IDENTIFICATION OF BACTERIAL ENDOPHYTES IN COTTON AND THEIR POTENTIAL FOR PLANT PROTECTION: A CASE STUDY OF NIGERIAN IMPROVED COTTON VARIETIES

Ajene I. J., Aba J. O and Kwanashie A. J.

Department of Crop Protection, Ahmadu Bello University Zaria, Nigeria

*Corresponding author: E-mail: inusaajene@yahoo.com

SUMMARY

Plant Pathogens cause severe economic losses to farmers worldwide. The control of plant diseases is based on frequent application of synthetic pesticides. The use of endophytes in the control of plant diseases creates opportunities for sustainable and eco-friendly alternatives. This study sought to identify bacterial endophytes present in the cotton varieties developed by the Institute for Agricultural Research, Samaru Zaria. The root samples of 6 improved cotton varieties (SAMCOTS 8, 9, 10, 11, 12, and 13) were screened for the presence of endophytic bacteria. The isolates were characterised by morphological and biochemical characteristics. Four genera were identified as *Pseudomonas, Bacillus, Azotobacter, and Azospirillum*. These four genera of endophytic bacteria were isolated from the root samples with each genus showing a different cell and colony morphology. The genus *Pseudomonas* was the most abundant amongst the isolates, while *Azotobacter* was the least abundant.

Keywords: Endophytes, Bacteria, Cotton

Endophytes are ordinarily characterised as microscopic organisms or parasites that dwell inside plant tissues. They can be isolated from the plant after disinfection, and cause no adverse impacts on plant development (1; 2), or any external sign of infection on their host (3; 4), and most plant species on the earth hosts one or more endophytes (5). studies on the endophytic biology has been carried out on only a few (6) hostplants; therefore, the potential for discovering beneficial endophytes among the diverse plant ecosystems is considerable. Plants are regularly involved in interactions with a wide range of bacteria. These plant-associated bacteria colonise the rhizosphere (rhizobacteria), the phyllosphere (epiphytes) and inside of plant tissues (endophytes). Endophytes are protected from environmental stresses and microbial competition by the host plant and have been isolated from a variety of plant parts in various plant species (7).

Some endophytic bacteria exert some beneficial effects on host plants such as stimulation of plant growth (8), accelerate seedling emergence, promote plant establishment under adverse conditions and bacterial nitrogen fixation, induction of resistance to plant pathogens (9), and also through the plant-microbe interactions they promote plant health and development; these have been the subject of considerable study. Recent work has also investigated their potential for the enhanced biodegradation of pollutants in soil, while many other studies have described the biocontrol potential of root-colonizing bacteria (10; 11; 12), prevention of disease development through endophyte-mediated *de novo* synthesis of novel compounds and antifungal metabolites (13). Investigation of the biodiversity of endophytic strains for new metabolites may have important implications for plant protection and other applications (5). Such metabolites have been shown to reduce the incidence of Fusarium wilt of cotton (induced by *Fusarium oxysporum* f.sp. *vasinfectum)* (9), cucumber anthracnose (induced by *Colletotrichum orbiculare*), and bacterial angular leaf spot (induced by *Pseudomonas syringae*pv. *lachrymans*) (14).

Due to the ability of endophytic bacteria to inhabit a variety of plants they have been isolated from different plant species, such as cotton, wheat, rice, sugarcane, and poplar tree (9; 15; 16).

Many studies have described the biocontrol potential of root-colonising bacteria (10; 11; 12; 14). *Pseudomonas fluorescens* 89B-61 is a root colonist which has been shown to reduce the incidence of Fusarium wilt of cotton induced by *Fusarium oxysporum* f.sp. *vasinfectum* (9).

Corn and cotton serve as a microbial habitat for a diverse bacterial endophytic microflora (17) and while cotton production in Nigeria is marred by low average yield that has resulted in low marginal profitability for small-scale producers, with yields falling within the range of 300 – 500kg/ha, the yield potential of popular Institute for Agricultural Research (IAR) varieties like SAMCOT-11 and SAMCOT-12 could be up to 1200 – 1500kg/ha (18).

