Assiut Journal of Agriculture Science 53 (4) 2022 (125-139) *Website:* http://ajas.journals.ekb.eg/
ISSN: 1110-0486 / EISSN: 2356-9840 *E-mail:* ajas@aun.edu.eg

(Original Article)

Isolation and Characterization of Local Azospirillum and Yeast Strains to Be Used for Wheat Grains Inoculation to Improve Plant Growth and Yield under Field Cultivation

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DOI: 10.21608/ajas.2022.155095.1165

Abstract

Four Azospirillum strains and three yeast isolates were isolated from rhizosphere soil of wheat plants grown on Soil of the Experimental Farm of Soil and Water Department, Assiut University, Egypt. There were studied the characteristic of both Azospirillum and yeast to be used as a biofertilizers in inoculation of wheat grains under field condition to study the response of growth and yield to inoculation. For Azospirillum, the isolation was on Nitrogen free bromothymol (Nfb) semisolid medium, their morphology (light microscope, scanning electron microscope), cultural characteristics to verity their identity to those reported on Bergey's Manual of Determinative Bacteriology (1994), and N2-fixing capacities, which were ranged from 13.8:42.5 mg N2-fixed/g malic acid. For yeast, the isolation was on Malt Extract Peptone Glucose (MEPG) medium, their morphology, cultural characteristics and acid production were determined, as well as the promotive stimulating effects of Azospirillum and yeast on grain germination and early seedling growth of wheat.

Keywords: *Rhizobacteria, Azospirillum, Yeasts, phosphate Solubilization, Hormonal effects.*

Introduction

Several research studies in the last 30 years have shown the beneficial effects of crop seed inoculation before sowing with selected beneficial PGPR that promote plant growth through: N2-fixation like those of the genera Rhizobium, Azotobacter, Azospirillum; or those producing hormones and cytokinines like yeasts; or those producing organic acids and chelating agents that increase the availability of P and other essential micro essential elements as Fe and Mn (Ahemad and Kibret, 2014).

The obtained research results have also shown that local isolated microbial strains were more active and produce faster and higher increase in plant growth and yield than those isolated from foreign habitat (Baldani *et al*., 1987; Salanture *et al*., 2006).

Received: 11 August 2022 / Accepted: 25 September 2022 / Published online: 1 October 2022

Also, Baldani *et al*., (1987) showed the superiority in inoculating wheat with wheat isolates, and maize with maize root isolates.

The aims of the present work are to isolate local Azospirillum and yeast strains from rhizosphere soil of wheat plants, and determine their morphological, cultural and physiological characteristics to verity their identity to those reported (Bergey's Manual of Determinative Bacteriology, 1994). The ideal isolated strains will then be used for wheat grains inoculation under field cultivation.

Materials and Methods

Preparation of rhizosphere soil sample of wheat roots

The rhizosphere soil sample was collected from soil particles strongly adhesive to roots of wheat plants grown on the clayey soil of the Experimental Farm of Soil and Water Department, Faculty of Agriculture, Assiut University.

The roots were vigorously shaken to remove all the loosely attached soil particles, then about 5gm of the roots with strongly adhesive soil particles were added to 100 ml sterilized water in Erlenmeyer flask, and vigorously shaken for 5 minutes to remove the sticky attached soil particles from roots surfaces and obtain rhizosphere soil suspension.

Isolation of microbial strains

Isolation of Azospirillum

Samples of 1 ml of the prepared rhizosphere soil suspension were taken and serially diluted using sterile distilled water up to 10-8 dilutions. From 10-6 to 10-8 dilutions for each sample it was taken 0.1ml of aliquot by using a sterile pipette and inoculated in screw caped tube containing Nitrogen free bromothymol (Nfb) semisolid medium of the following composition: Malic acid 5 gm, KH_2PO_4 0.4 gm, K_2HPO_4 0.1 gm, KOH 3.5 gm, Yeast extract 0.1 gm, $MgSO_4.7H_2O$ 0.2 gm, NaCl 0.1 gm, CaCl 0.2 gm, $FeSO_4.7H_2O$ traces, Sodium molybdate 0.002 gm, $MnSO_4.4H_2O$ 0.1 gm, Bromothymol blue 0.5 % 5 ml, Agar 1.75 g, and distilled water 1000 ml, (Dobereiner and Day, 1976).

