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The Effect of Fermented Broth of *Azospirillum spp.*Wild and Mutatnt Typeson *In-vitro* Propagation of *Paulowniatomentosa*

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Abstract: *Azospirillum spp. are considered to be important plant growth promotiverhizobacteria that can improve the growth and yield of several plant species. A collection of Azospirillum spp.isolates were isolated from different regions in Egypt. They were screened for their ability to produce phytohormones especially (IAA and cytokinins). Four isolates were selected depending on their capabilities in production of both IAA and cytokinins. The four isolates were identified depending on 16srDNA gene sequence. One isolate was selected for induction of mutations (K16) using physical mutagen (U.V. light), chemical mutagens (sodium azide). Five U.V. mutants and five sodium azide were selected and their capabilities in production of IAA and cytokinin were recorded. Filtrated fermented broth of two U.V. mutants (U.V.2 and U.V.4) and two sodium azide mutants (AZ3 and AZ4) were added in three concentrations (10, 20 and 40 ml/l MS) and the effect of this fermented broth on the in-vitro propagation of Paulownia tomentosa was studied. Results indicate that treatment with 40 ml/l MS fermented broth was the superior treatment followed by 20, and 10 ml/l MS respectively, fermented broth of U.V.4 (40 ml/l MS) was the best treatment in both root length and number of roots (2.03 and 12 respectively).*

Key words:*Azospirillum sp., fermented broth, U.V. mutagenesis, sodium azide mutagenesis, SDS PAGE, Paulownia tomentosa, In-Vitro propagation.*

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I. Introduction

Azospirillumspp. is an important genus among the plant growth promoting rhizobacteria (PGPR) that display direct beneficial effects on plants [1]**.** Azospirilla are Gram negative free-living nitrogen-fixing rhizosphere bacteria, they display a versatile C- and N- metabolism, which makes them well adapted to establish in the competitive environment in the rhizosphere. Ammonium, nitrate, nitrite and molecular nitrogen and amino acid can serve as N-sources [2]**.** Azospirilla have a worldwide distribution and occur in large numbers (up to 10^7/g) in rhizosphere soils and in association with the roots, stems and leaves of a large variety of different plants. Both *Azospirillumbrasilense* and *A. lipoferum* could be isolated from 30–90% of soil and rhizosphere samples collected all over the world [3]. *Azospirillum spp.* are members of the α-subclass of Proteobacteria, which harbors a large number of plant-associated and symbiotic bacteria, such as *Rhizobium spp.*, *Bradyrhizobium spp.*, *Agrobacterium spp.* or *Gluconacetobacter spp.*[4]**.**

Inoculation of plants with Azospirillum can result in a significant change in various growth parameters in different cereals such as an increase in plant biomass, plant height, root length, number of lateral roots, nutrient uptake, tissue N-content, leaf size, tiller numbers, and volume [5]. Mode of action of Azospirillum on plants are secretion of phytohormones, fixation of atmospheric nitrogen, reduction of nitrate and the enhancement of mineral uptake by plants [6].Corn seedlings inoculated with Azospirillum, relatively higher amounts of free active IAA, indole butyric acid detected when compared to non-inoculated controls [2].Azospirillum grown in culture are known to produce growth promoting compounds, such as gibberllin-like, cytokinin-like substances and auxins such as IAA from tryptophan [8].

Ultraviolet (UV) radiation is one of the simplest and most convenient ways to induce mutations in bacteria, it was used for enhanced ethanol production [9], induction of alcohol tolerance in yeast [10]. Azide is a well-known inhibitor of the terminal segment of the electron transport chain, but it has also been reported to have several effects on the growth of bacterial cells. For example, it was shown to interfere with DNA and RNA synthesis and to induce mutations. In addition, azide-resistant mutant cells are often defective in their control of cell division [11]**.** Sodium azide was used by [12]as a mutagen on *Azopsirillum spp.* to produce overproducer mutants in IAA.