The control of plant diseases is based on the frequent application of synthetic pesticides, which are known to pollute the environment. The essence of biological control is that the pests' natural antagonists are used for their control. The use of endophytes in the control of plant diseases create opportunities for sustainable and eco-friendly alternatives. The potential of plant growth beneficial endophytes to improve plant health has to a significant number of studies examined their applied use as inoculants, primarily in crops (19; 2; 20).

Bacterial endophytes can have beneficial effects such as plant growth promotion and reduction of disease symptoms induced by plant pathogens (9; 14; 8). Moreover, their unique ability to survive inside plants with little or no microbial competition makes them potential candidates for biological control. For example, endophytic bacteria may be constructed to carry genes for antibiotics and insecticides against pathogens and insects, respectively (21).

The potential for microbial inoculants to reduce the need for chemicals such as fertilisers or pesticides makes them essential in the development of sustainable agriculture; therefore, the need to screen for plant beneficial endophytes. This study, therefore, aims at identifying beneficial bacterial endophytes present in IAR cotton varieties for potential application in integrated pest management strategies.

MATERIALS AND METHODS

The study was conducted at the IAR field, Shika, and Bacteriology laboratory of the Department of Crop Protection, Faculty of Agriculture, Ahmadu Bello University, Zaria.

The varieties of cotton used in this study were SAMCOTS 8, 9, 10, 11, 12 and 13, which are improved varieties of the IAR, Zaria. The cotton seeds were sown at the rate of 4 plants per hole in the IAR research fields. Cotton plants were sampled (three plants per treatment) a month after sowing and secondly at boll development stages.

The roots of the plants were cut and washed with tap water to remove attached soil. Further sterilisation was carried out by washing samples in 50 ml of distilled water and 5 ml of sodium hypochlorite for 3 minutes in a beaker.

The sterilised samples were placed in a mortar and thoroughly macerated, following which 5 ml of distilled water was added into the mortar containing the samples. After diluting the suspension with distilled water, the extract was decanted into a sterilised Petri dish. It was then streaked on the already prepared media (agar media) for culture. The media used were: *Pseudomonas* agar, *Azospirillum* agar, *Azotobacter* agar, and *Luria-Bertani* agar. The cultures were incubated at 28°C. Sample cultures were taken at 24, 48 and 72 h after inoculation.

Identification and Quantification of Bacteria

The colonies obtained were then sub-cultured onto fresh media plates to obtain pure colonies. The bacterial colonies were harvested and suspended in sterile distilled water (SDW). The bacterial suspension was subjected to serial dilution. Twenty microliter aliquots of the 10^{-8} dilution were placed on the media plates and spread using sterile beads and incubated for 48 h, following which the colonies were counted using a colony counter (Gallenkamp, U.S.A). Colonies from the two representative isolates from each of the four groups of isolations were placed into groups on the basis of characteristics of the bacteria on nutrient agar and growth on selective media. Biochemical tests including Gram reaction, nitrate reductase, catalase reaction, oxidase test and starch hydrolysis were carried out on the isolates to ascertain their identities.

The nitrate reduction test was tested by using nitrate broth which was inoculated with the isolates and incubated for 48 h. 10 – 15 drops each of sulfanilic acid and N-naphthylethylene-diamide were added to each tube. Oxidase reaction was done by transferring bacterial colonies onto discs saturated in N-dimethyl-p-phenylenediamine (DMPD) using a wire loop and observing the disc for up to three minutes for colour change. Catalase reaction was carried out by smearing small amounts of the bacteria colonies onto a glass slide and applying drops of hydrogen peroxide to detect the presence of catalase enzyme through the formation of oxygen bubbles.

Data generated from the bacterial population were subjected to negative binomial regression analysis using the General Linear Model to ascertain the significance of the interactions of the variables, significant variables were subjected to Least-Square Means analysis, and the post-hoc analysis was done using Tukey's test in R version 3.4.4 (22).

RESULTS

Bacterial endophytes were found in the root samples of the six cotton varieties (SAMCOTS 8, 9, 10, 11, 12 and 13) screened. The bacterial genera *Pseudomonas, Bacillus, Azotobacter, and Azospirillum* were associated with all the varieties screened.

The interaction between the factors tested showed that the cotton varieties had no significant interaction while the bacterial species was highly significant (Table 1) and hence the quantification of the endophytic bacterial isolates (Fig 1) indicated that; *Pseudomonas sp.* was more abundant in the isolates with 3.56 × 10^{12}cfu/ml and *Azotobacter sp.* the least abundant with 1.23 × 10^{12}cfu/ml (Table 2).

