Antifungal activity of *Azotobacter nigrificans* against trichothecene-producing *Fusarium* species associated with cereals

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Abstract Antifungal efficacy of *Azotobacter nigrificans* on *Fusarium* infection, total seedlings mass, root and shoot length, and seed germination in maize, sorghum, and wheat were investigated. Antifungal efficacy of *A. nigrificans* against *Fusarium sporotrichioides*, *Fusarium graminearum*, *Fusarium poae* and *Fusarium equiseti* showed a significant reduction in growth and *Fusarium* infection incidence (up to 50%) in all the three treated cereals. However, challenge inoculation of *Fusarium* spp. to the three cereals showed 100% infection incidence. Total mass of the maize seedlings increased two fold by *A. nigrificans* treatment; however, only a slight increase was observed in sorghum and wheat seedlings. The highest vigour index recorded in maize was 1321 against *Fusarium crookwellense*, 1616.71 against *Fusarium sporotrichioides* in sorghum, and 1584.8 against *Fusarium acuminatum* in wheat treated with *A. nigrificans*. Highest germination incidence of 64% was in maize, 67% in sorghum, and 56% in wheat treated with *A. nigrificans*.

Keywords: *Azotobacter*, biocontrol, cereals, *Fusarium*, trichothecene

Introduction

Colonization of cereals by trichothecene-producing *Fusarium* species leads to economic losses and deteriorates food quality (1). Their colonization also implicates potential human and animal health risks (2). *Fusarium* species colonization is reported in a variety of cereal grains such as maize, sorghum, rice, oats, wheat, and millets, causing root, stem, and ear rot. Therefore, severe reduction in crop yield is observed, which is often estimated between 10 and 30% of the yield (3). Different management strategies have been developed for the control of this mycotoxigenic *Fusarium* spp. Over the past several years, research has clearly indicated that many microorganisms can be used as potential biocontrol agents (4,5).

Biocontrol of pathogens using microorganisms, especially, to suppress fungal colonization offers a powerful alternative to synthetic chemicals. Continuous use of chemical fertilizers disrupts soil ecology and environment; degrades soil fertility, which leads to harmful effects on human and animal health (6); and contaminates ground water resources (7). The use of chemical fertilizers and pesticides to control plant diseases and pathogens has encountered many problems such as the development of pathogen resistance to pesticides and inability of seed-treated pesticides to protect the roots of crop plants.

Rapid degradation of these chemicals and also requirement for repeated applications of hazardous fungicidal treatment to seed, seedlings, or soils have given impetus to search for alternative remedies (8). Hence, there is an increased concern among pathologists, ecologists, and consumers to switch over to safer alternative disease management and plant protection methods (9). Thus, biological control using antagonistic microbes has been given more stress in recent years as it is sustainable, safe, and environment friendly (10).

Among the different microorganisms used as biocontrol agents, the plant growth promoting rhizobacteria (PGPR) such as *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, and *Azotobacter* species have been used to reduce fungal colonization (11,4,5). Different PGPR strains have been used as potential biocontrol agents to control mycotoxigenic *Fusarium* species. However, there are limited studies conducted to control trichothecene-producing *Fusarium* species associated with maize, sorghum, and wheat by using PGPR strains.

The main goals of the present study were (I) to investigate the efficacy of *A. nigrificans* to control ten different trichothecene-producing *Fusarium* spp. such as *Fusarium sporotrichioides*, *F. crookwellense*, *F. graminearum*, *F. poae*, *F. sambucinum*, *F. culmorum*, *F. acuminatum*, *F. avenaceum*, *F. nivale*, and *F. equiseti*; (II) to evaluate the efficacy of *A. nigrificans* to promote the total mass of the

seedlings, root and shoot length, and seed germination (vigour index (VI)) in maize, sorghum, and wheat (III); and to evaluate the efficiency of *A. nigrkans* in rhizoplane colonization in the three cereals treated with different *Fusarium* spp.

