcohol (24:1) for further purification. DNA was re-precipitated in chilled ethanol (100%) and dissolved in $T_{10}E_1$ buffer. Purified DNA was checked for quality and quantity by 0.8% agarose gel electrophoresis using

uncut lambda (λ) DNA as a standard (300 ng μl^{-1}). Dilution of the DNA solution was done using $T_{10}E_1$ buffer to a concentration of approximately 25 ng μl^{-1} for use in PCR analysis.

DNA amplification by PCR

PCR amplification was carried out in 0.2 ml thin-walled PCR tubes using a T1 Thermocycler (Whatman Biometra, Goettingen, Germany). A total of 28 RAPD primers were screened. Twenty primers were Operon series A (Operon Technologies, Almeda, CA, USA) and eight primers were *Fusarium*-specific (primer 01, GTCACCCGGA; primer 02, GCGACGCCTA; primer 03, GCGGCATTGT; primer 04, AGTGGTCGCG; primer 05, CCAGACAAGC; primer 06, GATAGCCGAC; primer 07, GCTGGACATC; primer 08, GATCTCAGCG) custom-synthesized by Genetix Biotech Asia Pvt. Ltd., India. An unambiguous DNA profile was generated by 22 primers. A PCR mixture of 25 μl contained 25 ng of genomic DNA templates, 0.6 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India), 0.3 μM of decamer primer, 2.5 μl of 10× PCR assay buffer (50 mM KCl, 10 mM Tris-Cl, 1.5 mM $MgCl_2$) and 0.25 μl of pooled dNTPs (100 mM each of dATP, dCTP, dGTP and dTTP from Fermentas Life Sciences, MD, USA). PCR cycle conditions were as follows: initial denaturating step at 94˚C for 3 min, followed by 44 cycles of 92˚C for 1 min, 37˚C for 1 min and 72˚C for 2 min. In the last cycle, primer extension at 72˚C was provided for 7 min.

Documentation of gel and data analysis

PCR products were electrophoretically separated on 1.5% agarose gel containing ethidium bromide [at 1 μl (10 mg ml^{-1}) per 250 ml agarose solution] using 1× TBE buffer (pH 8.0) and a 60 V current that was run for three hours. The amplified product was visualized and photographed under UV lighting. O' Gene Ruller™ 100 bp DNA Ladder Plus (Fermentas Life Sciences) was used as a molecular weight marker.

DNA bands were scored as '1' for each primer-genotype combination that was present, and '0' for each primer-genotype combination that was absent. This binary data matrix was then utilized to generate genetic similarity data among the genotypes. Only unambiguous bands were scored, to estimate the genetic similarity between isolates using Jaccard's similarity coefficient. Based on these data dendrograms were generated using the SAHN clustering program selecting the unweighted pair group method with arithmetic mean algorithm (UPGMA) (Nei and Li, 1979) in NTSYS-pc (Rohlf, 1993). Support for the clusters was evaluated by bootstrap analysis with Win Boot software (Yap and Nelson, 1995). One thousand samples were generated by re-sampling with replacement of characters within the combined binary data matrix.

Biochemical characterization of *A. niger* aggregate isolates

Soil isolates of *A. niger* aggregate were characterized *in-vitro* with the following tests to identify isolates effective in mycotoxin production, fungus suppression and phosphate solubilization.

Ochratoxin A analysis and production. Ochratoxin A (OTA) was detected in the samples by high-performance liquid chromatography (HPLC), following the method of Scudamore and MacDonald (1998) with some modifications. Fifty grams of a finely grounded sample was added to a 250 ml Erlenmeyer flask containing a 100 ml mixture of methanol:water (9:1). The mixture was shaken for 30 min and filtered to remove any particulate matter. A 10 ml aliquot of the above extract was mixed with 40 ml of distilled water and filtered through a micro fiber filter. Ten ml of the filtered sample was taken and added to an immunoaffinity column (Ochra Test™, Vicam, Digen Ltd. Oxford, UK). The column was washed with 10 ml PBS containing 0.01% Tween 20, and then with 10 ml double distilled water. Ochratoxin A was eluted from the column with methanol (HPLC grade), again at a flow rate of 1–2 drops per second.