Table 1: Interaction between factors as obtained by Analysis of Deviance

Factor	Df	Deviance Df	Residual	Residual deviation	P (>Chi)	
Bacterial Species	3	695242		92	749	< 2e-16 ***
Cotton Variety	5	13		87	735	0.01982 *

Significance codes: 0 '***', 0.001 '**', 0.01 '*'

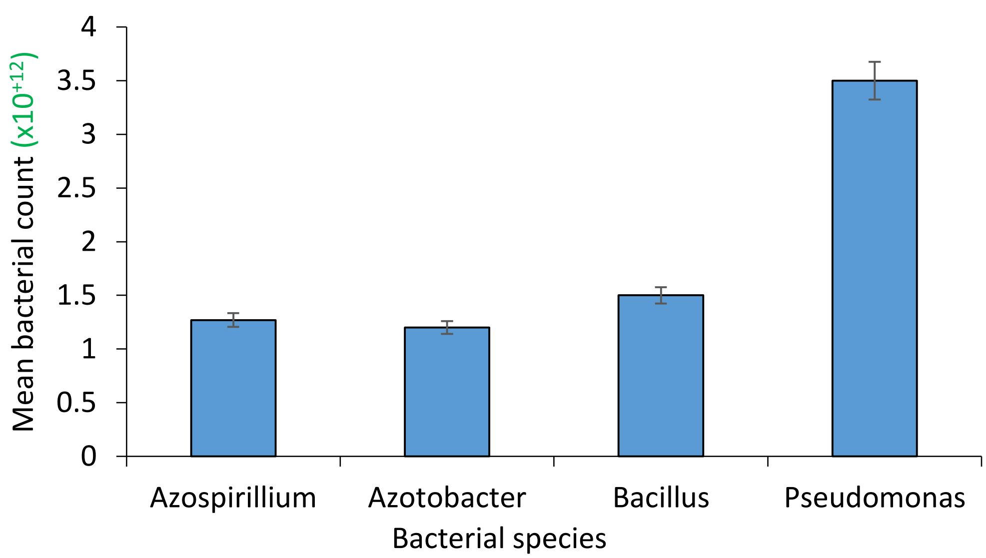

Figure 1: Population of bacterial endophytes from cotton

Table 2: Bacterial population from colony count

Bacteria	Bacterial population (cfu/ml)
Azospirillum sp.	1.27×10^{12a}
Azotobacter sp.	1.23×10^{12a}
Bacillus sp.	1.54×10^{12b}
Pseudomonas sp.	3.56×10^{12c}
SE	0.001098822

All means with the same letter show no significant difference at $p \leq 0.05$ level of probability

SE = Standard error

Morphological and Biochemical Properties of the Isolated Endophytic Bacteria

Morphological analysis showed that bacteria from three isolates sampled were gram-negative and one was gram-positive. All the isolates were catalase positive, two genera were able to reduce nitrogen while two did not, three genera were able to hydrolyse starch while one genus was not able to hydrolyse starch and each genus showed a different cell and colony morphology (Table 4).

Table 3: Morphological and Biochemical Properties of the Isolated Endophytic Bacteria

Morphological/Biochemical Properties	Bacteria Species			
	Bacillus sp.	*Pseudomonas sp.*	*Azotobacter sp.*	*Azospirillum sp.*
Gram staining	+	_	_	_
Oxidase	+	+	+	+
Catalase	+	+	+	+
Nitrate Reduction	_	_	+	+
Starch Hydrolysis	+	_	+	+

Cell Shape	Rod	Rod	Cocco-bacilli	Irregular
Colony Morphology	Dry, Irregular, dull white, motile	Slimy, mucoid, motile	Spherical, slimy, dull white, motile	Irregular, wrinkled, pink, motile

DISCUSSION

From an ecological perspective, biological pest control relies on two main forces: bottom-up (i.e., the effect of plants on herbivores) and top-down pest control (i.e., the effect of predators and parasites on herbivores) (23; 24). Since a myriad of soil organisms feed on root herbivores, they could be used as bio-control agents for root pest control in top-down control approaches. The result on number of bacterial isolates shows that all the IAR cotton varieties tested contained bacterial endophytes. The root of these plants serve as habitat for diverse bacterial endophytic microflora ; confirming earlier reports (5; 25) that endophytes are ubiquitous and have been found in a variety of plant species studied to date.