Materials and Methods

Strain details and maintenance The reference strains of trichothecene-producing *Fusarium* spp., namely, *Fusarium acuminatum* (NCBI GenBank Acc. No KJ371100), *F. avenaceum* (KJ371102), *F. crookwellense* (KJ371105), *F. culmorum* (KJ371104), *F. equiseti* (KJ371094), *F. graminearum* (KJ371099), *F. nivale* (KJ371097), *F. poae* (KJ371096), *F. sambucinum* (KJ371095), and *F. sporotrichioides* (KJ371098) were isolated from cereals collected from all over Karnataka state and all the *Fusarium* spp. were identified upto the species level by microscopic and molecular methods. Moreover, these strains have been used for antifungal assays. Further ability to produce different trichothecenes from these *Fusarium* spp. was also confirmed by thin layer chromatography and liquid chromatography and mass spectrometry (data not shown) (12).

Azotobacter nigrkans strain AZT 54 was isolated from paddy field soil samples. The strain was identified upto the species level by using cultural, microscopic, biochemical, and molecular methods. The identification of *Azotobacter nigrkans* strain AZT 54 was further confirmed with sequencing of the PCR products, and the sequence reads have been submitted at NCBI, USA (NCBI GenBank Acc. No JX262176) (13).

The test fungal and bacterial spp. were maintained in glycerol stock and were stored at 4°C. Fungal isolates were subcultured on potato dextrose agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India), and *A. nigrkans* were subcultured on Waksman selective media (Media composition: Mannitol: 10.0 g, CaCO₃: 5.0 g, K₂HPO₄: 0.5 g, MgSO₄: 0.2 g, NaCl: 0.2 g, MnSO₄: trace, FeCl₃: trace, Agar: 20 g, Distilled water: 1000 mL).

Bacterial and fungal spore preparation The spore suspension of *A. nigrkans* was standardized to 10⁹ CFU/mL of concentration by inoculating the fresh inoculum of *A. nigrkans* to 50 mL of Waksman broth and incubating for 24–48 h at room temperature. The bacterial cell pellet was harvested by centrifugation at 7,656x g for 15 min and resuspended in 0.1 M phosphate buffer solution, followed by serial dilution and haemocytometer studies.

Fungal spore suspension of all test *Fusarium* spp. was standardized to 10⁷ spores/mL of concentration by inoculating a 5 mm disk of freshly harvested *Fusarium* to PDA broth and incubating at 28°C for 5 days on an oscillatory shaker incubator at 100 rpm for 12 h. The spores were harvested using 5% NaCl and the concentration was adjusted using haemocytometer.

Bacterial and fungal seed treatments Freshly collected healthy

seeds of maize, sorghum, and wheat weighing approximately 100 g each were surface sterilized with 4% sodium hypochlorite and washed thrice with sterile distilled water to remove surface seed borne fungal pathogens. These seeds were subjected to four different treatments as detailed in (Table 1). Fungal and bacterial seed treatment (T2 and T4, respectively) was performed by taking each kind of seeds and soaking them separately for 2 h in 50 mL of fresh fungal/bacterial spore suspensions in 100 mL Erlenmeyer flasks and then incubating them at 28°C in incubator shaker at 100 rpm for uniform coating of test organisms. These seeds were used for the further studies. Combination of both bacterial and fungal treatment was performed by taking 25 mL of bacterial and 25 mL of fungal spore suspension. Moreover, mixed seeds and each kind of seeds were individually soaked for 2 h, and then, the same seeds were used for the further studies (T3). For control (T1) treatment, each kind of seeds were individually soaked for 2 h in 50 mL of sterile water.

Preliminary antifungal assay by dual culture technique Antifungal efficacy of *A. nigrkans* against *Fusarium* spp. was studied by observing the extent of inhibition of fungal mycelia by using a modified medium (Waksman media + Potato dextrose broth). Modified Waksman agar medium was prepared by adding 12 g of potato dextrose broth (HiMedia Laboratories Pvt. Ltd.) to 500 mL of Waksman agar and sterilized at 121°C for 20 min. This media was designed to facilitate the growth of both bacteria and fungi on same medium. *A. nigrkans* was horizontally streaked at the center of the plate touching two ends of the plate and incubated at 24±2°C for 42 h. After the development of bacterial colony over the media 5 mm of agar disk from freshly cultured *Fusarium* spp. was placed perpendicularly opposite on either side of the bacterial line and incubated at 28°C for 3 days. The zone of inhibition was recorded from the edge of the bacterial colony to the edge of fungal mycelia.

