Isolation, characterization and screening of bacterial endophytes from *Zea mays L. var. rugosa* (sweet corn) Sugar King variety with biotechnological potential in agriculture

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Abstract

This study aimed to determine the plant growth promoting activities exhibited by bacterial endophytes isolated from the roots of *Zea mays L. var. rugosa* (sweet corn) Sugar King. A total of eight different bacterial strains were isolated and characterized through Gram staining and screened for a positive reaction for nitrogen fixation, ammonia production and zinc solubilization through the use of Jensen's media, peptone water with Nessler's reagent, and zinc incorporated media, respectively. Results showed that Gram-negative bacteria were the dominant group. Colony characterization showed that circular forms comprised the majority. Similarly, the colonies with entire forms and either pulvinate or flat elevation were recurrent throughout the isolates. Cell characterization revealed that all isolates were rod in shape. The plant growth promoting screenings revealed that all isolates were plant growth promoters. All were found to be positive for ammonia production and zinc solubilization; however, none were found to be positive for nitrogen fixation.

Keywords: *endophytes, nitrogen fixation, ammonia production, zinc solubilization, Zea mays L. var. rugosa*

Introduction. Endophytes are organisms, often fungi and bacteria, that live between living plant cells [1]. They are defined as microorganisms that could be isolated from surface-sterilized plant tissues and do not visibly harm host plants. Endophytism is a universal phenomenon, and it is likely that all plants harbor endophytic bacteria [1]. There have been studies on the characterization and testing of endophytic bacteria for their beneficial effects on plants. It has been shown that some species of endophytic bacteria were able to produce indole-3-acetic acid which can promote plant growth [3]. Endophytic bacteria can also do nitrogen fixation in order to assist plants in obtaining nitrogen, which they use to promote growth development. Other plant growth enhancing capabilities were characterized by the ability to produce siderophores, ammonia, phytohormones, inorganic phosphate solubilization and biocatalyst like cellulase, amylase and protease [14]. Endophytic bacteria are also capable of being a biocontrol agent due to a mechanism of antibiosis which makes substances to be used against organisms that are harmful to the host plant [11]. In order for the endophytic bacteria and its host plant to have a symbiotic relationship, it is essential for the host plant to supply metabolites and nutrients to the endophyte and for the endophyte to produce auxins and do other plant growth promoting activities such as nitrogen fixation and phosphate solubilization. In order to be able to fully utilize these microorganisms, screening of endophytic bacteria having plant growth promoting abilities is necessary.

Corn is one of the staple food crops in the Philippines. Most of the corn sold in markets today are hybrids due to the advanced traits they have over natural corn. The *Zea mays L. var. Rugosa* (sweet corn) Sugar King variety was selected for the study due to its early maturity, uniform large cobs, good husk protection, and a strong plant habit designed for most weather conditions. These traits are considered as the possible benefits of the plant growth promoting activities that were screened for.

Using culture-dependent isolation techniques and standard procedures for the testing of plant growth promoting factors of the bacteria, the study focused on the characterization and screening of endophytic bacteria present in the roots of the corn hybrid variety *Zea mays L. var. rugosa* (sweet corn) Sugar King. The samples were characterized based on colony morphology, cell morphology and Gram stain. Bacterial strains from the isolated samples were not identified. Bacterial samples were inoculated into selective media to test for plant growth promoting activities. Jensen's media, peptone water, and zinc incorporated media were used to screen nitrogen fixing, ammonia producing and zinc solubilizing bacteria, respectively. The plant growth promoting activities associated with each cultured bacteria were also determined.

The study aimed to isolate, characterize, and screen endophytic bacteria present in the roots of *Zea mays L. var. rugosa* (sweet corn) Sugar King variety for plant growth promoting activities, which can be used as biofertilizers. It specifically aimed to:

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(i) isolate endophytic bacteria from Sugar King variety sweet corn using culture-dependent isolation methods;

(ii) characterize endophytic bacteria isolated from Sugar King variety sweet corn using culture-dependent isolation methods; and

(iii) screen the endophytic bacteria for plant growth promoting factors such as ammonia production, nitrogen fixation, and zinc solubilization.