Plant tissue culture techniques and micro propagation depend mainly upon synthetic plant growth regulators, e.g. auxins, cytokinins and gibberellins. Such compounds are known to be produced and released by various rhizospheric microorganisms [13].The genus Paulownia (Scrophulariaceae) includes 9 species of trees indigenous to Chine and East Asia [14].

II. Materials And Methods

1. Isolation of *Azospirillum spp.*

The technique used for isolation was enrichment culture technique described by [15].

2. Screening of isolates

2.1. Primary screening

Primary screening was carried out according to[16], depending on the ability of isolates to produce IAA.

2.2. Secondary screening

Secondary screening was carried out according to [17], depending on the ability of isolates to produce cytokinin.

3. Identification of isolates

Isolates were identified depending on sequence analysis of 16srDNA gene according to [18].DNA was extracted from the bacteria according to [19].

2.3. PCR conditions

The forward primer used (27F) (5′-AGAGTTTGATCCTGGCTCAG-3′)and the reverse primer (1512R) (5′-ACGGCTACCTTGTTACGACT-3′) were used to amplify the 16srDNA gene, the amplification of 16S rDNA was performed in a 50 μL final volume containing 10 μL of total DNA, using 0.2 mmol.L^{-1} of each primer from those listed inthe following table**,** 10 μL of 2.5 mmol·L^{-1} of each dNTP, and 0.05 U of Taq DNA polymerase. The reaction conditions were as follows: 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and primer extension at 72 °C for 2 min; followed by a final extension at 72 °C for 5 min. The reaction products were separated by running 5 μL of the PCR reaction mixture in 1.2% (*w*/*v*) agarose gel, and the bands were stained with ethidium bromide.

4. Mutagenecity

2.4. U.V. mutagenesis

This experiment was carried out according to [20]with some modifications**,**cells from exponential liquid cultures (50 ml)were harvested and centrifuged to pellet the cells, then cells were washed with sterilized distilled water, and diluted in NaCl 0.8 %, and the suspension was poured in 5 cm petri dish, the plate containing cell suspensions was placed on an adjustable platform below a UV germicidal lamp (254 nm) fixed in a chamber. The UV irradiation was carried out by adjusting the distance between UV lamp and culture plate at 20 cm and exposure times from 20 to 120 sec. In original protocol, distance of 37 cm was fixed between UV lamp and culture plate. Irradiated and control samples were immediately diluted serially at 4°C. Three aliquots from a particular dilution were irradiated (as three replicates) for each UV dose. About 0.1ml each from these dilutions (10^{-2}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}) was plated and viable colonies were counted after incubation made in dark at 37°C for 24 h. The frequencies of viable cells (CFU ml^{-1}) in the total bacterial population were determined by comparing the number of colonies to the volume of sample plated and dilution used. Five dilutions were used in the present study whereas previously only three dilutions (10^{-3}, 10^{-4}, 10^{-5}) were utilized for this purpose, the dilution giving the most suitable growth was selected.

2.4.1. Selection of mutants

Mutants were selected by plating the bacteria on Nfb solid media containing 5-flurotryptophan (0.01) mg/ml.

2.5. Sodium azide mutagenesis

This experiment was carried out according to [12]. Azospirillum K16 were overnight growing in Doberainer's medium using rotary shaker at 120 revolutions per min. (rpm) and 28°C giving finally 10^8 CFU/ml. One ml culture suspensionwas plated on Doberainer's agar medium supplemented with different concentrations of sodium azide and then incubated for six days at 28°C. Five single colonies from that appeared in every concentration were picked up and sub-cultured on Doberainer's slant agar medium. The resistant colonies obtained were retested and purified on the same medium containing the same concentrations of sodium azide.

5. In-vitro propagation of Paulownia tomentosa

This experiment was carried out according to[21]but with some modifications, the bioassay experiments were conducted with MS basal medium supplemented with improved strains of *Azospirillum spp*. and wild type supernatant with different concentrations (10, 20, 40 ml/l MS) as a source of phytohormones (Cytokinin and Auxin) at which the supernatant was filtered using syringe filter with (0.22 μm diameter of pores).