The HPLC apparatus (Hewlett Packard, Palo Alto, CA, USA) used for the determination of OTA was equipped with a spectrofluorescence detector (excitation, 330 nm; emission, 460 nm) and a C_{18} column (Supelcosil LC-ABZ, Supelco; 150×4.6 mm, 5 μm particle size), connected to a precolumn (Supelguard LC-ABZ, Supelco; 20×4.6 mm, 5 μm particle size). The mobile phase was pumped at 1.0 ml min^{-1} and consisted of an isocratic system as follows: 57% acetonitrile, 41% water and 2% acetic acid. Ochratoxin A was quantified on the basis of the HPLC fluorometric response compared with the OTA standard (Sigma Aldrich Co., St. Louis, MO, USA, purity > 99%). The lowest limit of detection was 1 ng g^{-1} . Each sample was analyzed three times.

Ochratoxin A production was tested on 16 isolates of *A. niger* aggregate. Ochratoxin A was determined using the method of Teren *et al*. (1996) with some modifications as follows: the isolates were grown in stationary cultures in 25 ml quantities of YES medium (2% yeast extract, 15% sucrose) at 30˚C for 10 days in the dark. After incubation, a portion (1 ml) of each culture medium was mixed with 1 ml of chloroform and centrifuged at 4000 *g* for 10 min. The chloroform phase was transferred to a clean tube, evaporated to dryness and re-dissolved in 0.5 ml of methanol. The OTA was quantified as described previously.

Hydrogen sulphide production. The test for production of hydrogen sulphide was performed following Deshmukh (1997).

Hydrogen cyanide (HCN) production. The test for production of hydrogen cyanide was performed following the method of Bakker and Schipper (1987).

Siderophore production. Siderophore production was detected using the chrome azurol assay (CAS) (Schwyn and Neilands, 1987).

Indole acetic acid (IAA) production. The IAA test was done using the standard method of Gordon and Weber (1951) and Brick *et al*. (1991).

Ammonia production. Organic nitrogen compounds are subject to dissimilation by a wide variety of heterotrophic micro-organisms to yield ammonia and other end-products. A peptone broth containing an organic nitrogen substrate was used to test the capacity of *A. niger* aggregate isolates to degrade proteins, causing ammonia to form. After incubation, the presence of ammonia, indicative of ammonification, was detected by using the standard method of Deshmukh (1997).

Phosphate solubilization. A loopful of *A. niger* aggregate was aseptically inoculated on solidified Pikovskaya's agar medium containing tricalcium phosphate [glucose 10 g, tricalcium phosphate 5.0 g, $(NH_4)_2SO_4$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $MnSO_4$ trace, $FeSO_4$ trace, yeast extract 0.5 g, agar 15.0 g, distilled water 1000 ml, and pH 7.2] in the centre of the dishes and were incubated at 25±2°C for 5–7 days. Colonies of the strains/isolates of *A. niger* aggregate showing a clearing or solubilization zone were considered phosphate solubilizers.

Antagonism tests and eggplant yield

The suppressive effects of the isolates on *R. solani* were tested *in vitro* using a dual-culture technique (Broadbent *et al.,* 1971) in which a 6-mm PDA disc of each isolate and an equal-sized disc of *R. solani* were placed 5 cm apart on PDA in each of 5 Petri dishes and incubated at 25±2°C. The radial growth of the fungal colonies was recorded 6, 7, 8, 9 and 10 days after inoculation.

The antagonistic effect of *A. niger* aggregate isolates on *R. solani* infecting eggplant cv. Pusa Kranti was examined *in vivo* in 15-cm clay pots. Each pot contained 1.5 kg of a steam-sterilized mixture of loam and compost (3:1). The top soil (6 cm) of one set of pots was inoculated with 3 g pot^{-1} sorghum seeds colonized by *R. solani*; this was followed by planting 3-week-old seedlings of eggplant two days later. The isolates of *A. niger* aggregate were cultured in potato dextrose broth in conical flasks for 10 days. The contents of each flask was filtered through Whatman filter paper no. 1 and the fungus mycelium and spores were homogenized in double distilled water. The suspension (10 ml) containing 1.5 g fungus was applied to the soil of each pot at planting. Two sets of pots with eggplant seedlings, one treated with *A. niger* aggregate, the other without *A. niger* aggregate, but all inoculated with *R. solani,* served as the controls for purposes of comparison. The soil population [colony forming units (CFUs)] of *R. solani* and *A. niger* aggregate was determined by the dilution plate method at planting (1 week), mid season (2 months) and harvest (4 months). Five pots were maintained per treatment. Four months after planting, plants were harvested and the number of fruits per plant and root rot were determined on a 0–5 scale (0, no root rot; 1, 1–19% rot; 2, 20–39% rot; 3, 40–59% rot; 4, 60–79% rot; and 5, 80–100% rot).