There were incubated at 37ºC for 72 hrs. After incubation, Azospirillum appeared in the inoculated tubes through forming characteristic thin dence, white pellicle few mm below the surface at the medium (Dobereiner, 1980). Then the pellicles were examined microscopically to study some characteristics like, the presence of gram negative, motile cells and vibroid. According to Krieg (1981), a loopful of the pellicle which developed in tubes was transferred to fresh semisolid Nfb medium, and the tubes were incubated at 37ºC, then transferred into the fresh semisolid Nfb medium thrice, each of transfer being made at 72 hrs. intervals, after incubation a loopful of the pellicle was streaked on the plates of Nfb solid medium (18g agar/L) and the plates were incubated at 37ºC for 72 hrs. Finally, the isolated were purified by streaking on the Congo Red agar medium (Rodrigues Caceres, 1982) of the following composition per liter of distilled water: K_2HPO_4 0.5 g; $MgSO_4.7H_2O$ 0.2 g; NaCl 0.1 g; yeast extract 0.5 g; $FeCl_3.6H_2O$ 0.15 g; DL-malic acid 5.0 g; KOH 4.8 g; and agar 18 g. pH of the medium was adjusted to 7.0 with

0.1 N KOH. After autoclaving and just before use, 1/400 congo red aqueous solution separately autoclaved was aseptically added at rate of 15 ml/ L.

Phenotypic properties and biochemical tests performed on isolated strains

The pure isolated strains were tested for the following phenotypic and biochemical tests, and were characterized according to Bergey's Manual of Determinative Bacteriology (1994):

1- Cell morphology: there were optically examined Gram-stained smears of the isolated strains, by light microscope, and also examined the cells by scanning electron microscope.

2- Cell motility by the hanging drop method in liquid cultures (48 hrs. old).

3- Catalase reaction with 10% solution of H_2O_2 by flooding colonies on Nfb agar plates.

4- The ability to utilize various C substrates (Fructose, Mannitol, Peptone, Cellulose, Sucrose, Lactose, Glucose, Malic acid, Gelatin, and Nitrate), at 0.5 % concentrations, as sole C source in Nfb semi solid medium in tubes.

5- Starch hydrolysis by cultures grown on Nfb agar medium (containing 0.5 % soluble starch).

Testing the isolates for N_2-fixing activity

The ability of the isolates Azospirillum strains to fix N_2 in culture medium was tested by 25 ml of the Nfb semisolid medium containing 0.5% of malic acid as a carbon source, then the culture grown for 10 days, then determining the total nitrogen content by the micro-Kjeldahl method (Jackson, 1973).

Isolation of yeast

For isolation of yeast, it had been by taking 0.1 ml of the enriched culture in flask to sterile petri plate and added 15 ml of the sterile cooled Malt Extract Peptone Glucose (MEPG) solid medium of the following composition: malt extract 3 g, yeast extract 3 g, peptone 5 g, D-glucose 10 g, distilled water 1000 ml, and agar 18 g; and pH 5.6. (Wickerham, 1951), to it then incubate for 48 hrs. to obtain yeast isolates colonies.

The isolated microbial strains yeast which isolated from rhizosphere soil of wheat plants have been purified then tested microscopically before study cultural characteristics of colonies on MEPG solid (18 g agar/L) medium, as well as determination the rate of growth and acid production.

Solubilization of tricalcium phosphate and calcium carbonate

The solubilization of tricalcium phosphate was tested on modified Bunt and Rovira agar medium (Abdel-Hafez, 1966) and the solubilization of calcium carbonate was tested on (MEPG) solid medium containing 0.5% calcium carbonate and inoculation with yeast isolates on petri plates, and the inoculated plates were incubated at 30ºC for 4 days. After the incubation period, the plates were observed for the presence of clear zone (Halo Zone) around the isolated colonies of yeast

which indicates the extent of phosphate solubilization, and the ability of isolated strains to solubilizing inorganic phosphate. And also, the isolated strains were inoculated on Pikovskayak's solid medium (modified by Sundara and Sinha, 1963) These media of the following composition: Glucose 10 gm, ammonium sulphate 0.5 gm, NaCl 0.2 gm, KCl 0.2 gm, $MgSO_4.7H_2O$ 0.1 gm, $FeSO_4.7H_2O$ traces, $MnSO_4.4H_2O$ traces, yeast extract 0.5 gm, Agar 18 gm, and distilled Water 1000 ml. Tricalcium phosphate 5 gm.

Promotive effect of isolated strains on wheat grain germination and seedling growth

1- Germination and seedling growth of grains inoculated with the isolated strains was tested on plates of Nfb semisolid (10 g agar/L) medium.
2- Control (uninoculated grains) soaked in water, for 2 hrs.
3- Grains soaked for 2 hrs. in isolated Azospirillum cultures.
4- Grains soaked for 2 hrs. in isolated yeast cultures.
5- Grains soaked for 2 hrs. in mixed culture of Azospirillum and yeast isolated strains.

