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Nitrogen Fixation Ability of Some Cyanobacteria Isolated of Maize Field (Case Study Iran)

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Abstract – **Nitrogen is generally considered one of the major limiting nutrients in plant growth. The biological process responsible for reduction of molecular nitrogen into ammonia is referred to as nitrogen fixation. Recently, cyanobacteria with nitrogen fixation ability were used as biofertilizer and for soil remediation. Thus, determination of their habitat, distribution and taxonomical uses would be very beneficial. The aim of the present work was a study of isolation and purification of nitrogen fixing cyanobacteria from Gorgan, Golestan province, maize fields. 20 Surface and rizosferic samples collected at 0-5 and 5-10cm depths .After isolation and purification by using BG11 media, totally 11cyanobacteria were isolated and evaluated for nitrogen fixation ability. 1 of them grow on medium without N source. All of isolate could growth on medium with insoluble phosphate. And one of the isolate indicate considerable ability to produce hydrogen cyanid. Overall results showed that cyanobacteria isolated from maize soils could be used as biofertilizer for increasing growth and yield of crops after isolation and purification. The MR7 seems to be an ideal choice for the preparation of microbial inoculant because of its ability to grow well and fix atmospheric nitrogen in the absence of combined nitrogen and soluble phosphates and produce hydrogen cyanide.**

Keywords – **Nitrogen Fixation, Diazotrophic Cyanobacteria, Sustainable Agriculture, Maize, Plant Growth Promoting Cyanobacteria.**

I. Introduction

Nitrogen (N) is an essential nutrient for organisms and can limit the production of organic matter. The input of reduced N compounds can take place via riverine in_ow and atmospheric deposition. Furthermore, it can be mediated by biological dinitrogen (N2) _fixation, which is the reduction of atmospheric N2 to ammonium (NH4+). N2_fixation can be performed by diazotrophic cyanobacteria, which comprise a diverse group of prokaryotes. Nitrogen (N) is the macronutrient required in high amounts by plants, and its availability in soils may change substantially at relatively short time intervals [1]. For rapid growth of all plants, nitrogen is probably the most common limiting Factor in soils. Hence, an adequate supply of N in agriculture field is also very important. The long-term field experiments showed that the use of only chemical fertilizers cannot be an efficient option to maintain and enrich the fertility of soils. However, some reports indicated that use of pyrite with cyanobacteria can be the very effective corrective measures in reclamation and enrichment of soils [2].

Diazotrophic cyanobacteria are the dominant microflora in rice agriculture, and are presently used as a supplement to chemical N fertilizers for rice cultivation in rice-growing countries. In order to significantly improve the efficient use of cyanobacteria as an N-based biofertilizer for rice cultivation, studies have been carried out in different dimensions both in the laboratory and in the field [3]. Although the fundamental investigations about growth and photosynthesis in cyanobacteria and field related studies on nitrogen fixation in rice agriculture are well documented [4, 5]. Cyanobacteria constitute the largest, most diverse and widely distributed group of prokaryotes that perform oxygenic photosynthesis and improve soil health [6]. The practical utility of these organisms as a source of biologically fixed nitrogen in paddies has been well recognized [7, 8]. Although, few reports exist on their ability to solubilize mineral P, not much attention has been paid to this attribute of these diazotrophs. Phosphorus (P) is second only to nitrogen as an essential mineral fertilizer for crop production, comprising *0.2% of plant dry weight. Further, soluble P is often the limiting mineral nutrient in soil and is conditioned by various factors. At any given time, a substantial component of soil P is in the form of poorly soluble mineral phosphates. These mineral phosphates are, in general, are biologically not available for nutritional transport and assimilation.

Cyanobacteria play an important role in various chemical transformations of soils and thus, influence the bioavailability of major nutrients like P to plants. Cyanobacteria and PSB have been used as biofertilizer to increase crop production [9]. Biofertilizers are organisms that enrich the nutrient quality of soil. The main sources of biofertilizers are bacteria, fungi, and cyanobacteria (blue-green algae). Plants have a number of relationships with fungi, bacteria, and algae. After the introduction of chemical fertilizers during the last century, farmers were happy of getting increased yield in agriculture. But slowly chemical fertilizers started displaying their ill-effects such as leaching out, polluting water basins, destroying flora and fauna including friendly organisms, making the crop more susceptible to the attack of diseases, reducing the soil fertility and thus causing irreparable damage to the eco system. In recent years, biofertilizers have emerged as promising components of the integrated nutrient supply system in agriculture. The cyanobacterial ability to mobilize insoluble forms of inorganic- P is evident from the finding of Kleiner and Harper (1977) who reported more extractable P in soils with cyanobacterial cover than in nearby soils without cover. Synergistic effects of efficient soil microbes and cyanobacteria, the excretion of organic acids to increase P availability and the decrease of sulphide injury by increased O2 content has also been reported [10]. Cyanobacterial biofertilizers mobilize nutritionally important Elements such as P from a non-usable to a usable form through biological processes [11]. They have been shown to solubilize insoluble Ca3(PO4)2, FePO4, AlPO4, hydroxyapatite (Ca5(PO4)3_OH) and rock phosphate [12, 13] in soils, sediments or in pure cultures. They are also known to solubilize organic sources of P [14].

