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Testing of biocontrol efficacy of *T. harzianum* against virulent isolate of *A. niger*

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Abstract
Among four isolates of *A. niger* viz., AN-I, AN-II, AN-III and AN-IV, the isolate AN-IV was found to be more virulent which gave higher mycelial growth after 48 hrs. (34.00 mm) in *in vitro* condition and caused much disease incidence (50.00%) under pot culture experiment. Protein molecular weights of isolates were estimated through SDS-PAGE. Isolate AN-III and AN-IV showed maximum three protein bands having molecular weight ranging from 29 kDa to 200 kDa. However, biocontrol efficacy of *T. harzianum* on virulent isolates of *A. niger* (AN-IV) showed effective inhibition in radial growth of *A. niger*, increased seed germination (91.43%) and caused least disease incidence (8.57%).

Keywords: Virulent isolate, *Aspergillus niger*, *Trichoderma harzianum*

Introduction
Groundnut (*Arachis hypogaea* L.*)* is an annual legume crop. It is world's largest source of edible oil and ranks 13th among the food crops as well as 4th most important oilseed crop of the world (Ramanathan, 2001) [13]. The seed (kernels) contains up to 50 % of a non drying oil, 40-50% fat, 20-50 % protein and 10-20 % carbohydrate (Mehta, 2002) [9]. *Aspergillus niger* Van Tieghem is an important pathogen causing seed rot and seedling blight. It occurs regularly in the cultivated crop due to its seed and soil borne nature. It may cause crop losses to the tune of 40 to 50% in terms of mortality of plants known as collar rot (Chohan, 1969) [2]. In Gujarat, Joshi (1969) reported 50% seedling blight in infected fields. The infection induces pre-emergence seed rot & seedling blight and post-emergence collar rot caused by *A. niger* (Misra and Ghewande, 1983) [11]. Different isolates of *A. niger* variable in degree of pathogenicity (Lashin *et al*., 1989). However, identification of variability within *A. niger* isolates by morphological characters is very difficult. Besides, biological control of plant diseases is gaining importance as an alternative means of crop disease management. *T. harzianum* was found to potential biocontrol agent that control several seed-soil borne plant pathogens (Rao and Sitaramaiah, 2000; Bhuneshwari and Rao, 2001; Kishore and Kulkarni, 2008) [14, 1, 6]. Keeping this in view, the present study attempts to identify the virulent isolate of *A. niger* and bio-efficacy of *T. harzianum* on virulent isolate.

Materials and Methods
Identification of virulent isolate of *A. niger* causing collar rot of groundnut
Groundnut seeds which failed to germinate and diseased seedlings were collected from farmers field and different groundnut experiment plot of AAU, Anand. The pathogen was isolated and pure culture of the pathogen periodically sub cultured and maintained.

In vitro studies on growth characteristics of the isolate of *A. niger*
Sterilized PDA i.e. 20 ml was poured aseptically in sterilized Petri plate of 90 mm diameter. Mycelial disc of (6 mm) seven days old actively growing culture of the four isolates of *A. niger* were cut separately with the help of sterilized cork borer and place on solidified PDA. The experiment was replicated for four times. All inoculated plates were incubated at 27 ± 1^0C temperature in Biological Oxygen Demand (BOD) incubator. The colony diameter of test pathogens were observed periodically and final observation was recorded after 24 and 48 hrs. of incubation.

In vivo* studies on collar rot incidence due to *A. niger

The pot culture experiment was carried out in glass house of Department of Plant Pathology at Anand Agricultural University, Anand. Different four isolates of *A. niger* were inoculated on seeds of groundnut (*A. niger* @ 10^7/ml). The observation on disease severity was recorded up to 50 days of inoculation. The virulent isolate of *A. niger* were ascertained on the basis of maximum disease incidence caused by the isolates.

Protein profiling of four isolates of *A. niger*

The protein content of isolates of *A. niger* was estimated by Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) method as described by Laemmli (1970) [8].

