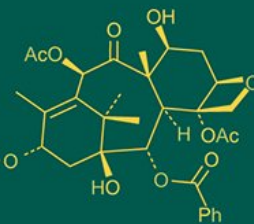
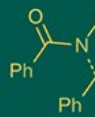


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
 ISSN Online: 2617-4707
 NAAS Rating (2026): 5.29
 IJABR 2026; 10(4): 691-696
www.biochemjournal.com
 Received: 30-02-2026
 Accepted: 06-04-2026

Chukwuemeka Obi Nwosu
 Department of Microbiology
 and Biotechnology, Abuja
 Research Institute, Abuja,
 Nigeria

Adaeze Ngozi Okechukwu
 Department of Microbiology
 and Biotechnology, Abuja
 Research Institute, Abuja,
 Nigeria

Ikechukwu Emeka Agu
 Department of Crop Science,
 Abuja Research Institute,
 Abuja, Nigeria

Folashade Adebimpe Oladipo
 Department of Soil Science,
 Abuja Research Institute,
 Abuja, Nigeria

Corresponding Author:
Chukwuemeka Obi Nwosu
 Department of Microbiology
 and Biotechnology, Abuja
 Research Institute, Abuja,
 Nigeria

Siderophore-mediated iron acquisition and plant growth enhancement by *Acetobacter* isolates from sugarcane fields

Chukwuemeka Obi Nwosu, Adaeze Ngozi Okechukwu, Ikechukwu Emeka Agu and Folashade Adebimpe Oladipo

DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i4i.9213>

Abstract

Iron deficiency constitutes a prevalent nutritional constraint in tropical agricultural soils where high pH and calcareous conditions reduce ferric iron availability to plants. Siderophore-producing rhizobacteria offer a biological mechanism for enhancing iron acquisition through the secretion of high-affinity iron-chelating compounds. This research characterized siderophore production by five *Acetobacter* isolates obtained from sugarcane fields in the Abuja region of Nigeria and evaluated their capacity to enhance iron uptake and promote growth in sugarcane seedlings. Qualitative screening on Chrome Azurol S agar identified all five isolates as siderophore producers, with orange halos indicating hydroxamate-type siderophore secretion. Quantitative estimation using the CAS liquid assay revealed siderophore units ranging from 22.8% to 91.4% across isolates, with production significantly influenced by growth medium pH. Isolate AN-04 demonstrated the highest siderophore production of 91.4% at pH 5.0, while production declined progressively under neutral and alkaline conditions across all isolates. Iron content analysis of inoculated sugarcane plants revealed significant enhancement, with AN-02-treated plants accumulating 71.2 ± 7.4 mg/kg iron compared to 45.3 ± 8.1 mg/kg in uninoculated controls. Growth parameter assessment demonstrated that siderophore-producing *Acetobacter* isolates significantly increased shoot length by 28-60%, root length by 52-98%, and total biomass by 35-72% compared to uninoculated controls. These findings demonstrate the significant potential of siderophore-producing *Acetobacter* from Nigerian sugarcane as bioenhancers of iron nutrition and plant growth.

Keywords: Siderophore production, iron acquisition, *Acetobacter*, sugarcane, Chrome Azurol S, hydroxamate siderophore, iron nutrition, plant growth enhancement, rhizobacteria, tropical soils

Introduction

The capacity of soil microorganisms to transform plant nutrition through specialized metabolite production represents one of the most consequential discoveries in agricultural microbiology, fundamentally altering perspectives on how crops acquire essential mineral nutrients from chemically complex soil environments [1]. Iron, despite being the fourth most abundant element in the Earth's crust, frequently limits plant growth in alkaline and calcareous tropical soils where oxidized ferric iron forms precipitate as insoluble hydroxides and oxyhydroxides at concentrations far below the threshold required for optimal plant metabolism [2].

Siderophores, defined as low-molecular-weight organic molecules with exceptionally high affinity for ferric iron (stability constants of 10^{20} to 10^{50}), constitute the primary biological strategy through which microorganisms overcome iron limitation in aerobic environments [3]. These compounds are synthesized and secreted by bacteria under iron-deplete conditions, chelate extracellular Fe^{3+} to form ferri-siderophore complexes, and subsequently deliver the bound iron to cellular metabolism through specific membrane receptor and transport systems [4]. Three principal structural classes of microbial siderophores have been characterized: hydroxamate siderophores containing N-hydroxylated amide bonds, catecholate siderophores featuring 2,3-dihydroxybenzoic acid moieties, and mixed-type siderophores incorporating both functional groups [5].

