

(Original Article)



Isolation and Characterization of Indole-3-butyric Acid-producing *Azospirillum* Bacteria from Grapevine Rhizosphere

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Abstract

Ten isolates of *Azospirillum* bacteria named from Azo-1 to Azo-10 have been isolated from the grapevine rhizosphere. *Azospirillum* isolates in Dobereiner medium with 0.01% tryptophan were evaluated for their ability to produce Indole-3-butyric acid (IBA). Indole butyric acid (IBA) was determined by colorimetric Salkowski reaction. As a result, of ten *Azospirillum* isolates collected from grapevine rhizosphere 3 isolates (30%) bio-synthesized IBA below 9 µg/mL, 5 isolates (50%) produced IBA from 9 to 17 µg/mL, and 2 isolates (20%) were recorded this value from 22 to 29 µg/mL. The results show a great variation among the tested *Azospirillum* isolates in production capacities of IBA (from 5 to 29 µg/mL). The results also show that the isolate Azo-4 produced the highest amounts of IBA (29 µg/mL). The highest five of *Azospirillum* isolates (Azo-1, Azo-4, Azo-6, Azo-8 and Azo-9), which are capable of producing IBA (22, 29, 15, 14 and 17 µg/ ml, respectively), are identified by the cell morphological, physiological and biochemical characteristics. Based on all the identification characteristics, select isolates were identified as *Azospirillum* spp.

Keywords: *Azospirillum*, Grapevine, Indole-3-butyric acid, Rhizosphere, Salkowski reagent.

Introduction

Azospirillum bacteria are a genus of Gram-negative bacteria that can live freely and fix N₂ in microaerobic conditions. The highly motile *Azospirillum* species have a mixed pattern of flagellation that allows them to move towards favorable nutrient conditions. They have been specifically isolated from the rhizosphere of cereals (Dobbelaere *et al.*, 2002). *Azospirillum* is a plant growth-promoting bacterium that can be discovered in cereals and other plant roots' rhizosphere and intercellular. The beneficial characteristics of this bacterium include nitrogen fixation, hormone production, improving water and nutrients uptake, and controlling pathogens. (Han *et al.*, 2018; Peng *et al.*, 2020; Zhang *et al.*, 2021).

The phytohormones produced by *Azospirillum* are effective in controlling respiration rate metabolism, growth, and root development, which leads to an increase in water and nutrient uptake in inoculated plants. (Holguin *et al.*, 1999). *Azospirillum* species are capable of synthesizing plant hormones, including indole butyric acid (IBA), gibberellins, and cytokinins (Bashan *et al.*, 2004). The efficiency of nutrient uptake through plants was probably enhanced by an increase in root surface adsorption after

inoculating with growth hormone-producing bacteria like *Azospirillum*. (Bashan *et al.*, 2004).

The current investigation was launched to isolate and characteristics *Azospirillum* strains from grapevine rhizosphere and assess their capability of producing phytohormones, Indole-3-butyric acid (IBA).

Materials and Methods

Isolation of *Azospirillum* isolates

Soil samples were collected from the rhizosphere of grapevine growing at the Experimental Farm of the Faculty of Agriculture at Assiut University in Egypt. The composition of the nitrogen-free bromothymol semi-solid medium (Nfb) used for isolation and subsequent cultivation is as follows.: Bromthymol blue solution 0.5% ; Malic acid $C_4H_6O_5$, 5.0 g; KH_2PO_4 , 0.5 g; K_2HPO_4 , 0.5 g; NaCl, 0.19; $MgSO_4$, 0.29, $MnSO_4 \cdot 7H_2O$, traces; Agar-Agar, 1.759 per liter of medium. Nfb medium, contained within screw-capped tubes, was inoculated with 0.1 ml of each sample. The inoculated tubes were then incubated at 37°C for a period of 72 hours. The tubes were inoculated with *Azospirillum* after incubation, resulting in the formation of a distinct thin, dense, white pellicle situated a few millimeters beneath the surface of the medium (Dobereiner, 1980). A segment of the pellicle was applied to Nfb-medium plates that had been solidified with 1.5% agar, and the plates were subsequently incubated at 37°C for duration of 72 hours. The isolates were ultimately purified by streaking onto the Rodriguez-Caceres (RC) medium supplemented with congo-red at a concentration of 37.5 mg/l (Rodriguez-Caceres, 1982). The solid Nfb-medium slants were used to incubate pure colonies at 37°C for 5 days, and then they were kept at 4°C.