Statistical analysis.

The data on the root rot indices and number of fruits per plant were subjected to analysis of variance and the LSDs at $P \leq 0.05$ were calculated to identify significant differences. Duncan's multiple range test was applied to identify significant effective isolates of *A. niger* aggregate.

Results

RAPD polymorphism

Among the 28 primers tested by PCR amplification, 22 primers showed clear and unambiguous

amplification while the remaining 6 primers did not amplify in any of the reactions tried, or produced only faint or fizzy lanes (Table 1). The 22 scorable RAPD primers led to the amplification of 727 fragments, ranging from about 3,500 bp (OPA11) to 200 bp (primer 06). Of the 169 bands, 167 were polymorphic, and two monomorphic (primer 02 and primer 06). Three amplicons produced by OPA16 were recorded as isolate-specific as 2300 bp by AnC2 and AnR3, and 2800 bp by AnC2 only. Most of the *A. niger* aggregate isolates had common bands of 0.4, 0.6, 0.7 and 1.7 kb size. The greatest number of amplified products was 13 and was produced by primer 01. Primer 03 produced 12 bands, followed by OPA17 with 11 bands, and OPA02 and OPA16 with 10 bands each. Primer OPA14 amplified only one (1) band. On average, 7.23 bands per primer were obtained, and 8 of the 22 primers (36.4%) used in the study produced DNA bands greater than the average,

Table 1. Summary of polymorphism produced by OPA and *Fusarium*-specific synthetic primers.

Primers	Total No. amplified bands (X)	No. polymorphic bands	No. (Y) and molecular weight of unique profile in base pairs (bp) with isolate	Percent uniqueness (Y/X)×100
OPA-01	-	-	-	-
OPA-02	10	10	4(300,AnPM3; 800,AnR4; 1400,AnL3; 2000, AnPM3)	40.00
OPA-03	7	7	-	00.00
OPA-04	5	5	3(500,AnPM2; 1600,AnPM2; 2100,AnC3)	60.00
OPA-05	-	-	-	-
OPA-06	-	-	-	-
OPA-07	-	-	-	-
OPA-08	7	7	4(600,AnM4; 750,AnL3; 800,AnPM2; 1300,AnPM3)	42.85
OPA-09	6	6	2(800,AnR1; 900,AnL3)	33.33
OPA-10	7	7	2(1700,AnPM1; 2700,AnPM3)	28.57
OPA-11	5	5	2(600,AnL1; 3500,AnL3)	40.00
OPA-12	4	4	1(700,AnPM2)	25.00
OPA-13	5	5	2(1300,AnR4; 1700,AnPM2)	20.00
OPA-14	1	1	-	00.00
OPA-15	8	8	2(500,AnL3; 2700,AnPM3)	25.00
OPA-16	10	10	2(1800,AnR4; 2800,AnC2)	20.00
OPA-17	11	11	2(600,AnL3; 2500,AnR1)	18.18
OPA-18	9	9	2(300,AnL3; 3400,AnPM3)	22.22
OPA-19	-	-	-	-
OPA-20	-	-	-	-
Primer 01	13	13	1(700,AnPM2)	7.69
Primer 02	9	8	-	-
Primer 03	12	12	2(300,AnPM2; 1650,AnR2)	16.66
Primer 04	6	6	1(1500,AnPM1)	16.66
Primer 05	7	7	4(200,AnPM2; 650AnL3; 800,AnL3; 1500,AnL3)	57.14
Primer 06	5	4	2(400,AnL3; 500,AnL2)	40.00
Primer 07	5	5	-	-
Primer 08	7	7	-	-