Results

Azospirillum strains

Morphological and cultural characteristics

Four strains have been isolated, formation of growth pellicle 1-2 mm below surface in the tube of Nfb semisolid medium and change of color of the medium to blue was considered as preliminary indication of growth of N_2-fixing microaerophilic micro-organisms (Figure 1). Part of the pellicle formed by the growing organisms was purified by reinoculation twice on sterile tubes of the Nfb medium before examining of cell morphology of the growing isolate (s).

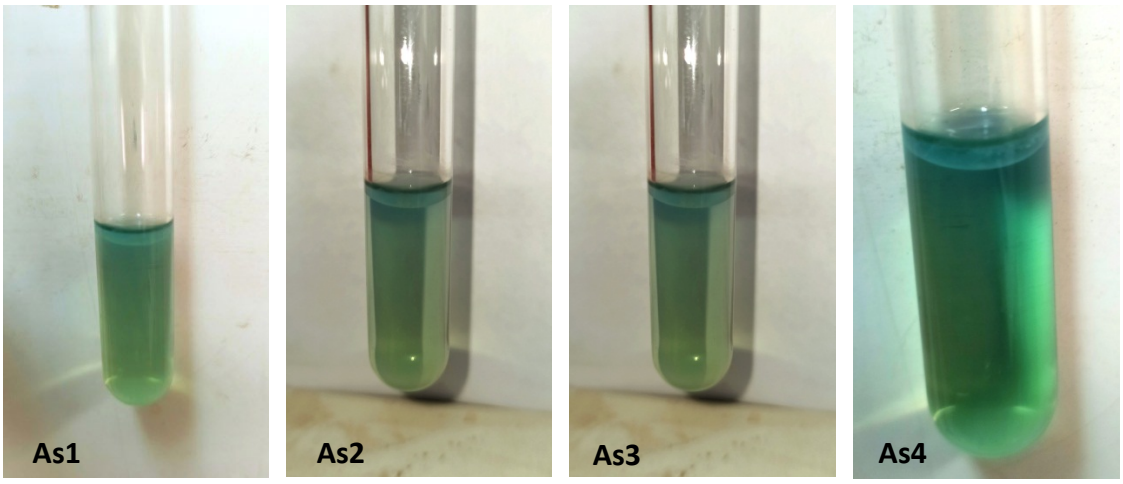

Figure 1. The under surface growth pellicle of four of the *Azospirillum* isolated strains; As1, As2, As3 and As4 on Nfb medium after 48 hrs. of incubation.

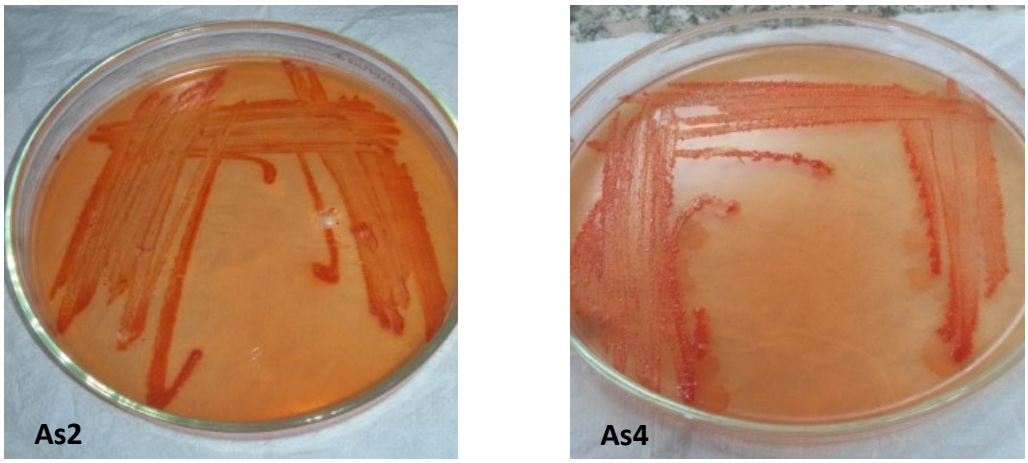

Figure 2. Dry red colonies of two of the isolated strains As2 and As4 formed on Congo Red agar medium.

The growth and type of the dry red colonies for the *Azospirillum*_isolated strains (Figure 2) which formed on Congo Red agar medium was examined in plates after 5 days of incubation and it was considered an important characteristic mark for Azospirillum strains.