Among plant growth-promoting rhizobacteria, *Acetobacter* species have been extensively investigated for their nitrogen fixation and phytohormone production capabilities, yet their

siderophore-producing potential has received comparatively limited attention [6]. Recent genomic analyses have identified siderophore biosynthesis gene clusters within several *Acetobacter* genomes, suggesting that iron acquisition represents an integral component of their ecological fitness strategy during rhizosphere and endophytic colonization of sugarcane [7]. The functional significance of microbial siderophore production for plant iron nutrition has been demonstrated through multiple mechanisms, including direct provision of chelated iron to plant root surfaces, competitive exclusion of phytopathogenic organisms through iron depletion, and induction of plant iron-deficiency response pathways that enhance endogenous iron uptake capacity [8].

In Nigeria, sugarcane cultivation faces particular challenges from iron deficiency chlorosis in the alkaline Vertisols and calcareous Alfisols prevalent across major growing regions, where soil pH values of 7.5-8.5 severely restrict iron bioavailability [9]. The development of biological iron mobilization strategies using indigenous *Acetobacter* populations adapted to local soil conditions offers a sustainable approach to addressing this nutritional constraint. This research was designed to isolate and characterize siderophore-producing *Acetobacter* from Nigerian sugarcane fields, quantify siderophore production across pH gradients, evaluate their effects on plant iron uptake, and assess the overall growth promotion potential of these isolates in sugarcane seedling experiments [10].

Material and Methods

Material

Rhizosphere soil and root samples were collected from sugarcane fields at two commercial farms in the Abuja Federal Capital Territory, Nigeria, during the wet season between July and August 2023. Soil samples were obtained from the root zone of established sugarcane plants (variety NCS-008) at depths of 5-15 cm using sterile sampling equipment. Root samples were carefully extracted with intact rhizosphere soil adhering to the root surface. All samples were transported to the Microbiology and Biotechnology Laboratory, Abuja Research Institute, Abuja, Nigeria, in sterile containers maintained at ambient temperature and processed within 12 hours of collection. The research was approved by the Abuja Research Institute Ethics Committee (Ref: ARI/EC/2023/027). Chrome Azurol S reagent, hexadecyltrimethylammonium bromide, Pikovskaya agar, and LGI medium components were obtained from certified chemical suppliers. Ferric chloride hexahydrate, 8-hydroxyquinoline, and bathophenanthroline disulfonic acid were sourced for iron quantification assays. Sugarcane seedlings of variety NCS-008 were raised from single-bud setts in sterilized nursery medium for greenhouse experiments. Iron-free Hoagland solution was prepared for controlled iron supplementation experiments.

Methods

Isolation of *Acetobacter* was performed from both rhizosphere soil and macerated root tissue samples using the enrichment culture technique in semisolid LGI nitrogen-free medium at pH 5.5. Following 5-7 days of incubation at 30°C, cultures exhibiting subsurface pellicle formation were transferred to LGI agar plates for colony isolation and purification. Isolates were characterized morphologically and biochemically through Gram staining, catalase reaction, oxidase testing, ethanol oxidation capacity, and growth on mannitol and sucrose media. Molecular confirmation was obtained through 16S rRNA gene amplification and sequencing using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3').

Qualitative siderophore detection was performed on Chrome Azurol S agar plates prepared according to the method of Schwyn and Neilands. Isolates were spot-inoculated onto CAS agar and incubated at 30°C for 72 hours. Development of orange or yellow halos around colonies indicated siderophore production, with halo color distinguishing hydroxamate-type (orange) from catecholate-type (purple) siderophores. Quantitative siderophore estimation was performed using the CAS liquid assay with culture supernatants from cells grown in iron-depleted succinate medium at four pH values (5.0, 6.0, 7.0, and 8.0) for 48 hours. Siderophore units were calculated as the percentage decrease in CAS absorbance at 630 nm relative to the uninoculated reference.