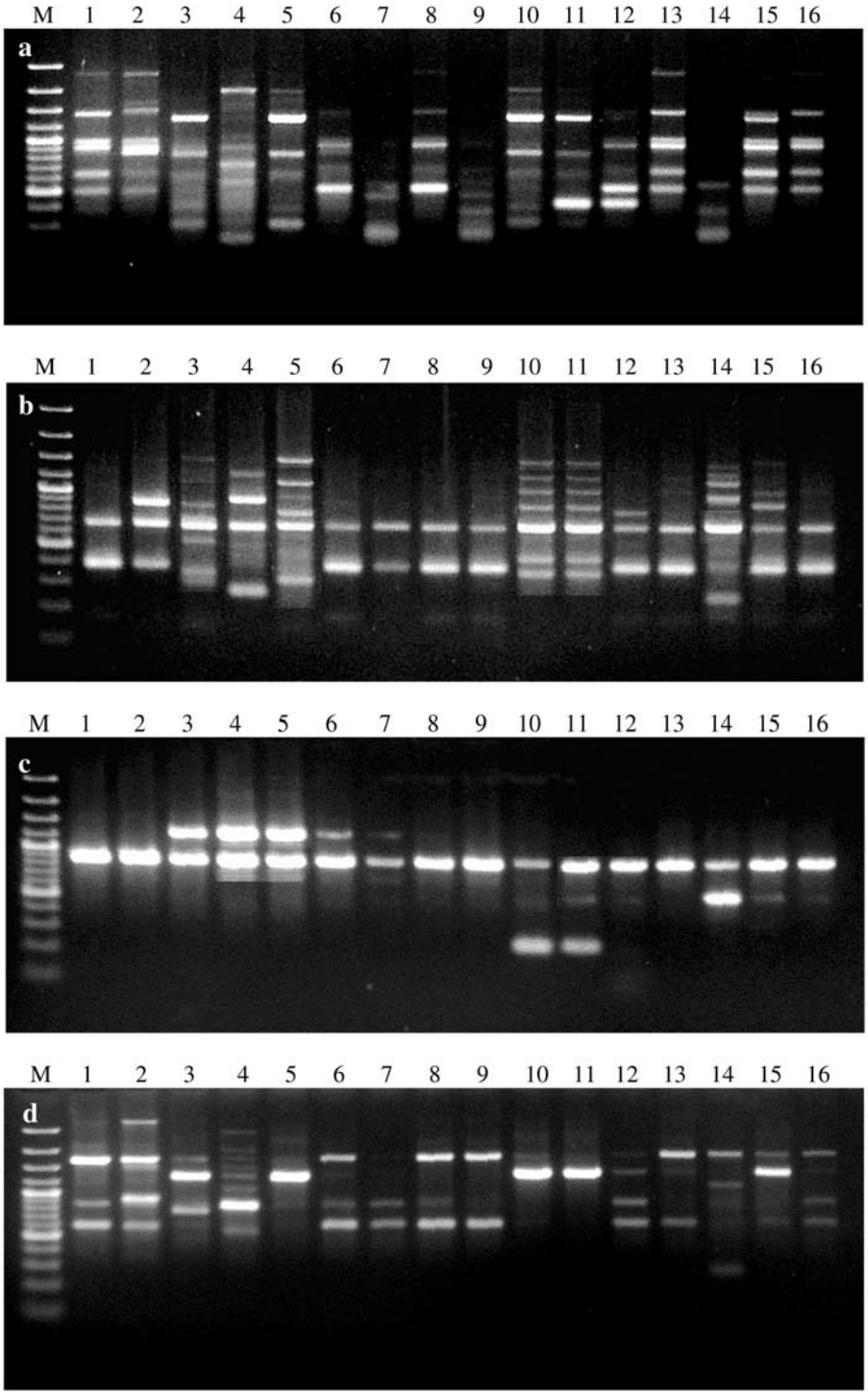

Fig. 1. RAPD profile of *Aspergillus niger* aggregate isolates obtained with various primers: primer 01 (a), primer 02 (b), primer 06 (c) and OPA 18 (d).

Lane M, 100 bp marker. Lane 1–16 different isolates of *Aspergillus niger* as: lane 1, AnPM1; lane 2, AnPM3; lane 3, AnPM4; lane 4, AnPM2; lane 5, AnC3; lane 6, AnC2; lane 7, AnC1; lane 8, AnR3; lane 9, AnR1; lane 10, AnR2; lane11, AnR4; lane12, AnL2; lane13, AnL1; lane14, AnL3; lane15, AnM4 and lane16, AnM1.

which was 7.2. DNA amplification patterns of the *A. niger* aggregate isolates as detected by some of the RAPD primers are shown in Fig. 1.