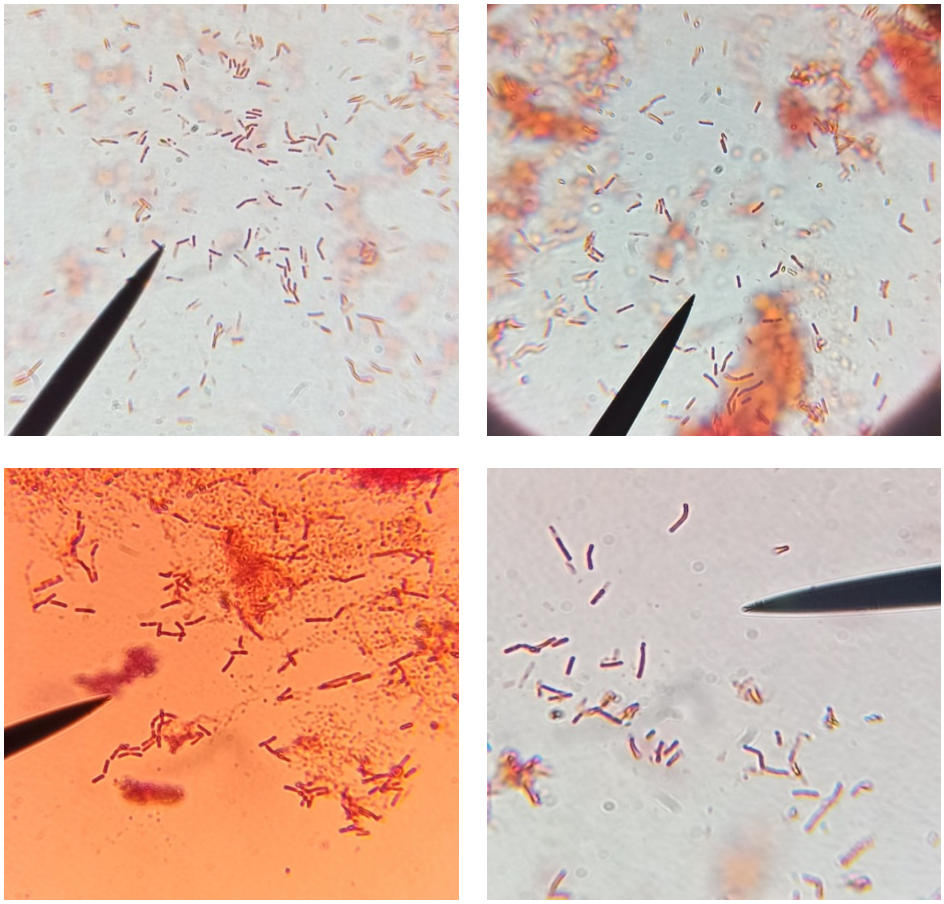

Figure 3. The curved rod-shape cells of isolated Azospirillum strains under compound light microscope magnified x1000.

All of Azospirillum isolates were microscopically observed for their cell shape and gram reaction. The curved rod shape cells for all of the isolated Azospirillum strains were spiral when it was examined under compound light microscope magnified x1000 (Figure 3), and all isolated strains were appeared as a Gram negative, and motile when it was examined in liquid cultures by the hanging drop technique for all isolated strains, and it also was examined by electron microscope magnified x5000 (Figure 4). Krieg and Dobereiner (1984) described the *Azospirillum* species as curved plump rods, 0.8-1.0 × 25 µm in size, and Pandiarajan *et al*., (2012) ranging it from 0.5–1 µm in length, exhibits spirillar movement and polymorphism, containing poly-β- hydroxy butyrate (PHB) granules and fat droplets, And Hall and Krieg, (1983) show that the cells motile with a flagellum. Besides that, all isolated strains were positive for catalase reduction.

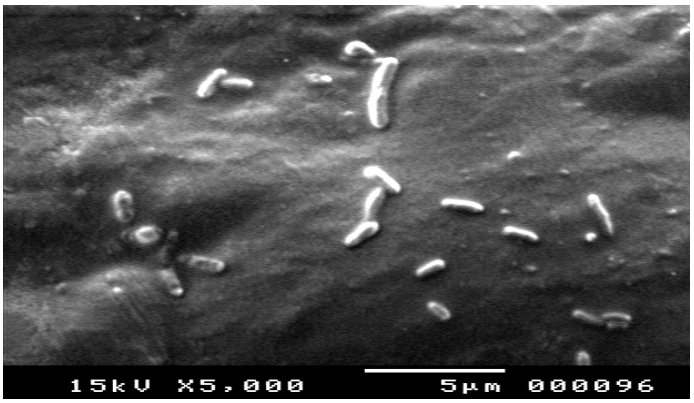

Figure 4. Electron microscope picture of *Azospirillum* cells, strains (As2), magnified x5000.