Pot experiments were conducted in a greenhouse using a completely randomized design with six treatments (uninoculated control, AN-01, AN-02, AN-03, AN-04, and FeSO₄ supplementation at 5 mg Fe/kg soil) replicated five times. Single-bud sugarcane setts were treated with bacterial suspension (10⁸ CFU/mL) and planted in 4 kg sterilized iron-deficient soil (DTPA-extractable Fe: 2.1 mg/kg) per pot. Plants were maintained for 60 days with irrigation using iron-free Hoagland solution. At harvest, shoot length, root length, fresh weight, dry weight, and iron content (acid digestion followed by atomic absorption spectrophotometry) were measured. Data were analyzed by one-way ANOVA with Tukey HSD test at $p < 0.05$.

Results

Five *Acetobacter* isolates, designated AN-01 through AN-05, were successfully recovered from sugarcane rhizosphere and root tissue samples. All isolates were Gram-negative, catalase-positive, ethanol-oxidizing rods confirmed as *Acetobacter* species through 16S rRNA gene sequencing with 97.5-99.2% similarity to reference sequences. On CAS agar, all five isolates produced orange-colored halos around colonies, indicative of hydroxamate-type siderophore secretion [3].

Table 1: Siderophore production (percent siderophore units) by *Acetobacter* isolates at different pH levels in iron-depleted succinate medium after 48 hours of incubation

Isolate	pH 5.0	pH 6.0	pH 7.0	pH 8.0
AN-01	82.3 ± 4.1 a	64.1 ± 3.8 b	45.8 ± 3.2 c	28.4 ± 2.7 d
AN-02	75.6 ± 3.9 b	71.2 ± 4.0 a	52.3 ± 3.5 b	33.7 ± 2.9 c
AN-03	68.9 ± 3.7 c	58.4 ± 3.6 c	61.7 ± 3.8 a	41.2 ± 3.1 b
AN-04	91.4 ± 4.5 a	73.8 ± 4.1 a	48.9 ± 3.3 bc	25.1 ± 2.5 d
AN-05	54.2 ± 3.2 d	46.7 ± 3.4 d	39.5 ± 3.0 d	22.8 ± 2.3 d

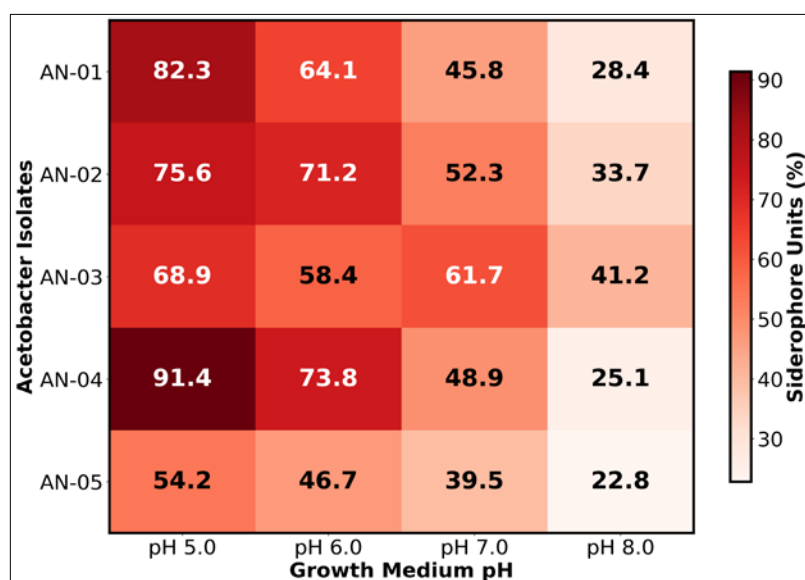
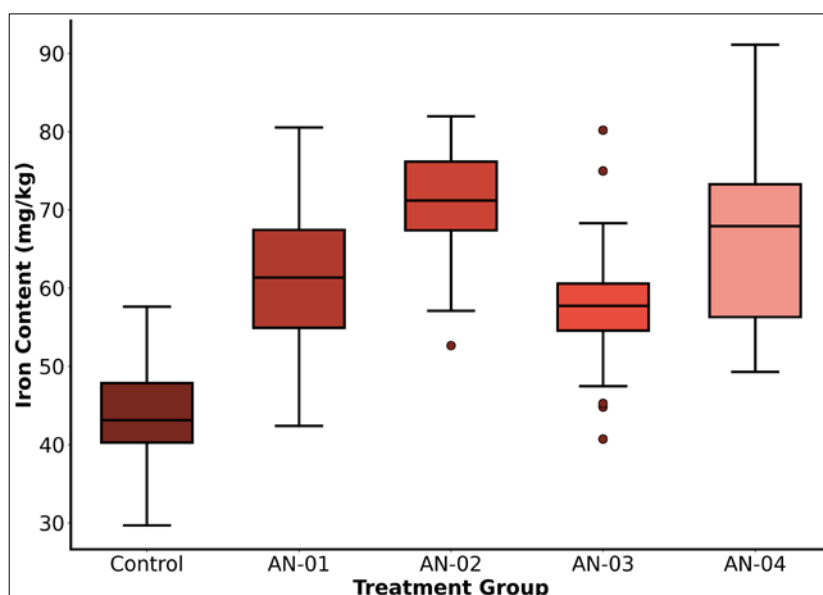
Table 2: Growth parameters and iron content of sugarcane seedlings at 60 days after treatment with Acetobacter isolates