The percent similarity coefficients between the *A. niger* aggregate isolates are shown in Fig. 2. The highest similarity (0.791) was measured between isolate AnC2 and AnR3. A high degree of similarity (>70%) was also seen between AnPM1 and AnR3; AnPM3 and AnM1; AnC2, AnR1 and AnM1; AnR3 AnR1 and AnM1; AnR1 and AnM1; AnL1 and AnM4; and AnM4 and AnM1. The lowest similarity (0.173) was found between AnC2 and AnR2. Isolates AnC1 and AnL3; AnR1 and AnR2; AnR2 and AnL2; AnR4 and AnL3, AnM1 also showed considerable diversity (22%). Multivariate (cluster) analysis of genetic similarity data clustered the isolates into three main groups (group I, group II and group III) (Fig. 2). Group I consisted of 10 isolates with 53.5 to 79.1% genetic similarity and all these isolates were effective fungus suppressors (Fig. 2). Two isolates, AnC2 and AnR3, positioned themselves separately from any sub-cluster of this group, and of all the isolates these were the most effective in suppressing root rot (Table 2). Group II consisted of two isolates, AnPM2 and AnL3, with 37.7% similarity. Group III consisted of two subgroups of two isolates each, AnPM4 and AnC3, with a genetic similarity 58.0%, and AnR2 and AnR4, with a genetic similarity of 60.0%. Bootstrap analysis used to evaluate the degree of support for clusters within the dendrogram detected that of the 13 groups and subgroups classified, the coefficient of probability for reproducibility ranged from 80 to 90% in groups I and III, and from 60 to 29% for group II, all subgroups of group III, and also all subgroups of Group I except three subclusters: AnPM1 and AnPM3; AnR1 and AnR3, AnC2; AnM1, and AnR3, AnC2. These three subclusters of group I had a 20-30% coefficient of probability (Fig. 2). In the present study the RAPD analysis clearly showed the relatedness of all the isolates.