Methods. Sweet corn gathered from Barangay Agutayan, Sta. Barbara, Iloilo were screened for possible activities on nitrogen fixation, ammonia production, and zinc solubilization. A total of nine samples were randomly selected and three mixed culture plates were generated from the gathered samples. The three mixed culture plates contained eight pure bacterial strains and each were then characterized based on their colony morphology, cell morphology and Gram stain. Each bacterial strain was tested for positivity in nitrogen fixation, ammonia production, and zinc solubilization through the use of selective media.

Preparation of Growth Media. Trypticase Soy Agar (TSA) and Trypticase Soy Broth (TSB) were used as growth media for the isolated bacterial endophytes. Three media bottles were each filled with 20 g of TSA and 500 mL of distilled water while another was filled with 6 g of TSB and 200 mL of distilled water. The mixtures were then boiled using a hot plate and continuously stirred using a sterile glass stirring rod until a clear solution was formed. After boiling, the solutions were sterilized in an autoclave at 121° C for 15 minutes, then was allowed to cool down for 3-4 minutes before being distributed. Sixty standard sized Petri dishes were filled with the TSA solution until the bottom of each dish was fully covered while 20 sterile test tubes were each filled with 10 mL of the TSB solution. The plates and test tubes were then allowed to rest for a few minutes before being stored at 2-8° C in a refrigerator.

Preparation of Selective Media. Jensens's media, peptone water, and zinc incorporated media were used for the screening of nitrogen fixation, ammonia production and zinc solubilization, respectively. In making Jensens's media, the procedure by Richard et al. [16] was followed. Twenty grams of sucrose, 1g of dipotassium phosphate, 0.500g of magnesium sulphate, 0.500g of sodium chloride, 0.100g of ferrous sulphate, 2g of calcium carbonate and 15g of agar were suspended in 1L of distilled water in a culture bottle. In making peptone water, the guideline reported by Jorgensen et al. [9] was followed. In a culture bottle, 10g of peptone and 5g of sodium chloride were suspended in 1L of distilled water. In making zinc incorporated media, the procedure by Kamran et al. [10] was followed. Half a gram of zinc chloride was suspended in 500 mL of TSA in a culture bottle. The three mixtures were then boiled using a hot plate and continuously stirred using a sterile glass stirring rod until a clear solution was formed. After boiling, the solutions were sterilized in an autoclave at 121° C for 15 minutes, then was allowed to cool down for 3-4 minutes, before being labelled and stored at 2-8° C in a refrigerator.

Sample Gathering. Samples of Sugar King variety sweet corn roots (SCR) were gathered from Brgy. Agutayan of Sta. Barbara, Iloilo. Nine root samples were gathered randomly and stored in polythene bags inside a cooler partially filled with ice, in order to minimize bacterial activity, before being transferred to the Microbiology Laboratory of PSHS-WVC.

Surface Sterilization. Following the procedure for surface sterilization by Youseif [18], samples were surface sterilized with 70% ethyl alcohol and bathed in 1% sodium hypochlorite for two minutes. The outside surface of the samples was inoculated onto TSA plates to check for sterilization efficiency. Respective samples from agar plates that showed no signs of microbial growth were gathered and smashed together using a mortar and pestle in preparation for the serial dilution.

Serial Dilution. Three sets of three test tubes, labelled 10^{-1},10^{-2}, and 10^{-3}, respectively, were filled with 9 mL of 0.9% saline solution. One gram of the sample was added to each of the test tube labelled 10^{-1}. Before the sample settled, 1 mL of the suspension was transferred into the test tubes labeled 10^{-2} using a micropipette. The procedure was repeated once again to achieve a concentration of 10^{-3}.