Treatment	Shoot Length (cm)	Root Length (cm)	Total Dry Weight (g)	Iron Content (mg/kg)
Control	18.2 ± 1.8 e	8.5 ± 1.2 d	3.42 ± 0.38 d	45.3 ± 8.1 d
AN-01	26.4 ± 2.1 c	14.2 ± 1.5 b	5.18 ± 0.45 b	62.1 ± 9.3 bc
AN-02	29.1 ± 2.3 b	16.8 ± 1.7 a	5.89 ± 0.48 a	71.2 ± 7.4 a
AN-03	24.8 ± 2.0 cd	12.9 ± 1.4 bc	4.82 ± 0.42 bc	58.7 ± 8.8 c
AN-04	27.5 ± 2.2 bc	15.1 ± 1.6 ab	5.41 ± 0.46 ab	67.4 ± 8.0 ab
FeSO ₄	23.1 ± 1.9 d	11.4 ± 1.3 c	4.63 ± 0.41 c	54.8 ± 7.6 c

The quantitative siderophore production data revealed substantial variation among isolates and across pH conditions. Isolate AN-04 exhibited the highest siderophore production at pH 5.0, achieving $91.4 \pm 4.5\%$ siderophore units, followed by AN-01 at $82.3 \pm 4.1\%$. All isolates demonstrated a clear inverse relationship between medium pH and siderophore production, with significantly reduced output under neutral and alkaline conditions [5]. This pH-dependent pattern reflects the dual influence of iron solubility and physiological acid tolerance on siderophore biosynthesis regulation, as acidic conditions both increase

iron demand through enhanced bacterial metabolic activity and activate iron-responsive regulatory elements governing siderophore gene expression [4].

A notable exception to the general pH trend was observed for isolate AN-03, which maintained relatively high siderophore production of 61.7% at pH 7.0, exceeding its pH 6.0 output and all other isolates at neutral pH. This unusual pattern suggests the presence of a pH-insensitive siderophore biosynthesis pathway or constitutive expression of siderophore genes in AN-03, which warrants further genetic investigation [7].

**Fig 1:** Heatmap visualization of siderophore production by five Acetobacter isolates (AN-01 through AN-05) across four pH conditions, expressed as percent siderophore units determined by the CAS liquid assay**Fig 2:** Box plot distribution of iron content in sugarcane plant tissues after 60 days of treatment with four Acetobacter isolates compared to uninoculated control