Accordingly another experiment was conducted using 40 ml/l MS supernatant in comparison with added synthetic hormones with concentration (1ml/l MS IBA and 3ml/l MSKinetine).
After 6 weeks the following parameters were determined on *Paulownia spp.* Number of shoots, shoot length, number of roots, length of roots, number of leaves and rooting percentage.

6.SDS-PAGE

SDS-PAGE was performed by the method described by[22], modified according to [23]. Total protein extraction was carried out to wild type bacteria, two mutant types of U.V., and two mutant types of sodium azide. Total proteins were electrophoratically separated based on molecular weight, as shown inFig.(4).

7.Statistical analysis

All data obtained were exposed to the proper statistical analysis according to[24] using Costat computer program V 6.303 (2004). LSR at 5% level as significance was used to differentiate between means.

III. Results and Discussion

1. Screening of isolates

Screening was achieved by testing the isolates for their capability to produce IAA (primary screening) and cytokinin (secondary screening). Quantitative determinations of IAA and cytokinin was carried out. Data represented in **Table (1)** shows the different capabilities of the best 10 isolates in the production of IAA and cytokinin. Isolate K16 showed the highest production of IAA (2.226 µg/ml), on the other side isolate K6 represent the superior isolate in the production of cytokinin (1.239 µg/ml).Four isolates were selected according to the screening for further studies, these isolates were (K12, K16, K18 and K28).

Table (1): Primary and secondary screening of the ten primary selected isolates.

Isolate code	IAA (µg/ml)	Cytokinin (µg/ml)
K1	0.991 i	1.182 h
K2	0.979 h	1.194 f
K5	0.987 j	1.203 d
K6	1.203 e	1.239 a
K8	1.181 f	1.214 c
K12	1.256 d	1.196 e
K16	2.226 a	1.169 i
K18	1.647 b	1.189 g
K24	1.029 g	1.221 b
K28	1.344 c	1.112 j
Reference strain	0.973 k	0.966 k

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level

2. Identification of isolates

The selected isolates were subjected to sequence and phylogenetic analysis. As shown in Fig (2) based on the results obtained from BLAST analysis of the nucleotide sequence of 16srDNA gene, the four isolates can be grouped in 3 clusters, all the three clusters represents *Azospirillumbrasilense.* The first cluster contains isolate K16, the second cluster contains isolates (K28 and K12), the third cluster contains isolate K18. These results are in agreement with those obtained depending on physiological and biochemical characterization (data not shown).

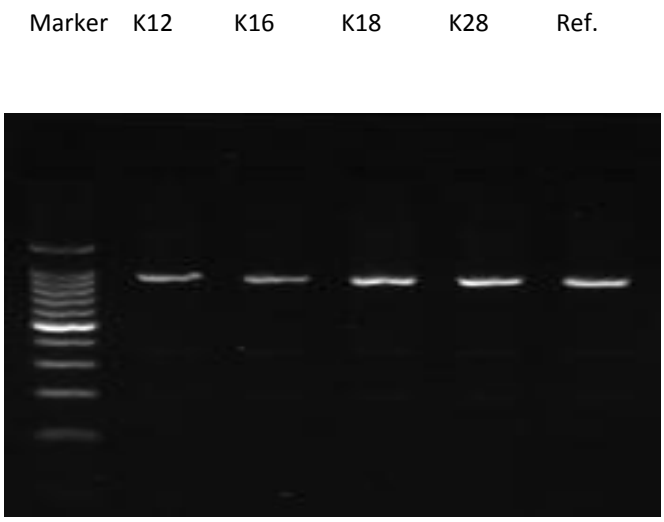

Fig (1) : Amplification of the 16srRNA gene for the selected isolates**.**

Fig (2): The phylogenetic tree showing the relationship between the four selected *Azospirillum spp.*

3. U.V. mutagenesis

Data illustrated in **Table (2)** and **Fig. (3)** represent the viability of *Azospirillum spp.* After exposure of the bacteria to different durations of U.V. rays 254 nm. This experiment was preliminary conducted to determine the lethal dose of ultra violet in mutagenesis of *Azospirillum spp.*. Data shows that the lethal dose (95-99% lethality) was recorded in 120 seconds (2 minutes) which gave 12 colony forming units (CFU) in comparison with control which gave 252 CFU. This dose was selected to induce U.V. mutations in *Azospirillum spp.* Data are in agreement with those obtained by [20]who applied the U.V. as a mutagen on *E.coli* and stated that the lethal dose was 120 seconds.