Isolation of Mixed Culture. One drop of the diluted samples from the test tubes labelled 10^{-3} was pipetted onto one side of an agar plate. Using an inoculating loop heat-sterilized using an alcohol lamp, the sample was streaked on the agar plate using the quadrant streak method. The agar plates were then incubated for 24 hours at 37° C. The plates were placed upside-down during the incubation period to avoid the interference of condensation in the growth of the microbes. After the incubation period, the colony morphology of the microbial plates was observed. Those with the same morphology were classified as the same colonies before the isolation of pure cultures to avoid multiple cultures of the same bacteria.

Isolation of Pure Culture (TSA). For the agar culture, a sterile inoculating loop was used to transfer bacteria from the mixed culture plate to another agar plate. The bacteria were then streaked using the quadrant streak method and incubated at 37°C for 24 hours.

Isolation of Pure Culture (TSB). A stock culture for each isolated colony was prepared. A sterile inoculating loop was gently touched to the surface of an existing colony to gather the bacteria. The loop was then inserted into the broth, moving the loop back and forth to ensure the inoculation of the bacteria to the media. The broth was then incubated at 37°C for 24 hours. Pure culture colonies were necessary for the biochemical testings as bacteria may react differently in isolation than when it is combined with other species.

Colony morphology. Colony morphology was observed before and after the isolation of pure culture bacterial colonies to ensure that the colonies isolated were correct. The shape, margin, elevation, size, color, and texture of the bacterial colony were identified. This determined the groupings, based on the similarities of their characteristics, for the isolation of pure culture colonies.

Cell morphology. Microscopy was conducted to determine the shape and arrangement of the bacterial cell.

Gram staining. From each pure culture colony grown, a bacterial smear was prepared. Smears were prepared by heat fixing bacteria onto a sterile glass slide. Following the procedure of Jorgenson et al. [9], the smears were then saturated with the following reagents: the primary stain crystal violet for 1 minute; Gram's iodine solution for 1 minute; 95% ethanol for 5 seconds; and the counterstain safranin for 1 minute rinsing the glass slide with distilled water after the addition of each reagent. During microscopy, purple-stained bacteria were considered Gram-positive while pink-stained bacteria were considered Gram-negative.

Screening Nitrogen Fixation. The procedure for nitrogen fixation screening by Ebeltagy et al. [5] was followed. Nitrogen-free malate agar (Jensen's media) was used in the screening for nitrogen fixation with bromothymol blue (BTB) acting as an indicator [8]. The isolates were then incubated at 37°C for 24 hours. Isolates with blue colored zones were marked as nitrogen fixers.

Screening Ammonia Production. The procedure for ammonia production screening by Richard et al. [16] was followed. Isolates were inoculated into 10 mL peptone water in separate test tubes, then incubated for 2-3 days at 28 ± 2 degrees Celsius. After the addition of 0.5 ml of Nessler's reagent, isolates that produced a brown-yellow discoloration were marked positive for ammonia production.

Screening Zinc Solubilization. The procedure for zinc solubilization screening by Kamran et al. [9] was followed. Zinc chloride ($ZnCl_2$) medium plates were used in the screening for zinc solubilization. The isolates were aseptically inoculated as a spot on the respective medium plates and covered with aluminum foil. They were then incubated in the dark at 28° C for one day. The presence of clear zones around the colony indicated the presence of zinc solubilizing strains.

Data Analysis. The basis for positive results in each screening was from past articles [9,10,16]. Evaluation of results were done through visual assessment and were verified by a licensed medical technologist.

Safety Procedure. While in the process of isolation and handling of bacteria, proper lab equipment such as lab gowns, sterile gloves, and masks were used to ensure the prevention of contamination and infection of bacteria. Used gloves, tissues, and other trash were properly disposed into designated disposal bins inside the Microbiology Laboratory of PSHS-WVC. Bacteria cultures were properly decontaminated with 10% sodium hypochlorite. Work areas were disinfected with 70% ethyl alcohol after working.

