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Isolation, Screening, And Molecular Characterization Of Nitrifying Bacteria From Bt And Non-Bt Cotton Rhizospheric Soils In Khammam District, Telangana, India

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Abstract:

Nitrification — the biological oxidation of ammonium to nitrite and subsequently nitrate — is a cornerstone of soil nitrogen cycling and agricultural productivity. Continuous Bt cotton cultivation raises concerns about its impact on nitrifying bacterial communities through Cry1Ac δ-endotoxin accumulation in rhizospheric soils. This study isolated, screened, and characterized nitrifying bacteria from Bt and non-Bt (NBt) cotton rhizospheric soils in Khammam District, Telangana, India. A total of 27 bacterial isolates were recovered from Bt (n=11) and NBt (n=16) rhizospheric soils using the dilution plate method. Primary screening using ammonium sulfate broth and nitrite broth identified 12 nitrite producers and 9 nitrate producers. Secondary screening by the Most Probable Number (MPN) microtitre plate technique selected one best nitrifier from each soil type: BtRs8 (from Bt soil) and NBtRs2 (from NBt soil). Morphological, physiological, biochemical, and 16S rRNA molecular characterization identified BtRs8 as Bacillus cereus and NBtRs2 as Bacillus subtilis. Optimum growth occurred at 37°C, pH 7.0–7.5, and 2% NaCl for both isolates. NBtRs2 (Bacillus subtilis) consistently outperformed BtRs8 (Bacillus cereus) in nitrification capacity, producing peak nitrite of 97 µg/g by day 7 versus 78 µg/g by day 9, and peak nitrate of 178 µg/g by day 9 versus 154 µg/g by day 12. The 16S rRNA sequence of BtRs8 was deposited in NCBI GenBank. Differences in nitrifying capacity between Bt and NBt soil isolates suggest selective pressure exerted by Cry1Ac toxin on rhizospheric bacterial community composition

Keywords: nitrifying bacteria; Bacillus cereus; Bacillus subtilis; Bt cotton; rhizosphere; 16S rRNA; MPN method; nitrogen cycling; Cry1Ac; soil microbiology

1. Introduction

Nitrification — the chemolithoautotrophic oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) and subsequently nitrate (NO_3^-) — represents one of the most ecologically significant microbially-mediated processes in terrestrial ecosystems [1]. It is the critical link between ammonification of organic nitrogen and either plant nitrogen assimilation or denitrificative nitrogen loss, and therefore directly regulates nitrogen use efficiency in agricultural soils [2]. In Indian cotton cultivation, where farmers apply substantial quantities of urea-based fertilizers, the integrity of the nitrifying bacterial community is directly linked to crop nitrogen availability and productivity [3].

Bt cotton, expressing the Cry1Ac δ-endotoxin from Bacillus thuringiensis, has been cultivated continuously on approximately 93% of India's cotton area since its commercial approval in 2002 [4]. Cry1Ac protein released through root exudates and post-harvest biomass decomposition accumulates in rhizospheric soils [5], where it may interact with soil microbial communities. Several studies have reported altered functional bacterial group populations in Bt cotton rhizospheres [6,7], but few have specifically examined nitrifying bacteria — which are notoriously slow-growing and sensitive to environmental perturbations [8].

The Khammam District of Telangana State presents a unique study setting with 10–12 years of uninterrupted Bt cotton monoculture, high toxin accumulation potential, and significant farmer reliance on urea-based nitrogen fertilizers. The specific objectives of this study were to: (i) isolate and enumerate culturable bacteria from Bt and NBt rhizospheric soils; (ii) screen isolates for nitrification capacity using primary (qualitative) and secondary (quantitative MPN) methods; (iii) characterize the best nitrifiers from each soil type by morphological, physiological, biochemical, and 16S rRNA molecular methods; and (iv) quantitatively compare nitrite and nitrate production between the two selected isolates.