Effect of *A. nigrkans* on infection incidence of *Fusarium* spp. on maize, sorghum and wheat grains The efficacy of *A. nigrkans* to reduce the infection incidence of *Fusarium* spp. when artificially inoculated to cereals was tested using standard blotter method (SBM) as per the protocol described by the Mathur and Kongesdal (14). For control (T1) treatment, each seeds were individually soaked for 2 h in 50 mL of sterile water, and then, randomly selected 100 seeds were subjected to SBM. Fungal and bacterial seed treatment (T2 and T4, respectively) was performed by taking each kind of seeds that were individually soaked for 2 h in 50 mL of fresh fungal/bacterial spore suspensions. Combination of both bacterial and fungal treatment was performed by taking 25 mL of bacterial and 25 mL of fungal spore suspension. Moreover, mixed and each kind of seeds were individually soaked for 2 h, and then, the same seeds were used for SBM (T3). All the treatments were performed in separate 100-mL Erlenmeyer flasks and incubated at 28°C in incubator shaker at 100 rpm for uniform coating of test organisms. Subsequently, the randomly selected 100 seeds were subjected to

SBM. The assay was performed in triplicates to know the infection incidence of *Fusarium* spp.

Treated seeds were placed over moistened blotter discs in a sterilized Petri plate, incubated at $23\pm 2^{\circ}\text{C}$ for 5 days in 12 h of alternate photo periods, and observed for infection incidence by *Fusarium* spp.

Influence of *A. nigricans* treatment on some growth characteristics of maize, sorghum and wheat *In vitro* evaluation of the ability of *A. nigricans* to influence the plant growth properties such as seed germination, root and shoot length, and total increase in VI of each kind of grain seedlings under moist conditions was performed. Maize, sorghum, and wheat grains were surface sterilized with 4% sodium hypochlorite, washed thrice, and subjected to four different treatments (T1, T2, T3, and T4). The seed treatments were performed as per the protocol described above.

A total of 100 seeds were randomly treated from each treatment and were placed between two moist germination sheets of grade 1 of 31 cm (width)×45 cm (length). Moreover, the seeds were tightly rolled and incubated in growth chamber with maximum availability of sun light in inclined position for 7 days. Moisture was provided to germination sheets at maximum to avoid any drying of seedlings. Shoot and root lengths of seedlings were measured using a centimetre scale. Moreover, the weight of the total mass of the seedlings was performed by taking 10 seeds from randomly selected seedlings that underwent all the treatments and analyzing the total mass of the seedling by weighing them using a scientific weighing balance (BSA223S; Sartorius AG, Göttingen, Germany). The growth parameters of each seedling such as germination percent, root length, shoot length, and total mass of seedlings after germination were studied and the VI was calculated using the following formula (15).

Vigour index $\text{VI} = (\text{mean root length} + \text{mean shoot length}) \times \% \text{ germination}$.

Isolation of *A. nigricans* and *Fusarium* spp. from maize, sorghum, and wheat rhizoplane The isolation was conducted as per the protocol described by Pereira *et al.* (16) with slight modifications. Twenty seeds of each cereal grain were subjected to T1, T2, T3, and T4 treatments as mentioned in table 1. All the treated seeds were sown in germination trays comprising 50 cups of 5×5 cm each, filled with field soil and coco peat, and incubated in a growth chamber at 28°C for 20 days. The germinated seedlings were uprooted, and one gram of roots representing the entire rhizospheric region of root

system along with rhizosphere soil was used for this study. The root samples of each cereal seedlings were washed in 10 mL of sterile PBS for 15 min. Moreover, from this, 1 mL of the sterile PBS was serially diluted up to 10^6 dilution, and 100 μL was inoculated on Waksman agar plates for the isolation of *A. nigricans* incubated at 34°C for 24–48 h. For isolation of *Fusarium* spp. 100 μL of spore suspension was inoculated on to PDA and incubated at room temperature. The total counts of bacteria and fungi were noted and expressed as Log_{10} CFU/g for each root sample.