Table 1. Response of Azospirillum to use different carbon sources

	As1	As2	As3	As4
Fructose	-	-	-	-
Mannitol	-	-	-	-
Cellulose	-	-	-	-
Sucrose	-	-	-	-
Lactose	-	-	-	-
Glucose	-	-	-	-
Peptone	+	+	+	+
Malic acid	+	+	+	+
Gelatin	-	+	+	+
Nitrate	-	+	-	+
Starch hydrolysis	+	+	+	+

The ability of isolated strains to utilize of the different C sources

It was observed that there was variability for isolated strains to utilize various C substrates, at 0.5 % concentrations, as sole C source in Nfb semi solid medium in tubes, for example, the isolated *Azospirillum* strains have been ability to utilize Peptone and Malic acid but unenabled to utilize Fructose, Mannitol, Cellulose,

Sucrose, Lactose and Glucose, while there were different between isolated strains in utilize Gelatin and Nitrate. But it was positive for all isolated strains for starch hydrolysis (Table1).

Determination of N_2 fixation

Nitrogen fixation ability of the 4 isolated strains was measured by micro Kjeldahl method. Among these 4 isolated were able to fix nitrogen. The range of nitrogen fixing ability was from 13.8 to 42.5 mg N_2 fixed/g malic acid (Table 2). Among them, the maximum nitrogen fixing ability (42.5 mg N_2-fixed/g malic acid) was recorded from As4 and minimum (13.8 mg N_2-fixed/g malic acid) was recorded in As3. Among these As1 was recorded 32.0 mg N_2-fixed/g malic acid, and As2 was recorded 37.2 mg N_2-fixed/g malic acid. The potential nitrogen producers were selected for the field experiment.

The highest efficiencies of nitrogen fixation first reported by Dobereiner and Day, (1976) 115 mg N_2 fixed/ g lactate which has not been reported in other studies, Okon *et al*., (1977) reported values of 20 to 24 mg N_2 fixed/ g substrate, while Lakshmi *et al*., (1977) recorded from 12 to 36 mg N_2 fixed/ g substrate, Nelson and Knowles, (1978) reached to 28 mg N_2 fixed/ g substrate, as well as Khan *et al*., (2001) recorded that the N_2-fixing potentials of *Azospirillum* isolated from wheat fields of Dhaka ranged from 15.12 : 22.16 mg N_2 fixed/g substrate, and *Azospirillum* isolated from Bangladesh was fixed nitrogen and ranged from 10.08 : 28.00 mg N_2 fixed/g substrate, Kanimozhi and Panneerselvam, (2010) ranged from 3.3 to 15.6 mg N_2 fixed/g substrate, while Hossain *et al*., (2014) ranging from 10.03 to 13.11 mg N_2 fixed/ g substrate (malate), finally Bharathiraja, (2019) ranged from 1.03: 42.30 mg N_2 fixed/g cell weight.

Azospirillum could convert the atmospheric nitrogen to ammonium under microaerophilic conditions, but the nitrogen levels should below through the action of the nitrogenase complex. Hartmann and Baldani, (2006) have been carried out most of the genetic and biochemical work of nitrogen fixation by Azospirillum to Azospirillum brasilense.

Table 2. Amounts of N_2-fixed/g of malic acid consumed by the four isolated Azospirillum strains grown on NFb semisolid medium

Isolates	mg N_2-fixed/g malic acid
As1	32.0
As2	37.2
As3	13.8
As4	42.5

As results of this characteristic the isolated strain of As4 and As2 were the best isolated strain and selected to be used as abiofertilizer for inoculation of wheat grains in field experimental.

Because of the Azospirillum benefits Soliman, (2018) show that the genus Azospirillum is considered one of the most genera used of plant growth promoting rhizobacteria (PGPR).

Yeast strains

Morphological and cultural characteristic of the isolated strains

Three strains of yeast have been isolated and examined under light microscope with safranine wet mount to describe the size, cell shape, bud formation and split division (Figure 5) of the three isolated yeast strain x1000.