Materials

- **Stock Acrylamide Solution (30%)**
  Acrylamide 30% : 30 g
  Bisacrylamide 0.8% : 0.8 g
  Water : 100 ml

- **Separating Gel Buffer**
  1.5 M Tris-HCl : 18.16 g
  Water : 100 ml
  pH: 8.8

- **Stacking Gel Buffer**
  0.5 M Tris-HCl : 3 g
  Water : 50 ml
  pH: 6.8

- **Polymerizing Agents**
  a. Ammonium persulphate 10% (APS): (0.1 g / ml, prepared freshly before use)
  b. TEMED (N,N,N', N'-Tetramethylethylene diamine): (Fresh from the refrigerator)

- **Electrode Buffer**
  0.05 M Tris : 12 g
  0.192 M Glycine : 28.8 g
  0.1% SDS : 2 g
  Water: 2 lit.
  pH: 8.2 – 8.4. NO adjustment required (May be used 2 – 3 times)
  Sodium Dodecyl Sulphate 10% Solution: (Stored at room temperature).

- **Sample Buffer**
  0.1M Phosphate buffer: 0.2 ml
  Mercaptaethanol: 0.1 ml
  Sample: 100 mg mycelia. Centrifuged for 15 min. at 10 x 1000 rpm. Collected 0.1 ml supernatant. Added 5 µl 10% SDS & boiled it for 2 – 4 min.

- **Protein Stain Solution**
  Coomassie brilliant blue R250: 0.1 g
  Methanol : 40 ml
  Acetic acid : 10 ml
  Water : 50 ml
  First, dissolved the dye in methanol and proceed. Used fresh preparation every time.
  Destainer: As above without the dye.

Method

1. Thoroughly cleaned and dried the glass plates and spacers, then assembled them properly. Hold the assembly together with bulldog clips. Clamped in an upright position. While petroleum jelly or 2% agar is then applied around the edges of the spacers to hold them in place and seal the chamber between the glass plates.
2. Prepared a sufficient volume of separating gel mixture (30 ml for a chamber of 18 x 9 x 0.1 cm by mixing the following)

   Separating Gel (10%)
   Acrylamide 30 % : 10 ml
   Tris – HCl 8.8 pH : 5 ml
   10% SDS : 300 µl
   Distilled water : 15.7 ml
   APS : 205 µl
   TEMED : 20 µl
3. Mixed gently and carefully poured the gel solution in the chamber between the glass plates. Layered distilled water on top of the gel and leave to set for 30 – 60 min.
4. Prepared stacking gel (4 %) by mixing the following solution:

   Stacking Gel (4 %)
   Acrylamide : 3.1 ml
   Tris – HCl 6.8 pH : 2.5 ml
   10% SDS : 150 µl
   Distilled water : 14.1 ml
   APS : 300 µl
   TEMED : 20 µl
5. Removed the water from the top of the gel and washed with a little gel solution. Poured the stacking gel mixture, placed the comb in stacking gel and allowed the gel to set for 30 – 60 min.
6. After the stacking gel has polymerized, removed the comb without distorting the shape of well. Carefully installed the gel after removing the clips, agar etc. in the electrophoresis apparatus. Then filled it with electrode buffer and removed the trapped air bubbles at the bottom of the gel. Connected the cathode at the top and turn on the DC – power briefly to check the electrical circuit. The electrode buffer and the plates were kept cooled so that heat generated during the run is dissipated and do not affect the gel and resolution.
7. Took up the required volume from the prepared samples in a micro syringe and carefully injected it into a sample well through the electrode buffer. Marked the position of the well on the glass plates and the presence of bromophenol blue in the sample buffer which facilitated easy loading of the sample. Similarly, loaded a few wells with standard marker proteins in the sample buffer.
8. Turned on the current to 10 – 15 mA for initial 10 – 15 min. until the sample travel through the stacking gel. The stacking gel helped concentrations of the samples. Then the run was continue at 30 mA until the bromophenol blue reaches the bottom of the gel which took about 3 hrs.
9. After the run is complete, carefully removed the gel inserted between the plates and immersed in staining solution for at least 3 hr. or overnight with uniform shaking so that the proteins absorb the coomassie brilliant blue.
10. Transferred the gel to a suitable container with at least 200 – 300 ml destaining solution and shaked gently and