Azospirillum isolates producing IBA screening

The *Azospirillum* isolates were cultivated in liquid Nfb medium supplemented with 0.01% tryptophan, under shaking conditions of 200 rpm at a temperature of 37°C for a duration of 5 days. The supernatant from the cultured suspension was obtained following centrifugation of the culture at 5000 rpm for ten minutes, in order to assess the capacity for IBA through a color reaction method. The colorimetric Salkowski reaction was employed to quantify Indole butyric acid (IBA) in the following manner: a volume of 2 ml from the prepared methanolic solution, corresponding to 100 ml of the culture, was combined with 4 ml of Salkowski reagent, which consists of 2.025 g of $FeCl_3$, 300 ml of H_2SO_4 , and 500 ml of H_2O . The solution was maintained in a dark environment for duration of 15 to 30 minutes prior to conducting a colorimetric analysis of the resulting pink hue using a Spectronic 20, Baush & Lomb, at a wavelength of 530 nm. A standard curve for authentic IBA was established, comprising solutions with varying concentrations of pure IBA ranging from 10 to 100 µg/mL. These solutions were treated with Salkowski reagent as outlined in prior studies and subsequently measured calorimetrically at a wavelength of 530 nm. The IBA content was determined following the methodology detailed by Glickmann and Dessaux (1995).

Characterization *Azospirillum* isolates

The top five *Azospirillum* isolates, which were likely to possess the ability to produce IBA, have been initially identified based on their cellular morphology, physiological traits, and biochemical properties. *Azospirillum* isolates cultivated on Nfb semi-solid and agar media were examined to assess cell morphology, dimensions, Gram reaction, cell motility, and colony characteristics. The isolates were also subjected to various tests, including the catalase test, oxidase test, starch hydrolysis test, and assessments of carbohydrate utilization to evaluate the physiological activities of the organisms. The development of the isolates was recorded through visual observation in every case. The identification of all chosen isolates was conducted in accordance with Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

Result and Discussion

Isolation of *Azospirillum* bacteria

The isolation of ten *Azospirillum* bacteria isolates named Azo-1 to Azo-10 from various grapevine rhizosphere samples has shown their ability to grow on nitrogen-free Nfb-specific medium. Table 1 presents the findings related to the morphological, cultural, and physiological traits of the ten *Azospirillum* isolates. All isolates developed subsurface pellicles in the Nfb semi-solid medium. The development of the pellicle occurred at a depth of 1-2 mm beneath the surface of the semi-solid Nfb medium (Fig.1). The colony morphology of isolates on Nfb agar medium was small to medium, white dense, spindle and transparent pale shiny white in color. Prior to the characterization of the bacteria, the IAA-producing capability of all isolates was assessed using Dobereiner medium enriched with 0.01% tryptophan.