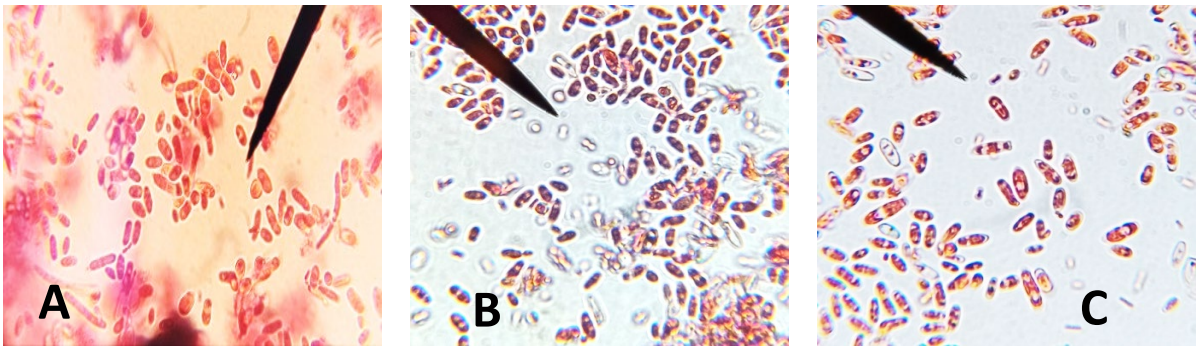

Figure 5. The three isolated yeast strain under compound light microscope magnified x1000.

The yeast isolated strains were examined for colony characteristics on MEPG solid medium. The colonies were appeared with round shape and white butterous colonies strains. It was formed after 72 hrs. The strains A and C were more soft and smooth than strain B.

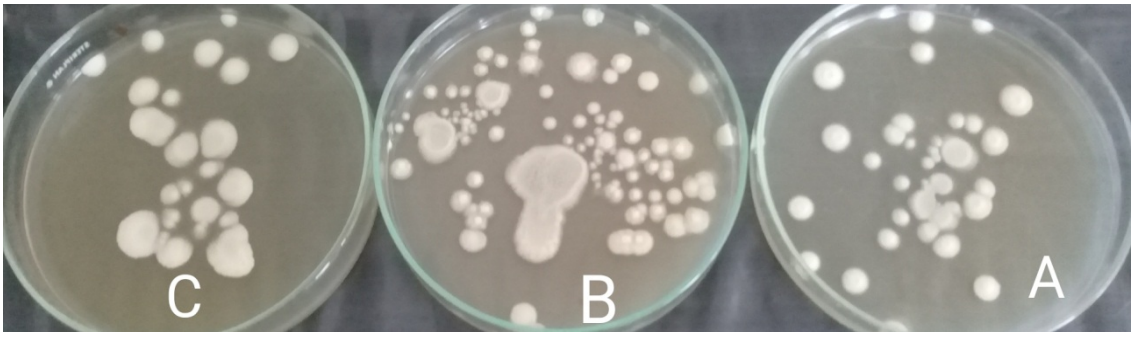

Figure 6. Colony characteristics of the three isolated strains A, B and C incubation on MEPG agar medium.

Production of acids

The production of acids by isolated strains which had due to change in pH of cultures which have been adjusted for pH 7 been determination after 48 and 72 hrs. It was ranged from 3.96 to 4.5 for isolated strains (Table 3). Strain B was considered the best in acids production reached to pH 3.95 after 48hrs. Beside of ability of this strain to solubilization of calcium phosphate and calcium carbonate, it was chosen to use in inoculation field experiments.

Table 3. change in pH of the yeast cultures by the three isolated strains A, B, and C

Strains	pH after 48 hrs.	pH after 72 hrs.
A	4.50	4.50
B	3.96	3.95
C	4.42	4.40

Solubilization of calcium phosphate and calcium carbonate

The Solubilization of tricalcium phosphate by the isolated strains A, B and C were determination on. The strain B was apple to solubilize the calcium phosphate in 3 colonies on the plates as appeared the Halo Zone around the colonies in Figure (7).

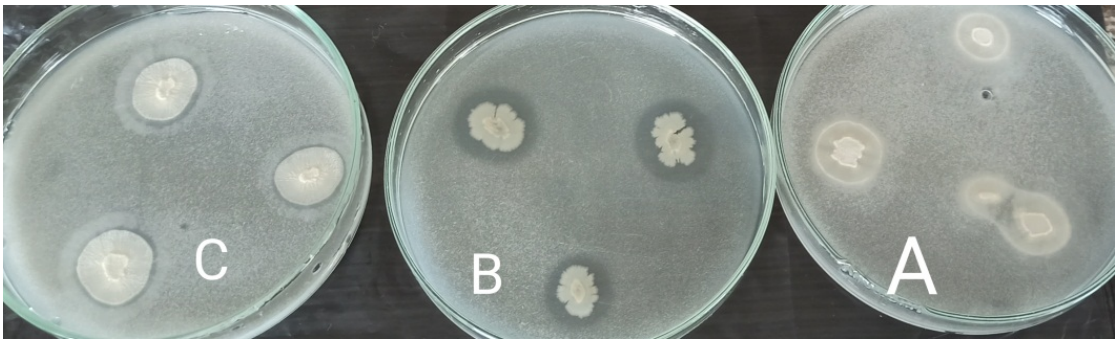

Figure 7. Solubilization of calcium phosphate by isolated strains

The Solubilization of calcium carbonate by the isolated strains A, B and C on the strain B was apple to solubilize the calcium carbonate in 3 colonies on the plates as appeared the Halo Zone around the colonies in Figure (8).