Table (2): Effect of U.V. exposure duration over the viability of *Azospirillum spp.*

Duration (Sec.)	CFU
Control	252
20	248
40	240
60	225
80	75
100	30
120	12

CFU; colony forming unit.

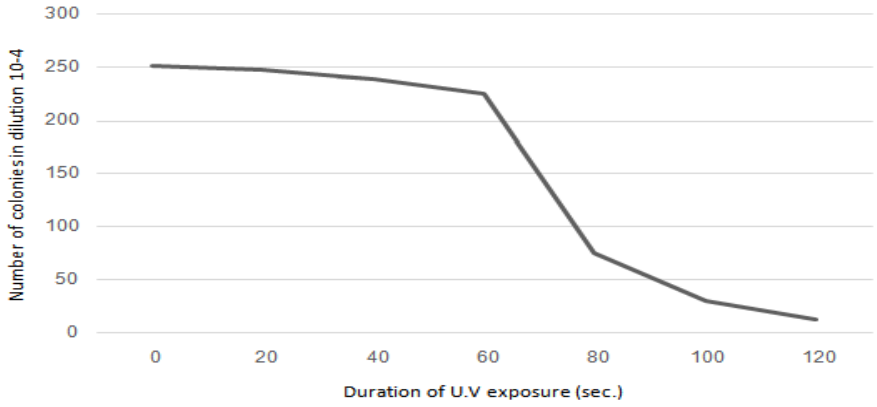

Fig (3): The effect of duration of U.V treatment on the viability of*Azospirillum spp.*

Data in **Table (3)** shows the different capabilities of U.V. mutant types of *Azospirillum spp.*in production of IAA and cytokinin. Data shows a significant difference between isolates at which the superior isolate in the production of IAA was U.V.4 (4.020 µg/ml), and in production of cytokinin was U.V.2 (1.81 µg/ml) compared with wild type (2.230 and 1.17 µg/ml) respectively.

Table (3): Effect of U.V. induced mutation over the production of IAA and cytokinine by *Azospirillum spp.*

Strain	IAA (µg/ml)	Cytokinin (µg/ml)
U.V.1	2.01 f	1.10 e
U.V.2	3.447 b	1.81 a
U.V.3	2.40 d	1.18 d
U.V.4	4.020 a	1.76 b
U.V.5	2.52 c	1.22 c
K16	2.230 e	1.17 d

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level

4. Sodium azide mutagenesis

An experiment was conducted on strain K16, with three concentrations (25, 27, 29 µg/ml) of sodiumazide, the concentration of 27 µg/ml was selected and five mutant colonies were chosen which then further studied for their abilities in the production of IAA and cytokinin. Data in **Table (4)** show the different abilities of the five mutant types of sodium azide in comparison with control. Results showed that there was a significant difference in the production of IAA at which the highest production was recorded in strain AZ4 with an increase 61 % than control, while the highest production in cytokinin was recorded in AZ3 with an increase 66 % than control. These data are in agreement with those obtained by [12], who obtained over producer of *Azospirillumbrasilense* of IAA.

Table (4): Production of IAA and cytokinin obtained from *Azospirillum spp.* K16 treated by sodium azide (27 mg/ml)

Strain	IAA (µg/ml)	Cytokinin (µg/ml)
AZ1	2.781 c	0.98 f
AZ2	2.470 d	1.62 c

AZ3	3.424 b	1.93 a
AZ4	3.632 a	1.88 b
AZ5	2.207 f	1.22 d
K16	2.251 e	1.16 e

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level

5. Analysis of total protein banding pattern

Total proteins were electrophoratically separated based on molecular weight. As shown in Fig. (4) and Different responses appeared as different banding pattern, either appearance or disappearance of bands was obtained. The maximum bands number was 25. Out of them; 15 bands were detected in all strains (wild and mutants) (monomorphic bands). The molecular weight of electrophoretic products are ranged from 7.5 to 130 KDa.