The morphological analysis of the isolates revealed that most of the isolated endophytes were Gam negative. Among the endophytic bacteria, Gram-negative bacteria out-number the gram-positive bacteria in most crops (26), as shown in this study. The biochemical characterisation revealed an important aspect required by the bacteria to reproduce and to avoid cellular toxicity which was the catalase positive nature of the bacterial genera identified. Some bacteria contain flavoproteins that reduce oxygen resulting in the production of hydrogen peroxide and superoxide, which are extremely toxic to the cell as they are potent oxidising agents and can destroy cellular components very rapidly (27). Since the bacterial endophytes isolated in this study were catalase positive, it means they possess the capability to protect themselves from these toxic effects. *Bacillus* species could be obligate aerobes or facultative anaerobes, and test positive for the enzyme catalase (28).

The nitrate reduction test was also performed to determine the ability of the isolates to reduce nitrates to nitrogen gas. This is an important factor to help maintain the nitrogen cycle in the three phases of the atmosphere, water, and the soil. 50% of the isolates tested positive for denitrification test. They can readily fix nitrogen by converting them to free nitrogen gas. The process of denitrification may produce molecular nitrogen through a series of intermediate gaseous nitrogen oxide products. The process is performed primarily by heterotrophic bacteria such as *Paracoccus denitrificans* and by various pseudomonads (29). The endophytic root pseudomonad identified in this research was able to reduce nitrate to nitrogen gas.

Interest in screening the endophyte for bioactive compounds is on the increase (30). A plethora of secondary metabolites have been extracted, isolated and characterised from endophytic microbes (5; 31). The metabolic properties as seen by the starch hydrolysis tests of isolated bacterial root endophytes, revealed that almost all the isolates hydrolyse starch. While characterising the bacterial endophytes associated with *Curcuma longa* L., and bacterial root endophytes, Kumar *et al*. (32) and Singh *et al*. (33) respectively observed a huge diversity in isolates for metabolising the various carbon sources

These strains of bacteria screened were reported in many studies as growth promoters. *Azospirillum* is one of the widely studied indo acetic acid (IAA) producers (34). Other IAA producing bacteria include *Azotobacter* (35), *Bacillus* (36), *Pseudomonas* (37) and *Rhizobium* (38) among others. *Pseudomonas* was also reported as auxin producer. Inoculation with IAA producing plant growth promoting rhizobacteria (PGPR) has been used to stimulate seed germination, to accelerate root growth and modify the architecture of the root system, and to increase the root biomass. In addition to stimulating root growth, IAA producing bacteria can also be used to stimulate tuber growth (36).

The variation observed in the population differences from the colony counts is attributed to many factors such as the physiological status of the host plants, the soil conditions, plant age, time of sampling, and environment (39; 40). The endophyte concentrations obtained from the cotton varieties in this study were relatively lower than previously reported (7); where it was advocated that natural endophyte concentrations can vary between 5.67×103 and 6.0 ×105 cfu per g for alfalfa, sweet corn, sugar beet, squash, cotton, and potato.

The critical roles of integrated pest management (IPM) are envisioned as to ease complete dependence on pesticides, encourage the use of natural enemies and parasites to suppress crop pests, and raise farmers knowledge about pests, agro-ecosystem and the surrounding environment (41; 42). The presence of these endophytes in the current study offer an opportunity for in-depth investigations into the cotton varieties tested as they are ubiquitous and have shown the ability to evade cellular toxicity thus making them stable and effective candidates for application in integrated pest and disease management strategies.

CONCLUSION

Plant bacterial endophytes are used as biological control agents and form a good tool in combating crop pests and diseases. In this regard, isolates from these varieties of cotton can be used as tools for plant protection by intercropping these varieties with other crop species that do not harbour bacterial endophytes. The endophytes obtained from these specific cotton varieties could be further screened via field trials for the enhancement of plant growth in cotton varieties. The identification of beneficial bacterial endophytes in the genera *Pseudomonas*, *Bacillus*, *Azotobacter*, and *Azospirillum* from IAR cotton varieties provide a new alternative for inclusion in integrated plant protection practices.

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