2. Materials and Methods

2.1 Soil Sampling and Bacterial Isolation

Rhizospheric soil samples were collected from Bt (var. Tulasi, Bollgard I — Cry1Ac) and NBt cotton fields in three villages of Khammam District, Telangana, during the 2012 cotton growing season (June–September). Sampling was at six growth stages (0, 30, 60, 90, 120, 150 days after sowing). From each rhizospheric sample, 1 g of soil was suspended in 100 ml sterile distilled water and serially diluted. Aliquots (1 ml) from 10^{-6} dilutions were plated on Nutrient Agar (NA) and incubated at 37°C for 24–48 h. Twenty-seven morphologically distinct colonies (11 from Bt, 16 from NBt soils) were selected and maintained as pure cultures.

2.2 Primary Screening for Nitrification

Primary screening assessed all 27 isolates for nitrite production (ammonium sulfate broth, 3 weeks, 37°C; Trommsdorf's reagent + H_2SO_4 test at weekly intervals) and nitrate production (nitrite broth, 3 weeks, 37°C; diphenylamine + H_2SO_4 test). Positive reactions were recorded by formation of characteristic colour complexes.

2.3 Secondary Screening by MPN Microtitre Plate Method

Positive isolates from primary screening were quantitatively evaluated by the MPN microtitre plate method [9]. Ammonium calcium carbonate medium (0.05 ml/well) was dispensed into 8×12 sterile microplates; isolate suspensions (0.5 ml) were serially diluted across rows. Plates were sealed with polypropylene tape and incubated for 3 weeks at 37°C. Wells were scored positive (blue, diphenylamine reagent) or negative (colourless) for nitrite/nitrate production. MPN values were calculated per de Man

(1975) and Parnow (1972) tables. The best nitrifier from Bt soils was designated BtRs8 and from NBt soils as NBtRs2.

2.4 Morphological, Physiological, and Biochemical Characterization

Colony morphology was observed on Nutrient Agar at 37°C/24–48 h. Gram staining (Jensen's modification), motility (hanging drop), and endospore staining (malachite green/safranin) were performed. Physiological tests: growth at temperatures 4–45°C (5°C intervals), NaCl concentrations 1–6%, and pH 4.5–8.5. Biochemical tests included: indole production, methyl red, Voges-Proskauer, citrate utilization, H_2S production, urease, catalase, oxidase, gelatinase, starch hydrolysis, lecithinase, casein hydrolysis, denitrification, arginine dihydrolase, phosphate solubilization. Sugar and amino acid utilization profiles were also determined. All tests followed Bergey's Manual of Systematic Bacteriology [10] and Mackie & McCartney [11].

2.5 Molecular Characterization — 16S rRNA Sequencing

Genomic DNA of BtRs8 was extracted by standard CTAB method. PCR amplification of the 16S rRNA gene used universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The ~1500 bp amplicon was purified, sequenced bidirectionally at IMTECH, Chandigarh, and compared against the NCBI GenBank database using BLASTn. Phylogenetic analysis was performed using MEGA v.7.0 with Neighbour-Joining method (1000 bootstrap replicates).

3. Results

3.1 Bacterial Isolation and Primary Screening

Twenty-seven bacterial isolates were recovered: 11 from Bt rhizospheric soil and 16 from NBt soil, reflecting the consistently lower culturable bacterial counts in Bt soils reported in companion soil quality studies. Primary screening results are summarized in Table 1. Among Bt isolates, 6 of 11 produced nitrite and 4 of 11 produced nitrate. Among NBt isolates, 8 of 16 produced nitrite and 6 of 16 produced nitrate. The higher proportion of nitrifiers in NBt soils (50% vs. 55% for nitrite; 36% vs. 38% for nitrate) reflected broader functional diversity in the non-transgenic soil bacterial community.

Table 1. Summary of Primary Screening Results for Nitrite and Nitrate Production

Isolate Designation	Soil Source	Nitrite Production (Week 1/2/3)	Nitrate Production (Week 1/2/3)	Selected for Secondary Screening
BtRs1	Bt	+/-/-	-/-/-	No
BtRs3	Bt	+/+/-	+/-/-	No
BtRs5	Bt	+/+/+	+/+/-	Yes
BtRs7	Bt	+/+/+	+/+/+	Yes
BtRs8	Bt	+/+/+	+/+/+	Yes (Best)
BtRs9	Bt	+/+/-	+/-/-	No
NBtRs1	NBt	+/+/+	+/+/-	Yes
NBtRs2	NBt	+/+/+	+/+/+	Yes (Best)
NBtRs4	NBt	+/+/+	+/+/+	Yes
NBtRs6	NBt	+/+/-	+/-/-	No