Results and Discussion. Results obtained from the study revealed that *Zea mays L. var. rugosa* (sweet corn) Sugar King variety harbors culturable endophytic bacteria. Eight bacterial strains were isolated from the sweet corn roots (SCR). All isolates were characterized based on their colony morphology, cell morphology, and Gram stain (Table 1). Five of the isolates (SCR 2, SCR 3, SCR 4, SCR5 and SCR 7) were Gram-negative (Figure 1) while three (SCR 1, SCR 6, and SCR 8) were Gram-positive (Figure 2).

Table 1. Morphological characteristics of individual strains.

Isolate*	Colony Morphology	Cell Morphology	Gram +/-
SCR 1	Circular, Lobate, Umbonate, Moderate, Brown, Smooth	Rod	Gram +
SCR 2	Rhizoid, Rhizoidal, Flat, Moderate, White, Rough	Rod	Gram -
SCR 3	Irregular, Entire, Flat, Small, White, Smooth	Rod	Gram -
SCR 4	Circular, Lobate, Raised, Moderate, Dark, Brown, Smooth	Rod	Gram -
SCR 5	Circular, Entire, Pulvinate, Moderate, Brown, Rough	Rod	Gram -
SCR 6	Circular, Entire, Pulvinate, Small, Orange, Smooth	Rod	Gram +
SCR 7	Circular, Entire, Pulvinate, Moderate, Brown, Smooth	Rod	Gram -
SCR 8	Irregular, Lobate, Flat, Moderate, Brown, Smooth	Rod	Gram +

*Nomenclature was given based on the crop and tissue type isolated which is sweet corn root (SCR).

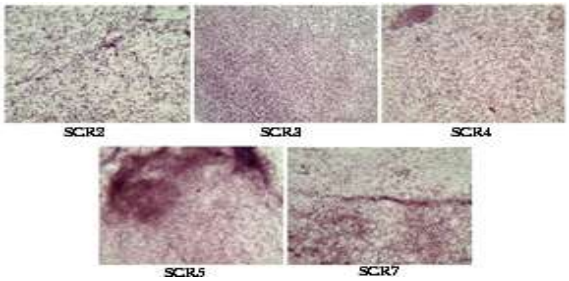

Figure 1. Isolates of the sweet corn root (SCR) that did not retain the crystal violet stain indicating it as Gram-negative.

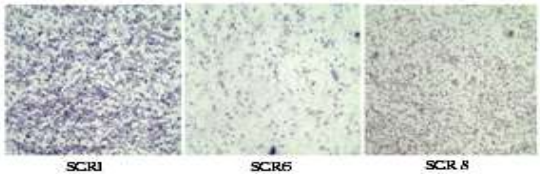

Figure 2. Isolates of the sweet corn root (SCR) that did retain the crystal violet stain indicating it as Gram-positive.

For the characterization of the bacteria based on colony and cell morphology as well as Gram Stain, results showed that the endophytic bacteria lcommunity of the Sugar King sweet corn was composed of both Gram-positive and Gram-negative bacteria. Gram-negative stain, circular form, entire margin, pulvinate and flat elevations, and rod shape predominated among all isolates.

The functional potentialities in relation to plant growth promoting activities of root isolates were assessed. Strains were tested in vitro for their ability to conduct three plant growth promoting activities such as ammonia production, nitrogen fixation and zinc solubilization (Table 2).

Table 2. Presence of plant growth promoting activities in endophytic bacteria from *Zea mays* roots.

Isolate*	Ammonia Production	Nitrogen Fixation	Zinc Solubilization
SCR 1	Present	Absent	Present
SCR 2	Present	Absent	Present
SCR 3	Present	Absent	Present
SCR 4	Present	Absent	Present
SCR 5	Present	Absent	Present
SCR 6	Present	Absent	Present
SCR 7	Present	Absent	Present
SCR 8	Present	Absent	Present

*Nomenclature was given based on the crop and tissue type isolated which is sweet corn root (SCR).