continuously. Dye that is not bound to proteins thus removed. Changed the destainer frequently, particularly during initial periods, until the background of the gel was colorless. As the proteins of minute quantities are stained frequently, de-staining process was stopped at appropriate stage to visualize as many bands as possible.

Efficacy of *T. harzianum* against virulent isolate of *A. niger*

***In vitro* studies**

Effect of *T. harzianum* was evaluated by dual culture technique against virulent strain of *A. niger*.

Observation

$$\text{Percent growth inhibition} = \frac{\text{Growth of } A.\ niger \text{ - Growth of } A.\ niger \text{ in In control plate presence of } T.\ harzianum}{\text{Growth of } A.\ niger \text{ in control plate}} \times 100$$

***In vivo* studies**

One hour before sowing, surface sterilized groundnut seeds were soaked in spore suspension of *A. niger* @ 10 ml/ 30 seeds for five minutes. The air dried seeds were again treated with conidial suspension of bioagent @ 30 ml/ kg of seeds in an Erlenmeyer flask and shaken well to get uniform coverage of the bioagent on seeds. The seeds were air dried and sown in earthen pots (30 cm dia.) containing sterilized soil. Seven seeds were sown per pot for each replication and five replications were maintained for each treatment. Seeds inoculated with *A. niger* alone, and uninoculated groundnut seeds served as inoculated and uninoculated controls, respectively. Per cent seed germination and collar rot incidence were calculated.

Results and Discussion

Identification of virulent isolates of *A. niger* causing collar rot of groundnut

To find out virulent isolates of *A. niger*, different isolates collected from farmers field and experimental plots were tested for mycelial growth in *in vitro* condition to ascertain their cultural characteristics on the basis of growth characteristics.

In vitro* studies on growth characteristics of the isolate of *A. niger

All the four isolates of *A. niger* were found significantly different for their mycelial growth (Table 1). The maximum mycelial growth was recorded in isolate AN-IV (34.00 mm) after 48 hrs. of incubation at 27°C. It was followed by AN-II (28.8 mm), AN-III (21.3 mm) and AN-I (18.0 mm).

In vivo* studies on collar rot incidence due to *A. niger

The four isolates of the pathogen was inoculated on seeds and the observation on collar rot incidence were recorded up to 50 days of sowing (Table 1, Figure 1). The result revealed that the four isolates of *A. niger* were found significantly different in causing the disease. Isolate AN-IV was found to cause maximum per cent disease incidence (50.00%) under pot culture study. It was followed by isolate AN-III and AN-II which caused 39.29 and 32.14 per cent disease incidence, respectively. Whereas, least disease incidence was recorded in isolate AN-I (21.43%) which indicated to be less virulent in degree of pathogenicity.

Table 1: Disease incidence and mycelial growth of four isolates of *A. niger*

A. niger Isolates	Disease incidence (%)*	Mycelial growth (mm)	
		After 24 hrs.	After 48 hrs.
AN - I	21.43 (27.25)	8.80	18.0
AN – II	32.14 (34.44)	12.5	28.8
AN – III	39.29 (38.73)	10.8	21.3
AN - IV	50.00 (44.98)	20.0	34.0
Control	00.00 (1.28)	--	--
S. Em. ±	2.16	0.42	0.76
C. D. (0.05)	6.51	1.30	2.33
C. V. %	14.72	6.47	5.94

* Figures in parentheses are Arcsine transformed values.