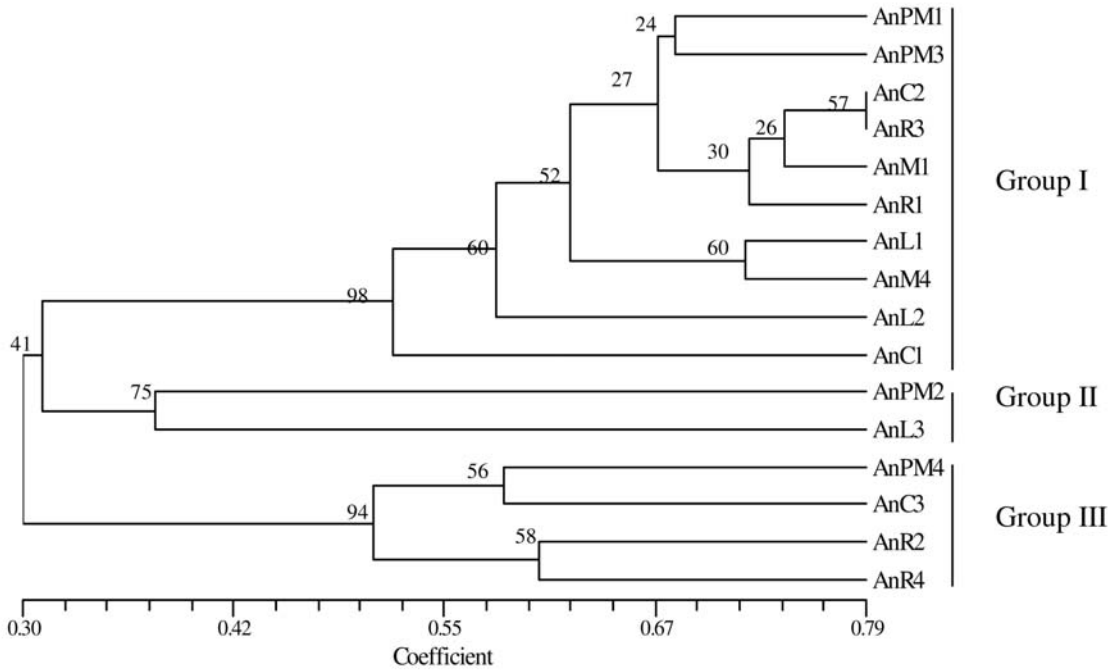

Fig. 2. Dendrogram of *Aspergillus niger* aggregate isolates, constructed using UPGMA with Jaccard's similarity Index based on 28 RAPD primers. Number at branch points indicate support for isolates clustered to the right of the number, values are percent of bootstrap sample that exhibit the cluster (no number at branch indicates support less than 10%). The major clusters are indicated on right margin. *Aspergillus niger* aggregate isolates were collected from different crop fields: AnPM1, AnPM2, AnPM3, AnPM4 from pearl millet; AnC1, AnC2, AnC3 from cauliflower; AnR1, AnR2, AnR3, AnR4 from rice; AnL1, AnL2, AnL3 from lentil and AnM1, AnM4 from mustard.

Table 2. Root rot and yield of eggplant treated with *Rhizoctonia solani* or a combination of *R. solani* and various *Aspergillus niger* isolates.

Treatment	Mean root rot index (0–5) [a]	No. of fruits plant^{-1} [a]
Rs (alone)	2.55	5.0
Rs+AnPM1	2.10^{c}	5.6^{b}
Rs+AnPM2	2.50	5.0
Rs+AnPM3	2.10^{c}	5.6^{b}
Rs+AnPM$_4$	2.45	5.4
Rs+AnC1	2.30^{d}	5.4
Rs+AnC2	0.50^{a}	6.4^{a}
Rs+AnC3	2.45	5.4
Rs+AnM1	1.90^{b}	6.0^{a}
Rs+AnM4	2.10^{c}	5.6^{b}
Rs+AnR1	2.00^{b}	5.6^{b}
Rs+AnR2	2.45	5.4
Rs+AnR3	0.60^{a}	6.4^{a}
Rs+AnR4	2.45	5.4
Rs+AnL1	2.10^{c}	5.6^{b}
Rs+AnL2	2.30^{d}	5.4
Rs+AnL3	2.50	5.0
LSD (P≤0.05)	0.109	0.45

[a] Each value is the mean of five replicates. Rs, *Rhizoctonia solani*, An, *Aspergillus niger*. Significant means followed by different superscript letters in the same column are different from each other (P≤0.05).

Biochemical characters of *A. niger* aggregate isolates

None of the isolates produced ochratoxin A (<1 ng g^{-1}), hydrogen sulphide or indole acetic acid. All isolates produced ammonia, siderophores (greatest amounts by AnC2 and AnR3, followed by AnM1, AnM4 and AnR1) and solubilized phosphates (greatest amounts by AnC2, AnR3, AnM1 and AnR1). Isolates AnC2, AnR3 and AnM1 produced hydrogen cyanide (Table 3).

Antagonism against *Rhizoctonia solani*, yield and soil population

In the dual-culture tests, no isolates overgrew *R. solani* for the first 6 or 7 days after inoculation. *R. solani* was overgrown by AnC2 and AnR3 after 8 days, by AnM1, AnM4, AnR1 and AnL1 after 9 days and by the remaining isolates after 10 days (Table 3). Eggplant cv. Pusa Kranti was quite susceptible to infection by *R. solani* and exhibited root rot lesions measuring 2.55 on the 0-5 scale (Table 2). A few *A. niger* aggregate isolates suppressed *R. solani* infection, leading to significantly lower root rot indices and they also significantly increased eggplant yield, especially AnC2 and AnR3, followed by AnM1, AnR1, AnPM1, AnPM3, AnM4, AnL1, AnC1 and AnL2 (Table 2).

The soil population of *R. solani* (CFU g^{-1} soil) drastically increased over time when *A. niger* aggregate isolates were absent, but when *A. niger* aggregate isolates were present, the *R. solani* population decreased to varying degrees. The population of *A. niger* aggregate isolates, however, increased and was greater in those treatments where the population of *R. solani* decreased. The greatest decrease in the population of *R. solani* with a corresponding increase in *A. niger* aggregate, was achieved with isolates AnC2 (*R. solani* population decreased from 17.6×10^6 to 1.1×10^2 CFUs, *A. niger* aggregate population increased from 10.7×10^4 to 6.8×10^8 CFUs) and AnR3 (*R. solani* population decreased from 17.6×10^6 to 1.1×10^2 CFUs, *A. niger* aggregate population increased from 10.5×10^4 to 4.5×10^8 CFUs), followed by AnM1, AnR1, AnM4, AnL1, AnL2 and AnC1 in pots inoculated with *R. solani*.