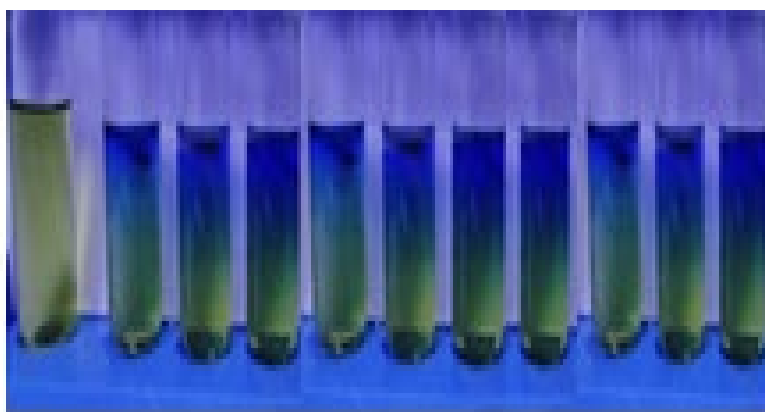


Fig 1. Growth of *Azospirillum* isolates on Nfb semi-solid medium, 72 hrs old culture showing growth pellicle formation and turn green color of bromothymol blue to brilliant blue.

Production of IBA by *Azospirillum* isolates

Dobereiner medium with 0.01% tryptophan was used to test *Azospirillum* isolates for the production of IBA. The data presented in Table (1) and Figure (2) demonstrate that all isolates have the ability to produce IAA in Dobereiner medium with 0.01% tryptophan. As a result, of ten *Azospirillum* isolates collected from grapevine rhizosphere, 3 isolates (30%) biosynthesized IBA below 9 µg/mL, 5 isolates (50%)

produced IBA from 9 to 17 $\mu\text{g/mL}$, and 2 isolates (20%) were recorded this value from 22 to 26 $\mu\text{g/mL}$. The findings indicate significant variability among the examined *Azospirillum* isolates regarding their production capacities of IBA, ranging from 5 to 29 $\mu\text{g/mL}$. The findings indicate that the isolate Azo-4 generated the greatest quantities of IBA, whereas the isolate Azo-7 yielded the least amounts. The detected amounts of IBA synthesized by the tested *Azospirillum* isolates in this investigation are in the range of those reported by Pham *et al* (2022), they also recorded variations in *Azospirillum* isolates for production of IAA (7 to 25 $\mu\text{g/mL}$). The level of IAA production was 41.5 $\mu\text{g/ml}$ for *Azospirillum* isolates (Yu62), 12.9 $\mu\text{g/ml}$ for *Azospirillum* isolates (Az39).

Table 1. Quantities of Indole-3-butyric acid (IBA) produced by *Azospirillum* isolates cultivated in liquid Nfb medium enriched with 0.01% tryptophan.

<i>Azospirillum</i> isolates	IBA content ($\mu\text{g/ ml}$)
Azo-1	22
Azo-2	7
Azo-3	9
Azo-4	29
Azo-5	13
Azo-6	15
Azo-7	5
Azo-8	14
Azo-9	17
Azo-10	7

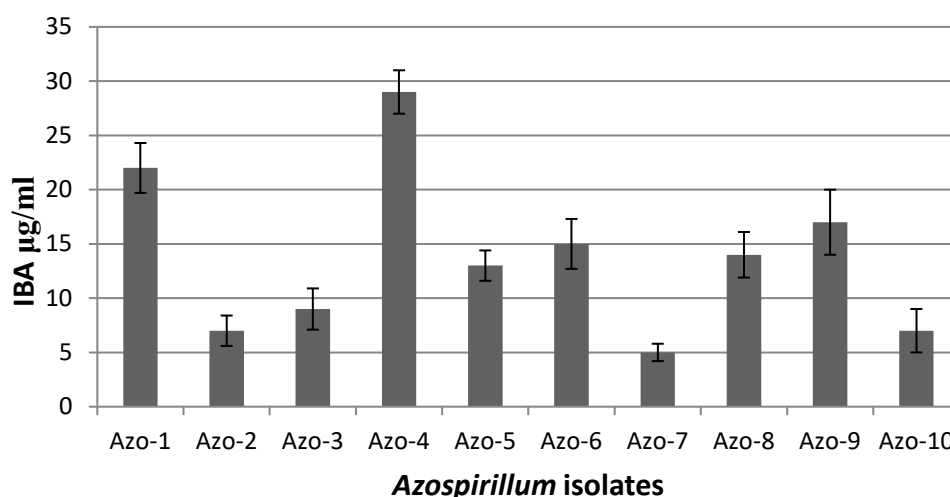


Fig 2. Amounts of Indole-3-butyric acid ($\mu\text{g/ ml}$) synthesized by *Azospirillum* isolates grown in liquid Nfb medium.