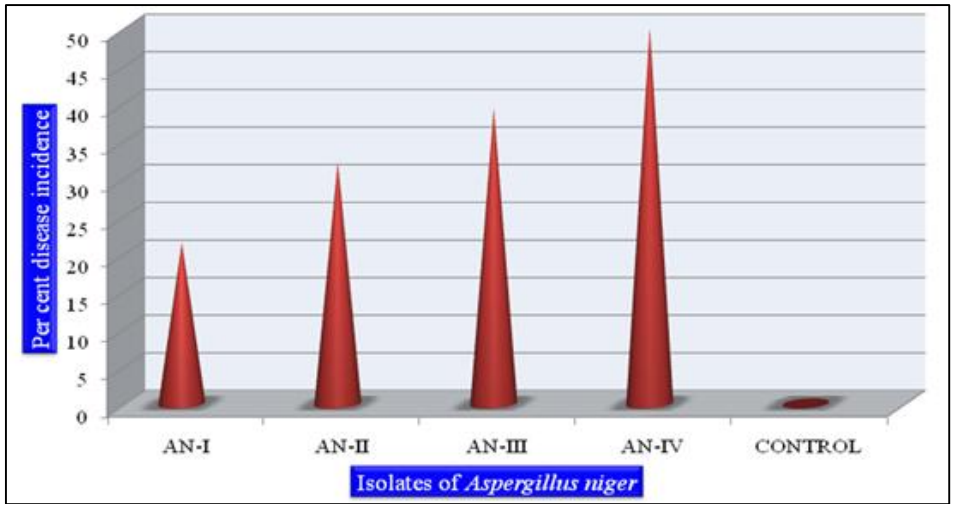

Fig 1: Per cent disease incidence caused by four isolates of *A. niger*

Thus, the results of present finding revealed that virulent isolate (AN-IV) have fastest growing nature (34.00 mm) after 48 hrs. as well much disease causing ability (50.00%).

The results of present investigation corroborate with the result reported by Lahsin *et al.*, (1989) [7], who found, isolates A_1, A_2 and A_3 of *A. niger* causing 60, 45 and 30 per cent collar rot of peanut, respectively. However, several workers also identify the virulent isolates of *A. niger* on the basis of degree of pathogenecity (Mishra and Raj, 1992; Shin Hyeongkwon *et al.*, 1996) [10, 15].

Protein profiling of the four isolates of *A. niger*

Four isolates of *A. niger* viz., AN-I, AN-II, AN-III and AN-IV were exposed for estimation of molecular weight of

protein through SDS-PAGE. These isolates differed in protein profiling estimated through SDS-PAGE (Plate-1, Table 2). The molecular weight of protein in the isolate ranged from 29 to200 kDa.

Among four isolates tested AN-III and AN-IV were recorded with maximum of three protein bands (Plate-1, well No. 3 & 4). Band one of 29 kDa to 67.0 kDa molecular weight, band two of 90.0 to 166.0 kDa and band three of 166.0 to 200.0 kDa. It was followed by isolate AN-II having two protein bands. One protein bands of higher molecular weight between 90 to 166 kDa and other having low molecular weight between 29 to 67 kDa. Whereas, only one protein bands of 29 to 67 kDa molecular weight was obtained in case of isolate AN-I.

It is clear from the result that much virulent isolates (AN-IV) have more protein bands with high molecular weight and caused maximum disease incidence i.e. 50.00 per cent. Further, these protein bands may act as a molecular marker for identifying the isolates of *A. niger* possessing higher virulency to cause disease.