Discussion

The sixteen *A. niger* aggregate isolates collected from different fields/crops showed considerable genetic variation with the 28 RAPD decamer primers. Variation ranged from 21% (between isolates AnC2 and AnR3) to 83% (between AnC2 and AnR2). Pekarek *et al.* (2006) reported 12–78% molecular variation among 89 isolates of *A. niger* aggregate using 31 RAPD markers. *A. niger* is classified as an asexual deuteromycetes (Raper and Fennel, 1965; Fennell, 1977). The findings suggest that many asexual fungi have higher than expected levels of genetic variation and are able to bring about genetic exchange in some way (Pekarek *et al*., 2006). The rate of mitotic recombination (crossing over and haploidization) of *A. niger* was calculated to be 100 times higher than the meiotic crossing over in the sexual species *A. nidulans* (Lhoas, 1967). The parasexual behavior of *A. niger* aggregate may account for the extensive genetic variation of the isolates (Debets, 1998). Some studies have shown experimentally that parasexuality in *A. niger* aggregate occurs under controlled

Table 3. Some biochemical characters of *Aspergillus niger* isolates and *in vitro* antagonism against *Rhizoctonia solani*. Each value is the mean of five replicates.

A. niger isolate	Phosphate solubilization[a]	Range of OTA[b] (ngg^{-1})	Production of biochemical compound					*In vitro* dual culture antagonism tests 6, 7, 8, 9 and 10 days after inoculation[d]				
			H_2S	Ammonia	HCN	Siderophore [c]	IAA	6	7	8	9	10
AnPM1	++	0	-	+	-	+ + +	-	+	+	++	+++	(+++)
AnPM2	++	0.3	-	+	-	+ +	-	+	++	++	+++	(+++)
AnPM3	++	0	-	+	-	+ + +	-	+	+	++	+++	(+++)
AnPM4	++	0.4	-	+	-	+ +	-	+	+	++	+++	(+++)
AnC1	++	0	-	+	-	+ + +	-	+	+	++	+++	(+++)
AnC2	+++	0	-	+	+	+ + + + +	-	++	+++	(+++)	(+++)	(+++)
AnC3	++	0.4	-	+	-	+ +	-	+	++	++	+++	(+++)
AnM1	+++	0	-	+	+	+ + + +	-	+	++	+++	(+++)	(+++)
AnM4	++	0	-	+	-	+ + + +	-	+	++	+++	(+++)	(+++)
AnR1	+++	0	-	+	-	+ + + +	-	+	++	+++	(+++)	(+++)
AnR2	++	0.3	-	+	-	+ +	-	+	+	++	+++	(+++)
AnR3	+++	0	-	+	+	+ + + + +	-	++	+++	(+++)	(+++)	(+++)
AnR4	++	0	-	+	-	+ +	-	+	++	++	+++	(+++)
AnL1	++	0	-	+	-	+ + +	-	+	++	+++	(+++)	(+++)
AnL2	++	0	-	+	-	+ + +	-	+	++	++	+++	(+++)
AnL3	++	0.4	-	+	-	+ +	-	+	++	++	+++	(+++)

[a] Width of halo: + <10mm, ++ 10–20mm, +++ >20mm.
[b] OTA, ochratoxin A.
[c] Width of yellow halo around the colony.
[d] +, visible growth of both fungi; ++, visible contact between fungi; +++, visible inhibition of *R. solani*; (+++) *A. niger* isolate overgrowing the entire Petri dish.

field conditions (Zeigler *et al.,* 1997; Souza-Paccola *et al.,* 2003). Genetic variation through parasexualism may be equal to or greater than that occurring with sexual reproduction (Agrios, 2005). On the basis of the genetic variation found among the 16 isolates, three groups were differentiated by RAPD markers, and some amplicons were identified as isolate-specific. This indicated that the RAPD test distinguished between the isolates of *A. niger* aggregate. This RAPD technique has also been successfully used to determine genetic variability and relatedness in the complex group of black Aspergilli (Megnegneau *et al*., 1993) and their genetic variations, and to identify the human-pathogenic isolate of *A. fumigatus* (Aufauvre-Brown *et al.,* 1992) and the plant-pathogenic isolate of *Fusarium solani* f. sp. *cucurbitae* (Crowhurst *et al.,*1991).