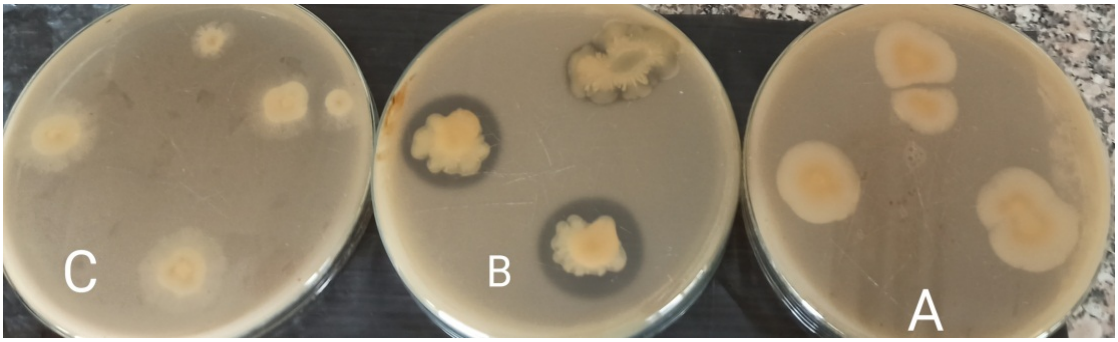

Figure 8. Solubilization of calcium carbonate by isolated strains

As results of acids production, solubilization of calcium phosphate, and solubilization of calcium carbonate (Figure 9) the strain B was the best isolated strain to be used as a biofertilizer for inoculation of wheat grains in field experimental.

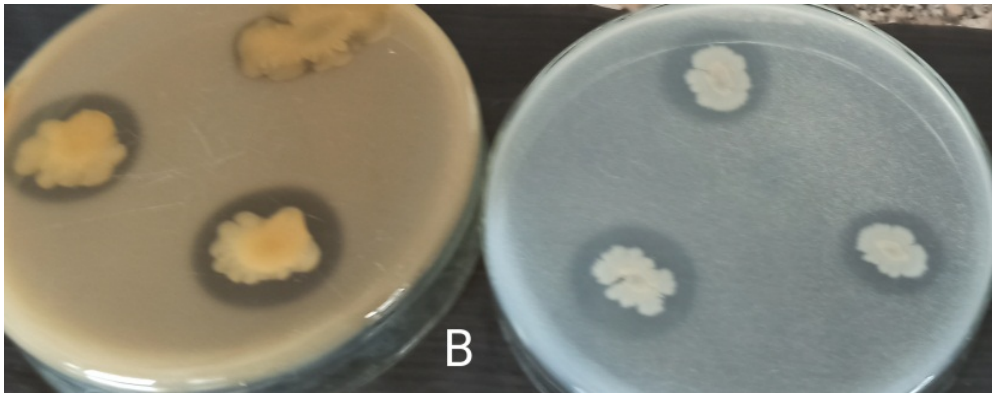

Figure 9. Solubilization of $CaCO_3$ and $Ca_3 (PO_4)_2$ by colonies of yeast strain B.

Ali, (2008) showed that there had many benefits gained to inoculation with yeast which attributed to many advantages, such as; supplying of plants with vitamins (B complex) and the growth regulating hormones (like IAA, Gibberillins, Cytokinins) which increase leaf area, photosynthetic pigments (Chlorophyll a, b), vegetative growth and flowering, and production of organic acids and chelating agents which increase mineral nutrient uptake (like P, Fe, Zn, Mn).

Effects of inoculation of isolated microbial strains Azospirillum and yeasts on wheat grain germination and seedling growth

In these pictures of Figures (10, 11 and 12) which show the effect for inoculation of wheat grains with *Azospirillum* (grains soaked in isolated *Azospirillum* cultures for 2 hrs.) Figure (10) or yeast (grains soaked in isolated yeast cultures for 2 hrs.) Figure (11) or *Azospirillum* + yeast (grains soaked for 2 hrs. in mixed culture of *Azospirillum* and yeast isolated strains) Figure (12) compared with control (grains soaked in water, for 2 hrs.) in all figures after 72 hrs. There was a response to inoculation featured in promoted earlier germination, formation of higher number of roots, longer shoot, size and average seed size after germination. The best of response was for treatment with *Azospirillum* + yeast isolates followed by treatment with *Azospirillum* isolate then treatment with yeast isolate compared with control.