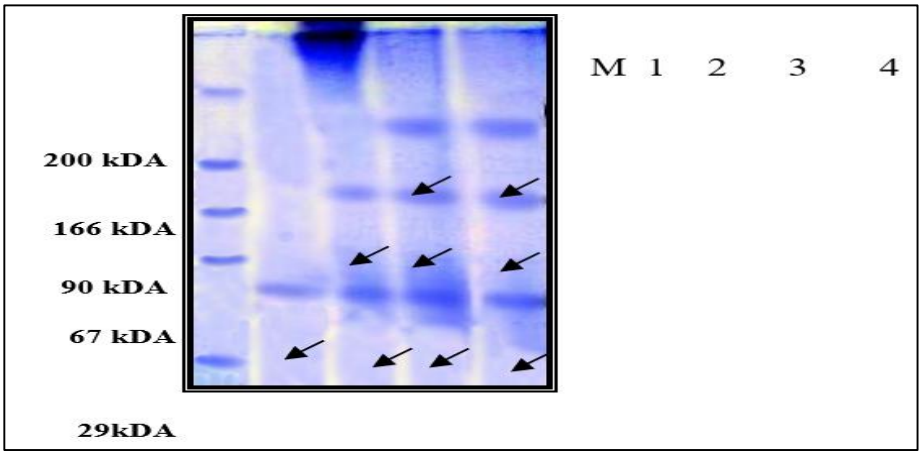

Fig 1: Protein profiling of four isolate of *A. niger*

M = Molecular weight ranging from 29 to 200 kDA
1 = *A. niger* isolate I (AN-I)
2 = *A. niger* isolate II (AN - II)
3 = *A. niger* isolate III (AN - III)
4 = *A. niger* isolate IV (AN - IV**)**

Table 2: Protein profile of four isolates of *A. niger* as studied by SDS – PAGE

S. No	*Niger* Isolates	No. of protein bands of different molecular weights in *A. niger* isolates				Total
		29.0-67.0 kDa	67.0-90.0 kDa	90.0-166.0 kDa	166.0-200.0 kDa	
1	AN-I	1	-	-	-	1
2	AN-II	1	-	1	-	2
3	AN-III	1	-	1	1	3
4	AN-IV	1	-	1	1	3

Different workers have found that *A. niger* produces extracellular enzymes. The molecular weight of xylanase from *A. niger* was found to be of 36 kDa (Coral *et al*., 2002a). Whereas, celllullase was found to be of 83 and 50 kDa (Coral *et al*., 2002b).

The above results indicated that the virulent strains AN-III & AN-IV of *A. niger* might be possessing the enzymes and toxin of higher molecular weight which are used for pathogenicity.

Testing of bio-control efficacy of *T. harzianum* against virulent strain of *A. niger* (AN-IV)

The pathogen showed 52.00 mm mycelial growth and 42.22 per cent growth inhibition in dual culture with *T. harzianum*. The bio-agent effectively checked the growth of the pathogen by covering more area than the pathogen.

In a study Sukanta and Raj (1998) [16] reported that in dual culture, *T. harzianum* inhibited the growth of two virulent isolates i.e. soil isolate 16 and seed isolate 24 of *A. niger* by 63 and 58 per cent respectively after 72 hrs of inoculation.

The results of the experiment carried out under pot study in glasshouse condition revealed that among three different treatments, seed treatment with *A. niger* along with *T. harzianum* showed 8.57 per cent disease incidence as well as higher seed germination (91.43%) (Table 3). Whereas, higher disease incidence was observed in seed treatment with pathogen alone (45.72%) along with 68.57 per cent seed germination.

Table 3: Effect of seed treatment with *T. harzianum* against collar rot in groundnut under pot culture conditions.

Sr. No	Treatments	Per cent seed germination	Per cent seed rot & collar rot incidence
1	*A. niger* + *T. harzianum*	91.43	8.57 (13.83)*
2	Inoculated control (*A. niger* alone)	68.57	45.72 (44.16)
3	Uninoculated control (Without *A. niger* & No biocontrol agent)	88.57	0.0 (1.28)
S. Em. ±		4.88	3.177
C. D. (0.05)		14.20	9.791
C. V. %		8.33	35.96

*Figures in parentheses are Arcsine transformed values.

** Disease incidence decreased over Inoculated control is 81.26 per cent.

The results are in conformity with the reports of Raju and Murthy (2000) [12], who found similar results that seed treatment with *T. harzianum* resulted in increase of seed germination and reduction in collar rot infection.

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