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CHARACTERIZATION AND MORPHOLOGICAL STUDY OF *AZOTOBACTER SP.* STRAIN

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Abstract: Plant growth promoting rhizobacteria (PGPR) are a group of free-living bacteria that colonize the rhizosphere and contribute to increase growth and yield of crop plants. PGPR possess many traits that make them well suited as biofertilizers and biocontrol agents, such as: rapid growth and utilization of seed and root exudates, colonization as well as multiplication in the rhizosphere. Among the PGPR group, *Azotobacter* is a free-living N2- fixer diazotroph that has several beneficial effects on the crop growth and yield. It helps in the synthesis of growth regulating hormones like auxins, cytokinin and giberellic acid (GA) and also stimulates rhizospheric microbes, protects the plants from phytopathogens and improves boost up nutrient assimilation.

Strain morphology and cultivation characteristics were investigated with a modification of Ashby's, Azotobacter and Jensen's media. Colony forming units per ml were used as main control parameter in the process of cultivation and were measured on Jensen media with bromophenol blue addition. The biochemical profile of *Azotobacter sp. strain* was determined by API 20NE and API ZYM systems (BioMericux, France). Protease, lipase, amylase productions and phosphate solubilization were investigated of the Azotobacter sp. strain to prove its plant growth promoting activity (PGP-activity). The strain was identified as *Azotobacter vinelandii* by molecular technique and the antagonistic effect was investigated on fungal phytopathogens *Fusarium, Aspergillus, Rhizopus* and *Penicillium*.

INTRODUCTION

Among the plant growth promoting rhizobacteria group (PGPR), Azotobacter is a free-living N2-fixer diazotroph that helps in the synthesis of growth regulating metabolites and also stimulates rhizospheric microbes and improves their

nutrient assimilation. About 95% of soil microorganisms can produce different metabolites, regulate all reactions in a plant, including stress response. They play an essential role in plants growth and development (Davies, 2004, Chobotarov *et al.,* 2017).

Azotobacter spp. are Gram negative, free-living, aerobic soil dwelling, oval or spherical bacteria. They form thick-walled cysts and are characterized by asexual reproduction under favorable condition. There are around six species in the genus *Azotobacter* some of which are mobile by means of peritrichous flagella, others are not. They are typically polymorphic and their size ranges from 2-10 μm long and 1-2 μm wide (Muthuselvan and Balagurunathan, 2013; Viscardi *et al.,* 2016).

Azotobacter vinelandii is also a plant growth promoting rhizobacterium, living near plant roots and potentially increasing crop yields. The strain can also increase plant growth by increasing the nodulation activity of local rhizobia around legume roots and by producing plant growth-promoting metabolites such as indole acetic acid (IAA), gibberellic acid (GA), siderophore production, phosphate solubilization. Some strains show potential for plant pathogen biocontrol via antifungal activity (Wani *et al.,* 2016; Noar and Bruno-Barcena, 2018). In order to guarantee the high effectiveness of inoculants and microbiological fertilizers, it is necessary to find the compatible partners, i.e. a particular plant genotype and a particular *Azotobacter* strain that will form a good association.

The aim of the research was to investigate different nutrient media and to determine the optimal media for *Azotobacter sp.* (an isolate), its biological effect, yield of cells and antagonistic effect on fungal pathogens.

MATERIALS AND METHODS

Microorganism and its cultivation media

For the purpose of the study, a culture of the genus *Azotobacter* was taken from the collection of the Department of Biotechnology, Faculty of Biology, Sofia University St. Kliment Ohridski and was morphologically, biochemically, molecularly and antagonistically examined.

Three different modified media were used in the experiments: Jensen's medium (g/l) (K_2HPO_4 – 1; $MgSO_4$ – 0,5; NaCl – 0,5; $FeSO_4$ – 0,1; trace of Na_2MoO_4; $CaCO_3$ – 2; Sucrose – 20); Ashby's medium (g/l) (K_2HPO_4 - 0,2; $MgSO_4$ – 0,2; NaCl – 0,2; $CaSO_4$ – 0,1; $CaCO_3$ – 10; Glucose – 10) and Azotobacter medium (g/l) (K_2HPO_4 - 0,9; KH_2PO_4 – 0,1; $MgSO_4$ – 0,1; $CaCO_3$ – 5; trace of Na_2MoO_4; $CaCl_2$ – 0,1; $FeSO_4$ – 0,01; Glucose – 5) (Chobotarov, A. *et al.,* 2017; Upadhyay, S. *et al.,* 2015). To isolate a single colony, the suspension of the sample is inoculated on the three different media and incubated at 30° C for three days.