Azospirillum not only fixes nitrogen, but it also produces auxins, such as IAA, that increase root hair production and improve nutrient uptake from the soil. IBA is another auxin that is linked to *A. brasilense* inoculation, and it can be detected in the roots of maize seeds that have been inoculated with this microorganism (Fallik *et al.*, 1989). The most significant phytohormone generated by *Azospirillum* is indole-3-acetic acid (IAA), as noted by Steenhoudt and Vanderleyden (2000). Various strains of *Azospirillum* can synthesize this hormone, contingent upon the culture medium and the availability of tryptophan, which serves as a precursor to auxins. The production of auxins by

Azospirillum species has a substantial influence on plant growth and development. Auxin-type phytohormones enhance root structure and facilitate the absorption of nutrients from the soil. Species of *Azospirillum* are capable of synthesizing plant hormones, including Indole-3-butyric acid, gibberellins, and ethylene (Bashan *et al.*, 2004).

Characterization *Azospirillum* isolates

The highest five *Azospirillum* isolates were (Azo-1, Azo-4, Azo-6, Azo-8 and Azo-9), which were capable of producing IBA (22, 29, 15, 14 and 17 µg/ ml, respectively), are identified by the cell morphological, physiological and biochemical characteristics (Table 2). The presence of *Azospirillum* in the culture tube was determined by the formation of a white, dense, and undulating fine pellicle, located a few millimeters beneath the surface of the semi-solid malate medium in screw-capped tubes, as described by Dobereiner (1980). He indicated that the pellicle formed by *Azospirillum* in Nfb medium contained in tubes can be consistently recognized after the researcher acquires adequate experience.

Table 2. Characteristics of *Azospirillum* isolates include their morphology, physiology, and culture.

Test	Azo-1	Azo-4	Azo-6	Azo-8	Azo-9
Gram reaction	-	-	-	-	-
Motility	+	+	+	+	+
Pellicle formation	+	+	+	+	+
Colony morphology	spindle	spindle	spindle	spindle	spindle
Colony Size mm	1-2 mm	1-2 mm	1-2 mm	1-2 mm	1-2 mm
Cell morphology	spiral	spiral	spiral	spiral	spiral
Biochemical properties					
Catalase test	+	+	+	+	+
Oxidase test	+	+	+	+	+
Starch hydrolysis	-	+	+	+	-
Gelatin hydrolysis	+	-	-	+	-
Carbon source					
Glucose	+	+	+	+	+
Sucrose	-	-	-	+	-
Mannitol	+	-	-	+	-
Lactose	-	-	-	-	-
Fructose	-	-	-	-	-
Maltose	+	-	-	+	-

There is still a significant possibility of the pellicle being contaminated by other microorganisms. Thin pellicles are less contaminated than thick pellicles and *Azotobacter*, Proactinomyces and protozoa are the common contaminants (Lakshmi-Kumari *et al.*, 1976). As a result, several transfers of the organism from the pellicles to a new semi-solid medium are necessary to obtain a pure culture. This necessity led to the execution of several such transfers throughout this investigation to obtain a pure culture.

The colony morphology of the isolates cultured on Nfb agar medium exhibited characteristics ranging from small to medium size, with a dense white appearance.

The colonies were spindle-shaped and displayed a transparent, pale shiny white coloration (Fig. 3). While the colonies of *Azospirillum* isolates on Rodriguez-Caceres (RC) medium were small, round, dry, compact and have undulated edge and were red in color (scarlet) due to the uptake of the congo red pigment (Fig.4). The morphology of all isolates was characterized by a spiral shape, and upon microscopic examination, it was determined that all isolates were Gram-negative (Fig. 5).