Biochemical tests, especially those to determine HCN and siderophore production and phosphate solubilization, also indicated variations between the *A. niger* aggregate isolates. The HCN-producing isolates AnC2, AnR3 and AnM1, belonging to group I, were differentiated by the RAPD test. These isolates also produced greater amounts of siderophore and solubilized phosphorus than the other isolates. This indicated that the genetic variability revealed by the RAPD test was authentic.

A pot trial to test the effectiveness of *A. niger* aggregate soil isolates against *R. solani* achieved varying results. Inoculation of eggplant with *R. solani* caused considerable root rot and loss of the lateral roots coupled with stunted growth and leaf chlorosis. These are the typical symptoms of root rot caused by *R. solani* (Lucas *et al*., 1993). A root rot index of 2.55 indicated that the inoculum of the fungus used was virulent and that the eggplant cv. Pusa Kranti was susceptible. Khan *et al*. (1994) reported that *R. solani* caused root rot with an index

value of 2.5–2.8 on various susceptible cultivars of cowpea. Soil application of *A. niger* aggregate isolates, especially AnC2 and AnR3, suppressed *R. solani* infection with a significant decline in the root rot index and the soil population of *R. solani,* and also a significantly increase in eggplant yield. *A. niger* aggregate isolates may have competed with *R. solani* in the soil or the root zone of eggplant by mycoparasitism and/or antibiosis. The dual-inoculation test with *R. solani* and *A. niger* aggregate isolates also indicated suppression of *R. solani* growth, especially with isolates AnC2, AnM1, AnM4, AnR1, AnR3 and AnL1. The antagonistic effect of *A. niger* aggregate on *R. solani* in dual culture has already been reported (Surulirajan and Kandhari, 2003). The inhibition operated by *A. niger* aggregate greatly decreased the soil population of *R. solani*, whereas the population of the *A. niger* aggregate isolates was correspondingly increased. This suggests that *A. niger* aggregate multiplied and sporulated efficiently in soil infested with *R. solani*. *A. niger* aggregate is a contact as well as an invasive necrotroph (Mondal and Sen, 1999; Abarca *et al.,* 2004) and grows and sporulates well on the mycelium of pathogenic fungi such as *R. solani* (Sen *et al.,* 1995). The production of NH_3, HCN and siderophore may also have contributed to the suppression of *R. solani* (Nair and Burke, 1988; Palakshappa *et al.,* 1989). *A. niger* aggregate isolates produced *in vitro* a prominent solubilization zone in Pikovskaya's medium. *A. niger* aggregate is an efficient phosphate solubilizer invariably found in natural soils (Gaur, 1990; Vassilev *et al.,* 1996). The greater availability of phosphorus to the eggplant may also have enhanced the tolerance of eggplant to infection with *R. solani* (Kirkpatrik *et al.,* 1964; François, 1984; Chadha, 2002). All isolates were ochratoxin A negative (<1 ng g^{-1}). The nephrotoxic and carcinogenic mycotoxin ochratoxin A was first reported in the black *Aspergillus* species by Ueno *et al*. (1991) on the species *A. foetidus*. This was later confirmed by Teren *et al*. (1996) for another isolate of *A. foetidus*. Abarca *et al*. (1994) first reported that *A. niger* (var. *niger*) produced ochratoxin A, and this was later confirmed by Teren *et al*. (1996). However, as mentioned by Varga *et al*. (2000) only some 3 to 6% (Leong, 2005) of *A. niger* aggregate isolates produce ochratoxin A.

The study demonstrated that the RAPD test and the construction of a genetic similarity dendrogram correctly identified genetic variations between *A. niger* aggregate isolates, dividing them into three groups. The greatest amount of HCN and NH_3 produced, of phosphate solubilization, of suppression of *R. solani*, and of root rot control and the greatest increase in fruit yield all occurred with isolates AnC2 and AnR3. Both these isolates had a 79% genetic similarity and belonged to the same subgroup of group I. All the isolates of group I were effective suppressors of *R. solani* at $P \leq 0.05$, whereas none of the isolates from the other two groups suppressed *R. solani*. This suggests that the RAPD technique is a reliable means to identify effective disease-suppressing and/or growth-promoting isolates of *A. niger* aggregate.

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