For the accumulation of biomass, the strain was cultivated in a liquid medium of different composition and amount of nutrients. Batch cultivation was performed in flasks which are incubated on a rotary shaker incubator at a

rotation speed of 250 rpm and 30° C for 72 hours. Biomass concentration was determined as colony forming units per ml. The main control parameter in the process of cultivation was cfu per ml and was measured on Jensen's media with bromophenol blue addition.

Characterization of Azotobacter sp.

Morphological characterization of *Azotobacter sp.* was investigated by microscopic method and the cell shape was determined. A biochemical test of catalase activity was carried out on the strain. The test showed the ability of some microbes to degrade hydrogen peroxide by producing the enzyme catalase. About 1 ml of 3 % of H_2O_2 was poured over *Azotobacter* culture on a microscopic slide. Intensive bubbling (*Azotobacter*) was noted as catalase positive, light bubbling was weakly positive; no gas production was catalase negative (Sandeep Upadhyay *et al.,* 2015).

Determination of biochemical activities *- API® ZYM, API® 20NE, protease, lipase, amylase productions and phosphate solubilization*

The biochemical properties of Azotobacter sp. strain was analyzed with API® 20NE and API® ZYM kits (Biomerieux, France) according to the manufacturer's recommendations. To characterize the microorganism, the enzyme activity profile was examined. The API® ZYM system (BioMericux, France) was used to qualify the enzyme profile of the test strain. API® 20NE determined the nitrate reduction, indole production, β-galactosidase and urease activities, hydrolysis of esculin and gelatin, and absorption of 12 substrates. Both analyzes were performed at 30°C and the time of cultivation of API® 20NE was 48 hours and API® ZYM - 4 h.

Protease production - An overnight bacterial culture was transferred to a selective medium, containing 15 g of skim milk, 0,5 g of yeast extract and 2% of agar. The bacterium was spotted on plates of SMA medium and incubated at 30°C for 48 h. Its ability to produce protease was determined by measuring the diameter of halo zones around bacterial colonies (Ghodsalavi *et al.* 2013).

Lipase production - An overnight culture was incubated on a selective medium, containing 10 g of peptone, 0,1 g of $CaCl_2$, 5 g of NaCl, 15 g of agar. The experiment was performed at 30°C for 48 h and the lipase activity was determined based on the presence of depositions around the bacterial colonies (Ghodsalavi *et al.*2013).

Amylase production- The test was performed to determine the capability of tested organism to use starch as carbon source. In this test, starch agar is inoculated with the *Azotobacter* strain. After incubation at 30°C temperature for 48 h, iodine was added to the surface of the agar. Absence of the blue-black color indicated that starch is no longer present in the medium. When a halo zone around the growing strain existed, that was a marker of the production of exoenzyme amylase (Bird *et al* 1954).

Phosphate solubilization - Phosphate-solubilizing bacterium (PSB) was identified on Pikovskaya's medium containing 10 g of glucose, 2,5 g of $Ca_3(PO4)2$; 0,5 g of $(NH_4)_2SO_4$; 0,2 g of NaCl; 0,1 g of $MgSO_4$ $7H_2O$; 0,2 g of KCl; 0,5 g of yeast extract; 0,002 g of $FeSO_4$ $7H_2O$; 0,002 g of $MnSO_4$ $7H_2O$ and 15 g of agar (Nautiyal 1999). Bacterium from an overnight culture was spotted on such a medium. After 7 days of incubation at 30°C, PSB was identified, based on the presence of a halo zone around the bacterial culture.

Identification of Azotobacter sp.

Molecular identification by determining the nucleotide sequences of 16S rRNA gene, was carried out on *Azotobacter sp*. strain. Isolation and sequencing were performed at Microgen Ltd, South Korea - Netherlands office. The sequences were processed by the Cromas program and by the BLAST algorithm, and compared with already stored sequences in GenBank DNA database. A phylogenetic tree was constructed by NCBI database sequences (https://www.ncbi.nlm.nih.gov/blast/treeview).