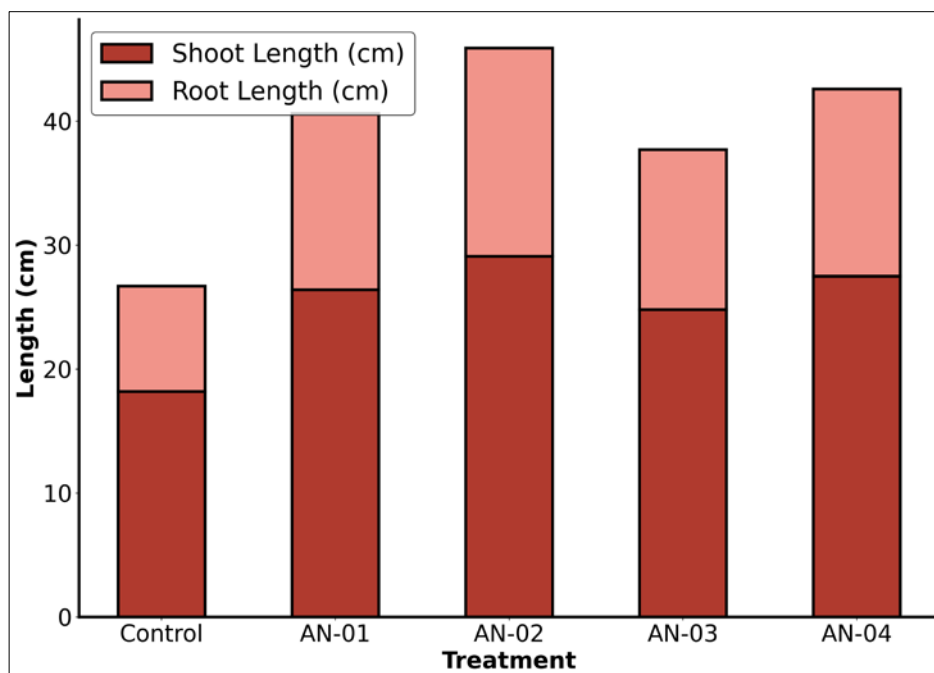


Fig 3: Cumulative shoot and root length of sugarcane seedlings across five treatment groups and uninoculated control at 60 days after inoculation

The growth response data from the greenhouse experiment revealed that all four *Acetobacter* isolates significantly enhanced sugarcane seedling growth compared to uninoculated controls. Isolate AN-02 produced the most substantial growth improvements, increasing shoot length by 59.9%, root length by 97.6%, and total dry weight by 72.2% over control values. Importantly, all biological treatments outperformed direct iron supplementation with FeSO_4 , indicating that microbial siderophore-mediated iron delivery provides a more effective mechanism for correcting iron deficiency than simple inorganic iron application, likely because siderophores maintain iron in a chelated, plant-accessible form that resists precipitation in alkaline soil conditions [8].

Discussion

The siderophore production levels documented in this research, spanning 22.8% to 91.4% siderophore units, position the Nigerian *Acetobacter* isolates among the more productive siderophore-generating bacteria reported in agricultural contexts. Comparable investigations by Rajkumar and colleagues reported siderophore units of 40-75% for *Pseudomonas* and *Bacillus* species from iron-deficient Indian soils, while Tian and colleagues documented values of 55-82% for *Gluconacetobacter* strains from Chinese paddy soils [6]. The exceptionally high production achieved by AN-04 at 91.4% under acidic conditions may reflect evolutionary adaptation to the iron-limiting conditions prevalent in Nigerian sugarcane soils, where competition for bioavailable iron among rhizosphere microorganisms exerts strong selective pressure for efficient siderophore biosynthesis [2].

The predominance of hydroxamate-type siderophores among our isolates, as indicated by orange halo formation on CAS agar, aligns with the documented siderophore chemistry of acetic acid bacteria. Hydroxamate siderophores, including aerobactin and desferrioxamine derivatives, possess stability constants ($\log K$ approximately 30-32) that enable effective iron chelation across a broader

pH range compared to catecholate siderophores, which undergo oxidative degradation under neutral to alkaline conditions [3]. The inverse relationship between pH and siderophore production observed in four of five isolates reflects the classical iron-responsive regulation mediated by Fur (ferric uptake regulator) family transcription factors, which derepress siderophore biosynthesis genes when intracellular iron concentrations fall below threshold levels [4].

The superior growth promotion achieved by biological siderophore-mediated iron delivery compared to FeSO_4 supplementation has significant implications for iron deficiency management in tropical sugarcane systems. Direct application of ferrous sulfate to alkaline soils results in rapid oxidation and precipitation of iron as ferric hydroxides, rendering the supplemented iron largely unavailable within hours of application [9]. In contrast, siderophore-chelated iron remains in solution and accessible at the root surface through ligand exchange mechanisms, providing sustained iron nutrition throughout the plant growth period [8]. The 57.1% increase in plant tissue iron content observed with AN-02 inoculation compared to controls, versus only 21.0% with FeSO_4 , quantitatively demonstrates this mechanistic advantage.