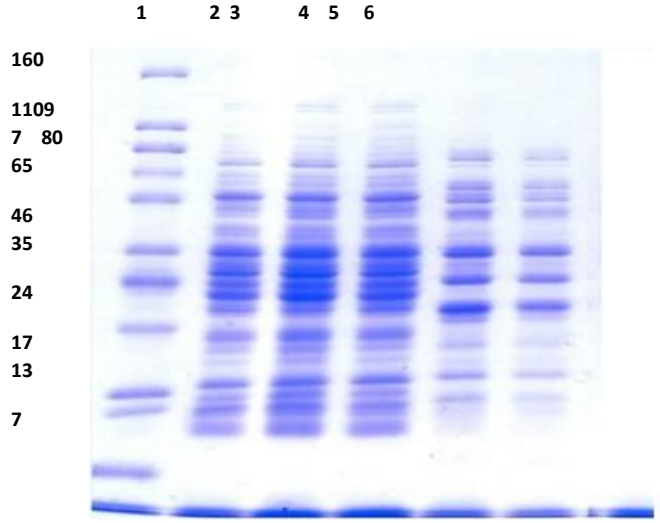

Fig (4): Electrophoretic separation for wild type K16(lane 2)**,** Mutants (U.V.2 lane3, U.V.4 lane 4, Az3 lane5, Az4 lane 6) and standard marker (lane 1).

6. In-vitro propagation of Paulownia spp.

Two experiments were carried out on *Paulownia spp.* To examine the effect of fermented broth of different *Azospirillum spp.* mutants (U.V.2, U.V.4, AZ3 and AZ4 and fermented broth of wild type K16, in comparison with the control (1ml/l MS IBA and 3ml/l MSKinetine). The first experiment was carried out to determine the effect of different concentrations of the fermented broth added for MS medium, and the second was conducted for screening the different effects of the mutants of *Azospirillumbrasilense* comparison with control. Data represented in Table (5)illustrate the effect of different concentrations of fermented broth of *Azospirillumbrasilense* (mutants and wild type) added. Data showed a significant difference between the three concentrations on root length, at which the concentration 40 ml/l MS gave the highest root length 1.56 cm, followed by concentration 20 ml/l MS 1.11 cm and finally the concentration 10 ml/l MS which gave 0.55 cm. Also significant difference was shown between the three concentrations on number of roots as the best result was in concentration 40 ml/l MS 9 roots, followed by 20 ml/l MS 6 roots and finally 3 roots in the concentration 10 ml/l MS. The results obtained on the number of shoots showed that there were no significant difference between concentrations of 20 ml/l and 10 ml/l MS as they gave mean number of 1.2 and 1 respectively, and there were significant difference in the concentration 40 ml/l MS as the mean number of shoots was 1.6. Results obtained on shoot length showed that there were no significant difference between the two concentrations 40 ml/l and 20 ml/l MS as they gave 4.69 and 4.54 cm respectively. Significant difference was shown in the concentration of 10ml/l MS 3.45 cm. Data obtained on number of leaves showed no

significant difference between 10 ml/l, 20 ml/l and 40 ml/l MS concentrations and results was 12 leaves per plant in the three concentrations.

Data in Table (6) represent the effect of using 40ml/l MS supernatants of wild type (K16) and mutants (U.V2, U.V4, AZ3 and AZ4) in comparison with control (1 ppm IBA and 3 ppmKinetine), and data showed no significant difference between used supernatants and control in rooting percentage. Fig (7)showed the different response of Paulownia plantlet for treatment with fermented broth of *Azospirillum spp.* mutants (U.V.2, U.V.4, AZ3 and AZ4) with concentration 40 ml/l MS compared with control (1 ppm IBA and 3 ppmKinetine).