Antagonistic effect of Azotobacter vinelandii on fungal strains

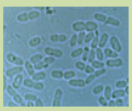
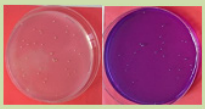
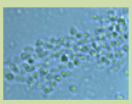
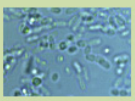
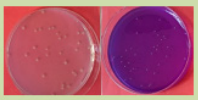
The modified protocol of agar-layer diffusion method was applied as follow (Tropcheva *et al.,* 2014): overnight culture of strain *Azotobacter vinelandii* was mixed with temperate PDA agar at 45°C in equal volumes and poured into plates. The agar plates, with sterile Jensen's media were prepared as a control. After drying of the plates, 3 µl of mold spores suspension of *Fusarium sp.* (an isolate), *Penicillium claviforme, Aspergillus flavus* and *Rhizopus* arrhizus were inoculated as a discrete spot into the center of the surface of the agar layer in each plate. The plates were incubated aerobically in an upright position at 30°C. Diameters of the growing mold colonies were measured daily until the mold in the control sample completely filled the volume of the Petri dish. Experiments were performed in triplicates.

RESULTS AND DISCUSSION

Characterization of Azotobacter sp. strain

Azotobacter is a free living bacteria, which grows on a nitrogen free medium. The main objective of the present study was to identify and characterize the plant growth promoting activities of the *Azotobacter sp.* strain. The investigated strain was cultivated in modified media, described in part "Materials and Methods". For appropriated visualization of the colony formation bromophenol blue was used. Different morphological and colony characteristics were observed and the results were presented in table 1:

Table 1. Characterization of *Azotobacter sp.*

MEDIA	AZOTOBACTER MORPHOLOGY	COLONI FORM OF AZOTOBACTER STRAIN	CATALASE ACTIVITY
Jensen's medium rod cells, paired, slightly elongated in form.			 +
Ashby's medium small and rounded cells, in pairs.			 +
Azotobacter medium elongated cells, in pairs			 +

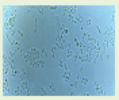
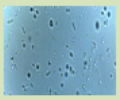
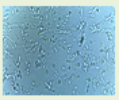
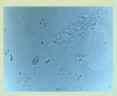
The formed colonies of *Azotobacter sp.* strain were small and difficult to elucidate. The modification of the nutrient media enhanced the differentiation of the cell colonies.

The *Azotobacter sp.* strain had rod cells with elongated form and cells in pairs. All probes were investigated for purity of the culture cells. The presence of catalase production was determined. All probes of the *Azotobacter sp.* strain demonstrated catalase activity. The result corelates the findings of Shamim Akhter *et al.,* 2012; Upadhyay *et al.,* 2015; Sadik *et al.,* 2016, Noar and Bruno-Barcena, 2018, where the strains of genus *Azotobacter* had catalase and oxydase production.

Biochemical characterization of Azotobacter sp. strain

Colony forming units per ml were used as main control parameter in the process of cultivation (table 2). The results from the different cultivation experiments demonstrated that the modified Jensen's media was optimal for culture growth.

Table 2. Growth characteristics and cell density during batch cultivation of *Azotobacter sp.* on modified Jensen's, Azotobacter and Ashby's media

MEDIA	MORPHOLOGY	cfu/ml	
Jensen's medium *with $CaCO_3$*	multiple rod-shaped cells, paired in irregular clusters; easy to differentiate	1,3 x 10^7	
Jensen's medium *without $CaCO_3$*	rod-shaped cells, paired in irregular chains; easy to differentiate	4,1 x 10^6	
Azotobacter medium *with $CaCO_3$*	elongated cells, in pairs	3,8 x 10^6	
Azotobacter medium *without $CaCO_3$*	elongated, rod-shaped cells in chains of different length	7,0 x 10^6	
Ashby's medium *with $CaCO_3$*	small round-shaped cells, paired; hard to differentiate	2,1 x 10^6	
Ashby's medium *without $CaCO_3$*	small cells, forming chains; hard to differentiate	3,2 x 10^6	

As a result of these experiments, it was found that the strain growth in all different media and Jensen's nutrient medium with $CaCO_3$ was optimal for biomass accumulation - 1,3x10^7 cfu/ml. The same results were obtained by Chennappa G. *et al.,* 2016, as the isolated strains of *Azotobacter* were cultivated in Jensen's N-free media. Various metabolites as catalase, phytohormones, siderophore and enzymes were produced. The Azotobacter medium was suitable for maintaining the strain as described in the catalogue of the National Bank for Industrial Microorganisms and Cell Cultures – Bulgaria, because the strain had biomass concentration - 10^6 cfu/ml.