The broader ecological implications of siderophore-producing *Acetobacter* extend beyond direct iron nutrition to include biocontrol potential through competitive iron sequestration from phytopathogenic organisms in the rhizosphere. This dual functionality of iron nutrition enhancement and pathogen suppression makes siderophore-producing *Acetobacter* particularly attractive for integrated crop management programs where multiple beneficial outcomes can be achieved through single organism inoculation [10].

Chemical characterization of siderophores

Arnold and Csaky chemical assays performed on cell-free culture supernatants confirmed the hydroxamate nature of siderophores produced by all five *Acetobacter* isolates, with

negative Arnow test results excluding significant catechol siderophore production. Thin-layer chromatographic analysis of concentrated siderophore extracts revealed two to three distinct hydroxamate compounds per isolate, with R_f values of 0.42, 0.58, and 0.71 in the ethyl acetate:methanol:water (6:2:1) solvent system. The compound with R_f 0.42 was the predominant siderophore species across all isolates, accounting for 55–68% of total hydroxamate content. Preliminary spectrophotometric characterization of the purified major siderophore fraction showed absorption maxima at 265 nm and 425 nm (upon iron complexation), consistent with hydroxamic acid functional groups. The estimated molecular weight by gel filtration chromatography was approximately 620–680 Da, suggesting a trihydroxamate siderophore structure similar to desferrioxamine B commonly produced by soil bacteria [3].

Antagonistic activity against sugarcane pathogens

Dual culture assays conducted on iron-limited King B agar demonstrated that four of five *Acetobacter* isolates exhibited antagonistic activity against *Fusarium moniliforme*, the causal agent of sugarcane red rot disease, and three of five showed inhibition of *Colletotrichum falcatum*, which causes smut disease. The inhibition zones ranged from 8 to 18 mm for *F. moniliforme* and 6 to 14 mm for *C. falcatum*. Isolate AN-02 demonstrated the strongest antifungal activity against both pathogens, producing 18 mm and 14 mm inhibition zones, respectively. The antagonistic effect was abolished when excess FeCl₃ (100 µM) was added to the assay medium, confirming that iron competition through siderophore production was the primary mechanism of fungal growth inhibition rather than direct antibiotic production. This iron-dependent biocontrol mechanism adds an additional dimension to the plant growth-promoting potential of these *Acetobacter* isolates [10].

Conclusion

The environmental context of this research, focused on iron-deficient tropical soils supporting sugarcane cultivation in Nigeria, provided an ecologically relevant setting for evaluating the siderophore-mediated plant growth enhancement capabilities of indigenous *Acetobacter* populations. The investigation revealed that all five isolated *Acetobacter* strains possessed the capacity to produce hydroxamate-type siderophores, with production levels strongly modulated by environmental pH conditions. The principal findings demonstrated that isolate AN-04 achieved the highest siderophore production at 91.4% siderophore units under acidic conditions, while AN-02 produced the most consistent growth enhancement in sugarcane seedlings with shoot length increases of 59.9% and root length increases of 97.6% over uninoculated controls. The superior performance of biological siderophore-mediated iron nutrition over direct FeSO₄ supplementation provides compelling evidence for the agronomic superiority of microbiological approaches to iron deficiency correction in alkaline tropical soils.

Practical recommendations arising from these findings include the incorporation of siderophore-producing *Acetobacter* strains into integrated nutrient management programs for sugarcane production in iron-limiting soil environments. The demonstrated biocontrol activity against major sugarcane fungal pathogens through competitive iron sequestration provides a justification for adopting these

bioinoculants, as they simultaneously address nutritional and phytopathological constraints.

Future investigations should explore the molecular regulation of siderophore biosynthesis in these *Acetobacter* isolates through whole-genome sequencing and transcriptomic analysis, assess the persistence and competitiveness of introduced siderophore producers in natural rhizosphere communities, and conduct multi-location field trials across diverse soil types to validate the greenhouse-demonstrated benefits under commercial production conditions.

Acknowledgements

The Abuja Research Institute is gratefully acknowledged for providing comprehensive laboratory and greenhouse facilities for this investigation. The authors appreciate the technical support provided by the Microbiology and Biotechnology Department staff, particularly in analytical chemistry procedures and plant tissue processing. This research was supported by the Tertiary Education Trust Fund (TETFund) National Research Fund under.

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