Figure 10. Microbial grains inoculation with Azospirillum isolated strain after 72 hrs. compared with control.

Figure 11. Microbial grains inoculation with yeast isolated strain after 72 hrs. compared with control.

Figure 12. Microbial grains inoculation with Azospirillum + yeast after 72 hrs. compared with control.

Backer *et al*., (2018) show that inoculation with PGPR is considered one of the most promising and safe strategy and it has quite significant role in alleviation of environmental changes under the context of climate extremes and excessive use of fertilizer in agricultural soils. As well as Galindo *et al*., (2022) said that the inoculation of these microbes is recognized as one of the best and alternative strategy for ecofriendly crop-management techniques as it could improve plant nutrition on other hand reducing the dependence of N_2 fertilizer application.

PGPR besides of direct biological nitrogen fixation it also, production of phytohormones and auxins especially IAA which considered as major growth promoting trait for plants (Ahemad and Kibret, 2014; Baggam *et al*., 2017, and Chari *et al*., 2015).

According to Zaheer *et al*., (2019) the *Azospirillum* have the ability to fix nitrogen, for economically important grasses through freely living in the soil or in association with roots, the also positive effects of inoculation with *Azospirillum* are mainly derived from production of phytohormone and from induced changes of morphological in plant roots, resulting in enhanced mineral and water uptake which increasing growth and yield (Burdman *et al*., 1997 and 2000).

Azospirillum brasilense has been reported to promote plant growth by increasing the production of phytohormones like gibberellins, auxins and cytokinins (Fukami *et al*., 2018, and Galindo *et al*., 2022).

Santi *et al*., (2013) showed that phytohormones produced by bacteria thus enhance root branching and root elongation, which in turn favour the uptake of soil water and minerals and has a positive effect on plant growth, and Gibberellin produced by *Azospirillum* was found to play an important role in the early stages of plant growth in Graminae by enhancing shoot and root growth and increasing root-hair density.

Salantur *et al*., (2006) explained that the Local isolates should had been preferred in the selection of rhizobacteria for inoculation, as they are adapted in the environment and it can be more competitive than the foreign rhizobacteria, and the enhancement of crop yields of cereals by inoculation with nitrogen fixing bacteria was observed in many experiments.

Finally, the inoculation of wheat with *Azospirillum sp*. increased the dry weight of roots and 1000-kernel weight, increase number of spikes per plant, number of grains/ spike and grain and straw yield, N-uptake and N-yield in wheat.

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عزل وتوصيف سلالات محلية من الازوسبيريليم والخميرة لغرض استخدامها في تلقيح تقاوي القمح لتحسين نمو النباتات والمحصول الناتج عند الزراعة الحقلية

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الملخص

تم عزل 4 سلالات من الازوسبيريليم و3 عزلات من الخميرة وذلك من تربة منطقة الريزوسفير لنبات القمح المنزرع في المزرعة البحثية لقسم الاراضي والمياه بكلية الزراعة بجامعة أسيوط. بعد ذلك تم دراسة صفات كل من الازوسبيريليم والخميرة وذلك بهدف ان يتم استخدامها لاحقا كأسمدة حيوية في تلقيح حبوب القمح تحت ظروف الزراعة الحقلية وذلك لدراسة استجابة نمو ومحصول القمح لعملية التلقيح. اولا بالنسبة للازوسبيريليم تم العزل على بيئة Nfbالشبه صلبة، ثم تم دراسة الشكل المورفولوجي (تحت الميكروسكوب الضوئي وكذا الميكروسكوب الالكتروني) وايضا الخصائص والصفات المزرعية ومطابقتها بتلك الواردة في Bergeys Manual of Determinative Bacteriology (1994) كما تم تقدير معدل تثبيت النيتروجين الذي تراوح من 13.8: 42.5 ملجم نيتروجين مثبت/جرام حامض ماليك. ثانيا بالنسبة للخميرة تم العزل على بيئة MEPG, ودراسة الشكل المورفولوجي والخصائص والصفات المزرعية وكذلك تم تقدير معدل انتاج الاحماض وانخفاض pH بواسطة العزلات، تلي ذلك دراسة التأثير المشجع لنمو حبوب القمح والانبات المبكر لحبوب القمح الملقحة بواسطة السلالات المعزولة.