The biochemical profile of *Azotobacter sp.* strain was investigated by API 20NE and API ZYM systems (BioMericux, France) (table 3).

Table 3 API 20NE and API ZYM tests of *Azotobacter sp.*

Azotobacter sp.							
API 20NE	*test results*	*API ZYM*	*activity*				
			1	*2*	*3*	*4*	*5*
NO_3	+						
TRP	-	Aklaline phosphatase					
GLU	+	Esterase (C-4)					
ADH	+	Esterase lipase (C-8)					
URE	+	Lipase (C-14)					
ESC	+	Leucine arylamidase					
GEL	-	Valine arylamidase					
PNG	+	Cystine arylamidase					
GLU	+	Trypsin					
ARA	+	α-Chymotrypsin					
MNE	+	Acid phosphatase					
MAN	+	Phosphohydrolase					
NAG	-	α-Galactosidase					
MAL	+	β-Galactosidase					
GNT	+	β-Glucuronidase					
CAP	+	α-Glucosidase					
ADI	-	B-Glucosidase					
MLT	-	N-Acetyl-β-glucosaminidase					
CIT	-	α-Mannosidase					
PAC	-	α-Fucosidase					

The ability of phosphate solubilization and enzymes productions as protease, lipase and amylase was determined (table 4). API 20NE was used to define the profile of assimilation and conversion of various substrates, suitable for the identification of the strain. The enzyme activity is determined by a color scale of 0 (lack of enzyme activity) to 5 (maximum enzyme activity) by API ZYM. Some low level of esterase (C-4), acid phosphatase and phosphohydrolase activities were detected.

The isolated strain was positive to fermentation D-glucose, produce enzymes to hydrolysis L-arginine, urea, esculin ferric citrate, 4-nitrophenyl-β-D galactopyranoside, assimilated D-glucose, L-arabinose, D-mannose, D-manithol, D-maltose, potassium gluconate and capric acid and production of esterase, acid phosphatase, phosphohydrolase, protease and amylase. The strain was negative for production of indole and did not hydrolyze gelatin. *Azotobacter sp.* assimilated N-acetyl-glucosamine, adipic acid, malic acid, trisodium citrate and phenylacetic acid and did not produced lipase enzyme.

For visualization of the production of amylase enzymes, the Petri dishes were inoculated with iodine solution and clear zones around the colonies were observed. Therefore positive result was obtained from the starch hydrolysis assay. This indicated that the isolate had the potential to hydrolyze starch, present in the medium.

Table 4 Phosphate solubilization and different enzymes productions of *Azotobacter sp.* Strain

ACTIVITIES OF AZOTOBACTER STRAIN	
Phosphate solubilization	
Protease production	
Lipase production	nd
Amylase production	

nd – not determined

It was observed that Pikovaskaya's agar, when inoculated with the isolate of Azotobacter sp. did show a clear zone of hydrolysis around and the bacterial growth. That indicated its ability to solubilize phosphates (table 4). Chennappa et al. (2016) showed that twelve of *Azotobacter* isolates demonstrated an ability of nitrogen fixation, indol acetic acid, gibberellic acid production and phosphate solubilization.

All PGPR activity - enzyme production, phosphate solubilization, antagonistic effect on phytopathogens were significant and all were related to their PGP efficiency.

Identification of Azotobacter sp.

The biochemical test showed that the isolate could be considered as *Azotobacter sp.* (table 3 and 4). For the confirmation of that result molecular identification was carried out 16S ribosomal coding gene identification. The Blast result revealed that obtained nucleotide base pairs showed 99 % similarity to *Azotobacter vinelandii* NBRC 102612. Furthermore, the sequence was used to construct phylogenetic tree, using related sequences (**figure 1**). Similar sequence of 16S rRNA c had been reported by Muthuselvan *et al.,* 2013.