The results demonstrated that the endophytic bacterial community isolated were all capable of producing ammonia, with SRC 2 and SRC 3 exhibiting maximum production. Marques et al. [13] suggested that the ammonia produced by endophytes is beneficial for the root and shoot elongation, consequently increasing plant biomass. Presence of these endophytes may have contributed to the large cobs and sturdy structure characterized by the sweet corn Sugar King variety. Nitrogen fixation is another biological process that plants undergo in order to take in nitrogen. It is an important biological process wherein bacteria convert molecular nitrogen, which plants cannot utilize, into usable forms like ammonia [15]. None of the strains isolated were able to fix nitrogen during the screening. This may be either due to all isolates being not really able to fix nitrogen or an error was conducted during the screening, specifically during the formulation of the selective media. All endophytic isolates of *Zea mays L. var. rugosa* were observed to be zinc solubilizers. So far, the *Bacillus spp.* and *Pseudomonas spp,* both of which are rods, are the only bacterial species from maize crops that were reported to be zinc solubilizers as they form a clear halo zone [10]. Reports from past studies have confirmed that zinc solubilizing strains significantly improved the activity of enzymes, plant growth, yield attributes and successfully biofortified maize grains aside from the increase in zinc content [9].

Endophtic bacteria are tissure specific, but not plant specific [18], meaning that the strains isolated from the study could be utilized as bio-fertilizers, which are becoming more and more popular in many countries, for many crops. Biofertilizers are natural products carrying living organisms derived from plant organs or soil itself, so they do not have any ill effects on soil health or the environment, unlike chemical fertilizers. In modern agriculture, chemical fertilizers have degraded the fertility of soil making it unsuitable for raising crop plants. In addition, the intensive use of these inputs has also led to severe health and environmental hazards such as soil erosion, water contamination, pesticide poisoning, falling groundwater table, water logging and depletion of biodiversity. Although chemical fertilizers are more commonly used, bio-fertilizers are more practical and beneficial to farmers and agriculturalists. Saeed et al. [17] reported that biofertilizers increased yield, yield components and growth promotion better than chemical fertilizers. Biofertilizers naturally activate the microorganisms found in the soil. Being cheaper, more effective and environmentally friendly, biofertilizers are gaining importance for use in crop production, restoring the soil's natural fertility and protecting it against drought, soil diseases and therefore stimulate plant growth. This shows that biofertilizers that utilize plant growth promoting endophytes are practically and economically better than chemical fertilizers. Endophytic bacteria isolated from *Zea mays L. var. rugosa* exhibiting capabilities for plant growth promotion (specifically ammonia production and zinc solubilization) could have potential use for biotechnological purposes in agriculture, in particular, as biofertilizers.

Limitations. The data presented in the study is exclusive to the sweet corn hybrid variety *Zea mays L. var. Rugosa.* The selective media used in the study were verified by only one licensed medical technologist. Due to the lack of resources, the researchers were not able to identify the isolated bacterial strains.

Conclusion. The present study showed that Zea *mays L. var. rugosa* (sweet corn) Sugar King variety is home for diverse plant growth promoting bacterial endophytes. Being able to showcase plant growth promoting activities, inoculation of maize plants with these endophytic representatives may result to a more stimulated plant growth and increased biomass production compared to uninoculated plants. The present study suggests the potential application of these endophytes in agricultural traits that could result in reduced input costs due to the use of agro-chemicals, better production yield and health, and in a way, may lead to improved soil quality and fertility. However, further field experiments and actual identification of the representative endophytes are encouraged to support the findings of the study.

Recommendations. It is recommended to add screenings for other important plant growth promoting activities such as indole acetic acid production and phosphate. Future researchers of similar field can use other corn hybrids as the plant sample to see differences in the resulting isolated bacteria. It is also recommended to consult experts on how to make the various growth media to be used for screening to further legitimize the results.

Increasing the sample size can improve the quality of the gathered data being a more accurate representation of the endophytic bacterial community from the sample site. For the success of biofertilizer technology and the utilization of endophytes, further research and development is needed to understand the mechanisms of action of various biofertilizers and to find out more competent endophytic strains and carrier materials to make agriculture practices more sustainable and economical. Finally, identification of bacterial strains is recommended to further strengthen the legitimacy of the study.

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