According to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994), considering all the identifying characteristics selected isolates were identified as *Azospirillum* spp.

Attitalla *et al.* (2010) successfully isolated a variety of *Azospirillum* strains from 23 different leguminous and non-leguminous plant species within the distinctive Mediterranean climate of eastern Libya.



Fig 3. Colonies of *Azospirillum* isolate on Nfb agar medium after 3 days of incubation.



Fig 4. Colonies of *Azospirillum* isolate on Rodriguez-Caceres (RC) agar medium after 3 days of incubation.



Fig 5. Vegetative cells of the *Azospirillum* isolates from 48 hrs old cultures on Nfb agar medium. ($\times 4000$)

The colony forming unit (CFU) counts varied between 1.1 and 130.2×10^3 CFU/g of soil, with the most elevated counts detected in the rhizosphere of leguminous plants. The identification of 15 strains of *A. lipoferum* was achieved through an analysis of cellular morphology along with cultural and biochemical traits. The rhizosphere of different crops in Korea was found to harbor N₂-fixing bacteria, which were isolated by Ki-Yoon *et al.* (2010) and subsequently evaluated for their capacity to reduce acetylene. A total of 13 isolates were examined and classified as members of the genus *Azospirillum*.

Conclusion

Ten isolates of *Azospirillum* bacteria named from Azo-1 to Azo-10 have been isolated from the grapevine rhizosphere. The highest five of *Azospirillum* isolates (Azo-1, Azo-4, Azo-6, Azo-8 and Azo-9), which are capable of producing IBA (22, 29, 15, 14 and 17 µg/ ml, respectively), are identified by the cell morphological, physiological and biochemical characteristics. Based on all the identification characteristics, select isolates were identified as *Azospirillum* spp.

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عزل وتوصيف بكتيريا *Azospirillum* المنتجة لحمض إندول-3- بيوتيريك من ريزوسفير نباتات العنب

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الملخص

في هذه الدراسة تم عزل عشر عزلات من بكتيريا *Azospirillum* من ريزوسفير نبات العنب المزروع بمزرعة كلية الزراعة جامعة أسيوط. وقد تم اعطاء العزلات ارقام من Azo-1 إلى Azo-10. وتم اختبار قدرة عزلات *Azospirillum* النامية على وسط Dobereiner المضاف إليه 0.01% تريبتوفان على إنتاج حمض إندول-3-بيوتيريك (IBA)، وتم تقدير حمض إندول-3-بيوتيريك (IBA) بواسطة تفاعل سالكوفسكي اللوني.

وقد أظهرت النتائج أنه من بين العشر عزلات *Azospirillum* أنتجت 3 عزلات (30%) حمض إندول-3-بيوتيريك بيولوجياً بتركيز أقل من 9 ميكروجرام / مل، وأنتجت 5 عزلات (50%) حمض إندول-3-بيوتيريك بتركيز من 9 إلى 17 ميكروجرام / مل، وسجلت عزلتان (20%) هذه القيمة من 22 إلى 26 ميكروجرام / مل. وتظهر النتائج تبايناً كبيراً بين عزلات *Azospirillum* المختبرة في قدرات إنتاج IBA (من 5 إلى 29 ميكروجرام / مل). كما تظهر النتائج أيضاً أن العزلة Azo-4 أنتجت أعلى كميات من IBA (26 ميكروجرام / مل).

ثم تم تحديد أعلى خمس عزلات من *Azospirillum* في إنتاج حمض إندول-3-بيوتيريك وهي العزلات: Azo-1، Azo-4، Azo-6، Azo-8، Azo-9 لدراسة بعض خصائصها المورفولوجية والفسولوجية والبيوكيميائية، تم تحديد العزلات المختارة على أنها تابعة لجنس *Azospirillum*.

الكلمات المفتاحية: البكتريا المشجعة لنمو النبات، الريزوسفير، إندول حمض البيوتيريك، عملية التجذير، عقل العنب.