Statistical analysis Root and shoot lengths of each cereal sample were analysed by one-way analysis of variance using (SYSTAT-Sigma Stat for windows version 3.1, SPSS 2.0, SPSS Inc., Chicago, IL, USA), and the significance was determined at $p \leq 0.05$, with standard error. Other parameters such as germination index, infection incidence, VI, and efficiency in rhizoplane colonisation are represented using graph pad prism 5.0 high efficient software with standard error.

Results and Discussion

There are many studies conducted to determine the ability of *Fusarium* spp. causing devastating diseases to economically important crops such as wheat, rice, maize, and sorghum. Many of the *Fusarium* spp. are also shown to produce many mycotoxins such as trichothecenes, fumonisins, and ergosterol. In view of this, in the present study, the efficacy of *A. nigricans* to control the ten different trichothecene-producing *Fusarium* spp. such as *F. sporotrichioides*, *F. crookwellense*, *F. graminearum*, *F. poae*, *F. sambucinum*, *F. culmorum*, *F. acuminatum*, *F. avenaceum*, *F. nivale*, and *F. equiseti* was tested using dual culture technique. Preliminary antifungal assays showed that all the tested *Fusarium* spp. were effectively inhibited by *A. nigricans*. Among all the *Fusarium* spp., *F. sporotrichioides*, *F. graminearum*, *F. poae*, and *F. equiseti* showed the highest inhibition followed by *F. crookwellense*, *F. culmorum*, and *F. sambucinum*, while *F. acuminatum*, *F. avenaceum*, and *F. nivale* showed minimum inhibition (Table 2). There are reports showing the decrease in the count of *Fusarium* pathogens following treatments with PGPR bacterial strains. Cavaglieri *et al.* (17) screened several PGPR bacteria for the biocontrol efficacy against fumonisin producing *Fusarium verticillioides* and concluded that the *Azotobacter* spp. have more ability to control. Chauhan *et al.* (18) tested 51 *Azotobacter* isolates against *Fusarium oxysporum* and *Rhizoctonia* spp. in cotton, tomato, and guar. Pereira *et al.* (19) used *Bacillus* spp. for the control of

Table 1. Different seed treatment used

Treatment	Treatment type
T1	Sterile water
T2	Suspension of each <i>Fusarium</i> strain of approximately 10^7 spores/mL
T3	Suspension of each <i>Fusarium</i> strain of approximately 10^7 spores/mL (T2) along with <i>A. nigricans</i> strain of 10^9 CFU/mL
T4	<i>A. nigricans</i> strain of 10^9 CFU/mL

Table 2. Preliminary screening of *A. nigrkans* for their anti fusarial activity

Sl. No	Test organisms	Zone of Inhibition	Dual culture assay
1. A	<i>F. crookwellense</i>	++ ¹⁾
2. B	<i>F. sporotrichioides</i>	+++	
3. C	<i>F. culmorum</i>	++
4. D	<i>F. graminearum</i>	+++	
5. E	<i>F. acuminatum</i>	+
6. F	<i>F. poae</i>	+++	
7. G	<i>F. sambucinum</i>	++
8. H	<i>F. equiseti</i>	+++	
9. I	<i>F. nivali</i>	+
10. J	<i>F. avenaceum</i>	+	

¹⁾Good Inhibition (+++), Moderate inhibition: (++) , Less inhibition: (+)

fumonisin producing *Fusarium* spp. in maize agro ecosystems. Treatment of *Pseudomonas* reduced the growth of *Fusarium* spp. associated with maize (11). The present study targets the trichothecene-producing *Fusarium* spp. associated with maize, sorghum, and wheat, because there are only limited studies conducted. Dual culture assay showed that the known trichothecene producers such as *F. sporotrichioides*, *F. graminearum*, *F. poae*, and *F. equiseti* were significantly inhibited.

The infection incidence assay was performed to find out the ability of *A. nigrkans* to reduce the *Fusarium* spp. colonization when coated artificially to cereals and thus reduce the fungal infection on cereals. Results in the study revealed the significant decrease of infection incidence by *Fusarium* spp. in seeds treated with *A. nigrkans* (T3). The control T1 treatment of maize and sorghum showed 15% and 13.7% fungal colonization, respectively. Seeds with all the ten trichothecene-producing *Fusarium* spp. spores (T2) treatment to all the cereals showed 100% infection to all the *Fusarium* spp. The T3 treatment with *A. nigrkans* showed significant reduction of infection incidence in the three cereals tested. T3 treatment to maize, sorghum, and wheat showed the reduction of infection severity in the range of 31-50, 30-44, and 40-48.8%, respectively. As in case of

Fig. 1. Effect of *A. nigrkans* on infection incidence of *Fusarium* spp. artificially inoculated on cereal grains, (A) maize, (B), sorghum and (C) wheat.