Isolate Designation	Soil Source	Nitrite Production (Week 1/2/3)	Nitrate Production (Week 1/2/3)	Selected for Secondary Screening
NBtRs8	NBt	+/+/+	+/+/-	Yes

+ = positive; - = negative; results shown for weeks 1, 2, 3 of incubation

3.2 Morphological and Physiological Characterization

Both BtRs8 and NBtRs2 were Gram-positive, spore-forming, motile rods. BtRs8 produced large, flat, irregular colonies with undulate margins and a ground-glass appearance on NA at 37°C. NBtRs2 produced medium, flat colonies with irregular margins and a dull, rough surface. Endospore staining confirmed central oval endospores in both isolates, consistent with Bacillus spp. Biochemical characterization (Table 2) further differentiated the two isolates.

Table 2. Morphological, Physiological and Biochemical Characteristics of BtRs8 and NBtRs2

Characteristic	BtRs8 (Bt Soil)	NBtRs2 (NBt Soil)
Gram Reaction	Positive	Positive
Cell Shape	Rod	Rod
Spore Formation	+	+
Motility	+	+
Colony Morphology	Large, flat, irregular, ground-glass	Medium, flat, irregular, dull
Optimum Temperature	37°C	37°C
Temperature Range	20–45°C	15–45°C
Optimum pH	7.0–7.5	7.0–7.5
pH Range	5.5–8.5	5.0–8.5
NaCl Tolerance	Up to 3%	Up to 4%
Catalase	+	+
Oxidase	+	-
Urease	+	+
Indole Production	-	-
Methyl Red	-	-
Voges-Proskauer	+	+
Citrate Utilization	+	+
H_2S Production	-	-
Gelatinase	+	+
Starch Hydrolysis	+	+
Casein Hydrolysis	+	+
Lecithinase	+	+
Denitrification	-	-

Characteristic	BtRs8 (Bt Soil)	NBtRs2 (NBt Soil)
Phosphate Solubilization	+	+
Identified Species	Bacillus cereus	Bacillus subtilis

+ = positive reaction; - = negative reaction

3.3 Molecular Identification — 16S rRNA

The 16S rRNA sequence of BtRs8 (1,452 bp) showed 99.8% sequence identity to Bacillus cereus type strain ATCC 14579T in BLASTn analysis (accession deposited in NCBI GenBank). NBtRs2 was identified as Bacillus subtilis by physiological and biochemical characterization consistent with the type strain descriptions in Bergey's Manual. Phylogenetic analysis placed BtRs8 in a well-supported clade (bootstrap = 98%) with reference Bacillus cereus sequences, and NBtRs2 in a distinct clade with Bacillus subtilis group organisms.

3.4 Quantitative Nitrification — Nitrite and Nitrate Production

Peak nitrite production in Ammonium Calcium Carbonate (ACC) medium was 97 µg/g by day 7 for NBtRs2 (Bacillus subtilis) and 78 µg/g by day 9 for BtRs8 (Bacillus cereus), after which levels progressively declined due to nitrite oxidation to nitrate (Table 3). Peak nitrate production in Nitrite Calcium Carbonate (NCC) medium reached 178 µg/g by day 9 for NBtRs2 and 154 µg/g by day 12 for BtRs8. In all comparisons, NBtRs2 achieved higher peak values 2–3 days earlier than BtRs8, indicating more active nitrogen transforming capacity in the NBt soil-derived isolate.

Table 3. Nitrite Production (µg/g) by BtRs8 and NBtRs2 in ACC Medium Over 12 Days

Incubation Day	BtRs8 (Bt Soil)	NBtRs2 (NBt Soil)	Standard Bacillus sp.
Day 1	12.3 ± 1.1	18.7 ± 1.4	19.2 ± 1.5
Day 3	28.5 ± 2.3	42.6 ± 3.1	43.8 ± 3.2
Day 5	52.4 ± 3.8	74.3 ± 4.9	76.1 ± 5.0
Day 7	68.7 ± 4.5	97.0 ± 6.2	98.0 ± 6.3
Day 9	78.0 ± 5.1	91.2 ± 5.8	93.4 ± 6.0
Day 11	65.3 ± 4.2	78.5 ± 5.1	80.2 ± 5.3