Significantly highest number of shoots was obtained in AZ3 which was 4 shoots, and there were no significant difference between other treatments as they gave 1 shoot. The highest number of leaves was obtained in AZ3 treatment which was (18 leaves) followed by both U.V2 and U.V4 (13 leaves), followed by control (12 leaves) and finally AZ4 and wild type K16 both gave (9 leaves).

The highest shoot length was obtained in treatment AZ3 which was 6.4 cm followed by control 5.97 cm, 5.5 cm for U.V4 and 5.3 cm for U.V2. Thus there were no significant difference between these treatments. But there were significant difference between those four treatments and treatments of K16 and AZ4 (3.67 and 3.13 cm respectively).

The superior treatment in root length was obtained in U.V4 (2.03 cm) which gave a significant difference in comparison with other treatments, followed by u.v.2, az3, control, az4 and k16. Their results were (1.8, 1.7, 1.57, 1.57 and 1.07 respectively).

Data obtained in number of roots gave significant difference between treatments at which the highest root number was obtained in treatment U.V.4 followed by U.V.2, AZ3, AZ4, control and finally K16. The results were (10.33, 9.67, 9, 7.67 and 5.67 respectively). These observations indicate the possibility of large-scale use of such bacterial supernatant for propagation of *Paulownia spp*.These data are in agreement with those obtained by [21].

Table (5): Effect of different concentrations of the fermented broth of *Azospirillumbrasilense*K16 mutants and wild type added on development of Paulownia plants grown *in vitro.*

Concentration	N.O of roots	Root length (cm)	N.O of Shoots	Shoot length (cm)	N.O of leaves
10 ml	3 c	0.55 c	1 b	3.45 b	12 a
20 ml	6 b	1.11 b	1.2 b	4.54 a	12 a
40 ml	9 a	1.56 a	1.6 a	4.69 a	12 a

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level

Fig (5): Plantlet produced from treatment with concentration 10 ml/ l MS fermented broth of mutant types and wild type of *Azospirillum spp.* K16

U.V.2 **U.V.4A.Z.3** **A.Z.4** **K16**

Fig (6): Plantlet produced from treatment with concentration 20 ml/ l MS fermented broth of mutant types andwild type of *Azospirillum spp.* K16.

U.V.2 **U.V.4A.Z.3** **A.Z.4** **K16**

Fig (7): Plantlet produced from treatment with concentration 40 ml/ l MS fermented broth of mutant types and wild type of *Azospirillum spp.* K16

Table (6): The effect of addition of 40 ml/l MS fermented broth of *Azospirillumbrasilense* mutants and wild type in comparison with control (1ml/l MS IBA and 3ml/l MS Kinetine) on development of Paulownia plants grown *in vitro*.

Treatment	N.O of roots	Root length	% of Rooting	N.O of shoots	Shoot length	N.O of leaves
AZ3	10 ± 1.15 b	1.70 ±0.10 b	100	4 ± 1.73 a	6.40 ± 1.44 a	18 ± 1 a
AZ4	9 ± 1 bc	1.57 ± 0.15 b	100	1 ± 0 b	3.13 ± 0.86 b	10 ± 3.5 b
U.V.2	11 ± 0.58 ab	1.8 ± 0.10 b	100	1 ± 0 b	5.33 ± 0.58 a	13 ± 2.3 b
U.V.4	12 ± 0.58 a	2.03 ± 0.15 a	100	1 ± 0 b	5.5 ± 0.87 a	13 ± 2.3 b
K16	6 ± 1.52 d	1.07 ± 0.12 c	100	1 ± 0 b	3.67 ± 1.04 b	10 ± 1.15 b
Control	8 ± 1.15 c	1.57 ± 0.11 b	100	1 ± 0 b	5.96 ± 0.15 a	12 ± 0.58 b

Values in the same column followed by the same letter do not significantly differ from each other, according to Duncan's at 5 % level

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