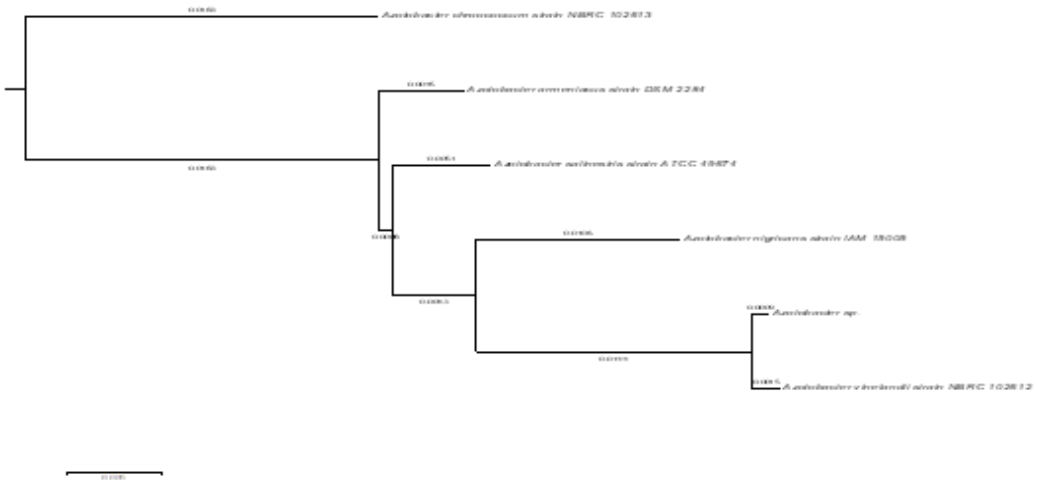
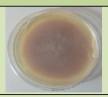
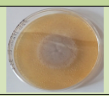
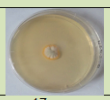
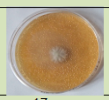
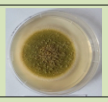
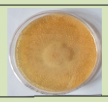
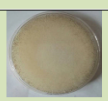


Fig. 1. Phylogenetic tree of strain *Azotobacter sp*

Antagonistic effect of Azotobacter vinelandii on fungal strains

The strains of genus *Azotobacter* were evaluated for their ability to control plant root fungal pathogens such as *Fusarium sp., Aspergillus, Alternaria, Mucor* and ext. The antagonistic properties of *Azotobacter vinelandii* were tested against plant fungal pathogens such as *Fusariurm sp.* (an isolate), A*spergillus flavus, Penicillium claviforme* and *Rhizopus arrhizus* by dual culture technique. It was found that the *Azotobacter vinelandii* inhibited the tested fungal pathogens *Fusarium sp.* and *Aspergillus flavus*, as the percentage of inhibition was around 50% (table 5). The same results were obtained by Muthuselvan *et al,* 2013

Table 5 Antifungal activity of *Azotobacter vinelandii*

FUNGAL TEST	DIAMETER OF CONTROL /144 H/	ANTIFUNGAL ACTIVITY OF AZOTOBACTER VINELANDII
Fusarium sp.(an isolate)		
	71 mm	37 mm
Penicillium claviforme		
	17 mm	17 mm
Aspergillus flavus		
	67 mm	27 mm
Rhizopus arrhizus		
	80 mm	80 mm

The strain *Azotobacter vinelandii* demonstrated inhibitory effect effect against all fungal strains, % at day 6, against *Aspergillus flavus* - 59,7 % and against *Fusarium sp.* - 47,9%. Antifungal effect was not observed on *Rhizopus arrhizus* and *Penicillium claviforme*, which are fungal pathogens on fruits and food. Many researchers also reported antifungal activity of *Azotobacter sp.,* isolated from different soil samples as they studied the effects of *Azotobacter sp.* on *Fusarium sp.* which was isolated on the root colonization. The inhibition effect on *Aspergillus terrus, Alternaria alternata, Aspergillus flavus* and F*usarium oxysporum* by *Azotobacter chroococcum*, isolated from rhizosphere soil, was evaluated by Bhosale *et al,* 2013; Muthuselvan and Balagurunathan, 2013.

These results indicated that *Azotobacter vinelandii* culture have antifungal properties and could control major plant pathogens by producing antifungal metabolites.