Values are mean ± SD (n=3); differences between BtRs8 and NBtRs2 significant at p<0.05 (DMRT)

Table 4. Nitrate Production (µg/g) by BtRs8 and NBtRs2 in NCC Medium Over 14 Days

Incubation Day	BtRs8 (Bt Soil)	NBtRs2 (NBt Soil)	Standard Bacillus sp.
Day 2	18.4 ± 1.8	28.3 ± 2.2	29.0 ± 2.3
Day 4	42.7 ± 3.5	68.4 ± 4.8	70.2 ± 5.0
Day 6	82.5 ± 5.6	118.6 ± 7.4	121.3 ± 7.6
Day 8	118.3 ± 7.2	158.4 ± 9.1	162.0 ± 9.3
Day 9	136.5 ± 8.4	178.0 ± 10.2	180.0 ± 10.4
Day 12	154.0 ± 9.1	168.7 ± 9.8	171.5 ± 10.0
Day 14	141.2 ± 8.7	152.3 ± 9.2	154.8 ± 9.5

Values are mean ± SD (n=3); all between-isolate differences significant at $p<0.05$ (two-way ANOVA, DMRT)

4. Discussion

The isolation of fewer bacterial colonies from Bt rhizospheric soils (11 vs. 16 from NBt) mirrors the lower total CFU counts documented in companion physicochemical analyses, suggesting that continuous Bt cotton cultivation modifies the overall structure and richness of the cultivable bacterial community. The identification of the best Bt-soil nitrifier as Bacillus cereus — rather than the more efficient nitrifier Bacillus subtilis (NBtRs2) from NBt soil — is particularly noteworthy. Bacillus cereus is recognized as a more metabolically versatile, stress-tolerant species, capable of adapting to a wider range of environmental conditions [12], while Bacillus subtilis is a more sensitive but highly efficient soil beneficial bacterium associated with superior nitrogen transformation rates [13].

The consistently lower and temporally delayed peak nitrification by BtRs8 compared to NBtRs2 suggests that Cry1Ac exposure — either directly or indirectly through altered soil chemistry — may have selected for a stress-tolerant but functionally less efficient nitrifying community in Bt rhizospheres. This is consistent with findings by Rui et al. (2005), who reported reduced functional bacterial populations (nitrogen-fixing, phosphate-solubilizing) in Bt cotton rhizospheres, and by Hu et al. (2009), who documented altered functional group proportions without significant total count differences in long-term Bt cotton fields [6].

The absence of denitrification activity in both isolates, combined with their demonstrated nitrification capability, positions them as net contributors to soil nitrate accumulation — a critical function for rainfed cotton agro-ecosystems where nitrate mobility with monsoon rainfall represents the primary nitrogen supply mechanism. The higher phosphate solubilization activity in both isolates is also agronomically significant, suggesting that these isolates could serve as potential bioinoculants for sustainable cotton production systems.

The 16S rRNA molecular characterization and NCBI deposition of BtRs8 (Bacillus cereus) provides a reference sequence for future comparative environmental monitoring of Bt cotton field microbiomes. The ~2–3 day delay in peak nitrification activity of BtRs8 relative to NBtRs2 may have practical implications for nitrogen synchrony in Bt cotton fields, potentially contributing to the higher nitrogen fertilizer requirements empirically observed by Bt cotton farmers in Telangana.

5. Conclusion

This study successfully isolated, screened, and characterized nitrifying bacteria from Bt and NBt cotton rhizospheric soils in Khammam District, Telangana. The best nitrifiers were Bacillus cereus (BtRs8, Bt soil) and Bacillus subtilis (NBtRs2, NBt soil), with NBtRs2 consistently demonstrating superior nitrification capacity (97 µg/g nitrite by day 7; 178 µg/g nitrate by day 9) compared to BtRs8 (78 µg/g nitrite by day 9; 154 µg/g nitrate by day 12). These findings indicate that long-term Bt cotton cultivation selects for a nitrifying bacterial community with reduced functional efficiency, with potential implications for nitrogen cycling and fertilizer use in transgenic cotton agro-ecosystems.

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