T4 with only *A. nigrkans* treatment, less than 8% of infection incidence was recorded in all the seed samples (Fig. 1). The results presented here clearly demonstrate that *A. nigrkans* reduces the ability of *Fusarium* spp. to infect and colonize all the cereals tested. This may also decrease the possible trichothecene contamination problem associated with cereals as there is no initial fungal colonization. Several researchers have reported the high incidence of *Fusarium* spp. associated with maize and sorghum grains (20,21). González et al. (22) reported that the genus *Fusarium* spp. was the most prevalent species associated as a seed borne component with sorghum grain.

Along with growth promotion and reduction in infection incidence, positive influence of *A. nigrkans* treatment on maize, sorghum, and wheat on other parameters is also essential. Improvements in cereal crop plants growth and disease resistance covering a broad array of

Fig. 2. Root and shoot length of maize, sorghum, and wheat. (A) Measurement of root length of untreated seed samples. (B) Measurement of shoot length of untreated seed samples. (C) Measurement of root length of seed samples treated with *Fusarium* + *A. nigrkans*. (D) Measurement of shoot length of seed samples treated with *Fusarium* + *A. nigrkans* (T3) and (T4). R.L, root length; S.L, shoot length.

phytopathogens can be accomplished using PGPR strains of *Azotobacter* spp. (23-25). In the present study, the PGPR efficacy of *A. nigrkans* on maize, sorghum, and wheat was evaluated when treated with trichothecene-producing *Fusarium* species. The VI of maize, sorghum, and wheat had significantly increased with T3 treatment compared with that of T1 and T2 treatments. However, T4 treatment recorded three fold increases in its root and shoot lengths and the VI of all cereals tested. This may be due to PGPR activities of *A. nigrkans* and absence of pathogenic *Fusarium* spp. colonization (Fig. 2).

In T1 treatment, the VI of maize, sorghum, and wheat were 1186.8, 1572.5, and 2145.41, respectively. In T2 treatment, the VI of maize was 161.6-488.4, sorghum was 455.2-620.88, and wheat was 367.54-670.95. In T3 treatment, the highest VI reached by maize was 1299.48 when treated with *F. poae* and the lowest was 637 when treated with *F. culmorum*. In sorghum, the highest VI was 1616.71 when treated with *F. sporotrichioides* and the lowest was 1132.61 when treated with *F. avenaceum*. In wheat, the highest VI was 1582.16 when treated with *F. poae* and the lowest was 1232.11 when treated with *F. graminearum*. In T4 treatment maize, sorghum, and wheat showed a VI range of 3211-3705.91, 2521.8-2887.59, and 3495-4818.06, respectively (Fig. 3). The effect of *A. nigrkans* and trichothecene-producing *Fusarium* spp. on the total mass of seedlings was also evaluated. In T1 control treatment, maize, sorghum, and wheat had a total mass of 5.674, 2.148, and 2.216 g, respectively. In

T2 with *Fusarium* spores, the total mass of seedlings was 2.495-4.702 g for maize, 1.025-1.452 g for sorghum, and 0.015-1.126 g for wheat. In contrast, the T3 treatment with *A. nigrkans* showed significant increase in the total mass of seedlings that is 7.740-12.518 g in maize, 2.324-3.624 g in sorghum, and 2.484-2.808 g in wheat seedlings. As in T4 treatment, maize, sorghum, and wheat had the seedlings mass of 16.452, 8.462, and 6.532 g, respectively (Fig. 4). The results clearly draw the importance of *A. nigrkans* with a prominent attribute of PGPR properties, thus leading to increase in root and shoot lengths, the VI, and the total mass.