CONCLUSIONS

The modification of the three media enhanced the differentiation of the cell colonies by bromophenol blue additions. For batch cultivation of *Azotobacter vinelandii* was used optimal Jensen's N-free media and various metabolites were obtained. The investigated strain produced different enzymes – catalase, protease, amylase activities, phosphate solubilization which were important properties and all were related to their PGP efficiency. The molecular result, obtained from the investigated strain showed 99 % similarity to *Azotobacter vinelandii.* The strain had antifungal capacity to inhibit the growth of *Aspergillus flavus* and on *Fusarium sp.* Further investigation of strain *Azotobacter vinelandii* is necessary to develop PGP capacity and investigate the production of microbial metabolites and their applications in various fields.

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CONFLICT OF INTERESTS: The author declares no conflict of interests. E.Y. has written the manuscript, made the experiments and discussed the results.

REFERENCES

1. Bhosale, H. J., Kadam, T. A., Bobade, A. R., 2013. Identification and production of Azotobacter vinelandii and its antifungal activity against Fusarium osporum. *Journal of environmental biology,* 34, 177-182.
2. Bird R, Hopkins R.H., 1954.The action of some alphaamylases on amylase, *Biochem. J.* 56: 86–99.
3. Chennappa, G., Naik, M. K., Adkar-Purushothama, C. R., Amaresh, Y. S., Sreenivasa, M. Y., 2016. PGP potential, abiotic stress tolerance and antifungal activity of Azotobacter strains isolated from paddy soils, *Indian journal of experimental biology,* vol. 54, 322-331

4. Chobotarov A., Volkogon M., Voytenko L., Kurdish I., 2017. Accumulation of phytohormones by soil bacteria Azotobacter vinelandii and Bacillus subtilis under the influence of nanomaterials, *J Microbiol Biotech Food Sci,* 7 (3), 271-274
5. Davies, P. J., 2004. Plant Hormones - Biosynthesis, Signal Transduction, Action! Berlin, New York: Springer-Verlag.
6. Ghodsalavi B, Ahmadzadeh M, Soleimani M, Madloo PB, TaghizadFarid R., 2013. Isolation and characterization of rhizobacteria and their effects on root extracts of Valeriana officinalis, *Aust J Crop Sci,* 7: 338–344
7. Jesse D. Noar and Jose M. Bruno-Barcena, 2018. Azotobacter vinelandii: the source of 100 years of discoveries and many more to come, *Microbiology*, 164: 421–436, DOI 10.1099/mic.0.000643
8. Md. Shamim Akhter, Sheikh Julfikar Hossain, SK. Amir Hossain, Rakesh Kumar Datta, 2012. Isolation and characterization of salinity tolerant Azotobacter sp, *Greener Journal of Biological Sciences,* ISSN: 2276-7762, Vol. 2 (3), 043-051
9. Muthuselvan, I. and Balagurunathan, R., 2013. Siderophore production from Azotobacter sp. and its application as biocontrol agent, *International Journal of Current Research and Review,* 5(11), 23.
10. Sadik, A. S., El-Khamash, N. A., Mohamed, S. H., 2016. Isolation and characterization of free-nitrogen fixer bacterial strains (Azotobacter sp.) and their phages from maize rhizosphere-soil at Taif.,Pak. *J. Biotechnol,* 13(1), 31-37.
11. Sapna C., Kunal W., Manjula V., Neeru N., 2012. Potential of Azotobacter spp. as biocontrol agents against Rhizoctonia solani and Fusarium oxysporum in cotton (Gossypium hirsutum), guar (Cyamopsis tetragonoloba) and tomato (Lycopersicum esculentum), *Arch Agro Soil Sci;* 58 (12): 1365-1385.
12. Upadhyay, S., Kumar, N., Singh, V. K., Singh, A., 2015. Isolation, characterization and morphological study of Azotobacter isolates, *Journal of Applied and Natural Science,* 7(2), 984-990.
13. Viscardi S., Ventorino V., Duran P., Maggio A., Pascale De S., Mora M.L., Pepe O., 2016. Assessment of plant growth promoting activities and abiotic stress tolerance of Azotobacter chroococcum strains for a potential use in sustainable agriculture, *J. Soil Sci. Plant Nutr.,* vol.16 no.3, http://dx.doi.org/10.4067/S0718-95162016005000060
14. Wani, S. A., Chand, S., Wani, M. A., Ramzan, M., & Hakeem, K. R., 2016. Azotobacter chroococcum–a potential biofertilizer in agriculture: an overview, In *Soil Science: Agricultural and Environmental Prospectives*, 333-348