The effect of *A. nigrkans* and trichothecene-producing *Fusarium* spp. on germination incidence of maize, sorghum, and wheat was evaluated. In control T1 treatment, the germination percent of maize, sorghum, and wheat were 76, 85, and 86.3%, respectively. In T2 of only *Fusarium* spp. Treatment, all cereals showed nearly 55% reductions in germination. The T3 treatment with *A. nigrkans* showed significant increase in germination in all cereals that were up to 64% in maize, up to 66% in sorghum, and 56.1% in wheat. In case of T4 only *A. nigrkans* without any *Fusarium* pathogens treatment, germination had gone up to 98% (Fig. 5).

It was proved that *Azotobacter* spp. can positively influence plant growth (26,13). Significant increase in plant VI (length of shoot and root and weight of shoot and root), germination percent, and the total mass of the seedlings were observed by *A. nigrkans* (T4) in the present study. However, similar response was also observed in the

Fig. 3. Vigour index of cereal grains, (A) maize, (B) sorghum, and (C) wheat.

combination of *A. nigrkans* and *Fusarium* spp. (T3) treatment when compared to *Fusarium* alone without *A. nigrkans* application in (T2) treatment. Among three cereals tested for VI, sorghum has responded well to *A. nigrkans* application, followed by wheat and maize. As in case of the effect on total mass of the seedlings, maize showed better response than the sorghum and wheat in T4. Up to 98% of germination incidence was recorded in all the cereals. Similar type of results was observed by Biari *et al.* (26), where he reported that treatment of *Azospirillum* and *Azotobacter* significantly increased plant VI, germination index, and the total mass of the maize seedlings. Akhtar and Siddiqui (27) reported the treatment of *Bacillus* and *Pseudomonas* spp. for the control of *Fusarium* wilt on lentil plant. However, in his study, treatment with *Fusarium* spp. showed negative impact on the growth parameters of all cereals tested. These results are in accordance with the results obtained by Pereira *et al.* (16).

Fig. 4. Effect of *Azotobacter* on the total mass of (A) maize, (B) sorghum, and (C) wheat.

The concept of introducing PGPR bacterial strains into the rhizospheric region of maize, sorghum, and wheat was to provide resistance against trichothecene-producing *Fusarium* spp. before and after transplanting the plants in the field. Total fungal count obtained in *Fusarium* treated seeds of (T2) treatment was in the ratio of 8×10^7 CFU/mL. However, along with *A. nigrificans* (T3), there was a reduction up to 4×10^7 CFU/mL in fungal count. Total fungal count obtained in T3 treatment in each cereal was in the range of 2×10^7 CFU/mL to a maximum of 6×10^7 CFU/mL, and the least among all *Fusarium* spp. was *F. sporotrichioides* in maize and had 2×10^7 CFU/mL and 3×10^7 CFU/mL in sorghum and wheat, respectively, from rhizo plane soil. However, the total *A. nigrificans* count obtained in T4 treatment was 8×10^7 CFU/mL and either bacterial or fungal presence were not observed in control (T1) treatment of rhizoplane soil sample from all the cereals (Fig 6). Pereira *et al.* (16) reported that *F.*

Fig. 5. Effect of *Azotobacter* treatment on the germination incidence of cereal grains, (A) maize, (B) sorghum, and (C) wheat.

verticillioides count was significantly reduced at the rhizospheric and inner roots regions of maize. They also observed an increase in CFU of the PGPR strains. Our results are in agreement with the results obtained the Pereira *et al.* (16).

It is concluded that *A. nigrkans* is a potential biocontrol agent with an additional antifungal activity beside its plant growth promoting activity. It can be used successfully to control the potential mycotoxigenic *Fusarium* spp. associated with cereals. However, it is very necessary to validate the results further at the field level for large-scale application and to optimise the application methods.

Disclosure The authors declare no conflict of interest.

Fig. 6. Total count of bacteria and *Fusarium* spp. isolated from rhizoplane soil of cereal grains: (A) Maize, (B) Sorghum, and (C) Wheat. *Azt*, *A. nigrkans*; *F. spo*, *F. sporotrichioides*; *F. cro*, *F. crookwellense*; *F. gra*, *F. graminearum*; *F. poe*, *F. poae*; *F. sam*, *F. sambucinum*; *F. cul*, *F. culmorum*; *F. acu*, *F. acuminatum*; *F. ave*, *F. avenaceum*; *F. niv*, *F. nivale*; *F. equ*, *F. equiseti*

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