Based on the above information, it may be deduced that the application of biofertilizers like cyanobacteria can be a potential agent to provide essential nutrient and organic matter to soils. Keeping all these attributes in mind the present study was designed to isolate, identify and screen native plant growth promoting cyanobacteria.

II. Methods and Materials

A. Sample collection and cyanobacteria isolation

Soil sample was collected from maize fields at two depths (0-5 and 5-10cm), totally 20 samples, at Gorgan town, Golestan province, Iran. Then transferred under aseptic conditions to the laboratory and stored in an ice pack at 4°C in the laboratory.1gr of soil samples strewed on BG-11 liquid medium in big prescription bottle. The cyanobacteria cultivated under identical conditions on BG 11 media, pH 7 supplemented with 100mg/L cyclohexamid at 25°C and continuous light provided by cool–white fluorescent lamps (200µEm$-m^{-2}$–s^{-1}) for 15 days [15]. After growth in prescription bottle 1ml of liquid media containing cyanobacteria plated To BG11 plate incubated at 25 ± 2°C. This process was repeated three to four times until axenic culture was obtained. Cyanobacteria growth was monitored on weekly basis to achieve axenic culture and get isolated colonies of isolates. All strains were grown for minimum periods of 3 months under the unchanged culture conditions before the analysis were conducted.

B. Estimation of Total chlorophyll;

Chlorophyll was estimated by the method of Mackiney [16]. A known volume (10 mL) of homogenized cyanobacterial suspension was taken and subjected to centrifugation (4000g, 10 mins.). The chlorophyll was extracted from pellet with similar volume of methanol (95%) in a water bath (600C, 30 mins.). The suspension was centrifuged and the absorbance of the supernatant was read at 650 and 665 nm against 95% methanol as blank. The concentration of chlorophyll was calculated using the formula:

Total chlorophyll (mg/mL) = 2.55 _ 10-2 E650 + 0.4 _ 10-2 E665

Where, E650= Absorbance at 650 nm
E665= Absorbance at 665 nm

C. Plant growth promoting traits

Cyanobacteria isolates were evaluated for different growth promoting traits such as phosphate solubilisation, nitrogen fixation, hydrogen cyanide (HCN). Phosphate solubilisation was determined qualitatively by streaking strains on BG-11 agar plates containing tri-calcium phosphate (0.3%) as sole P source. Development of a clear zone around the growth of cyanobacteria was recorded after incubation at 24±2°C for 15 days [17]. Nitrogen fixation potential was examined by growing them in nitrogen free media (BG-11 without NaNO3) [17]. HCN production was determined as described by Lorck [25].

III. Results and Discussion

A. Isolation and purification

The cyanobacteria strains were collected from soil located in Golestan province, Iran. The unicellular strains were isolated and purified in laboratory of biology, soil science department, Tehran University. Totally 11 cyanobacteria strains has ability to grow on BG-11 medium and were subjected to PGP test.

Table 1: Phisico-chemical properties of soil

Soil property	Maize filed
Soil texture	clay
pH	8.4
Electrical conductivity ($dS.m^{-1}$)	1.3
Organic carbon (%)	1.03%
Organic matter (%)	1.78%
Total N (%)	0.15%
Absorption able P ($mg.Kg^{-1}$)	6.9
Absorption able K ($mg.Kg^{-1}$)	8.9

Table 2: Purified isolate from maize field

Surface	-	MS2	MS3	-	-	-	MS7	-	MS9	MS10
Rizosphere	-	-	MR3	MR4	MR5	-	MR7	-	MR9	MR10

M: maize field, R rizosfer soil, S surface soil

B. Total Cholorophyll contant:

Total Cholorophyll of isolate showed in fig.1

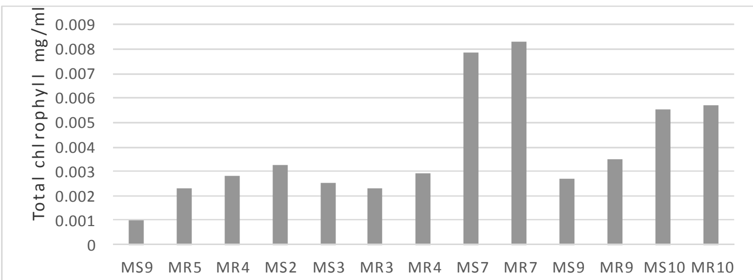

Fig.1. Total Chlorophyll content of isolate

C. Plant growth promoting traits

Nitrogen fixation

After 15days of inoculation of cyanobacteria strains on BG-11 medium. Criteria for nitrogen fixation ability were to growth and colony formation on BG-11 medium without N source. After 15days, MR7 could grow on N-free BG-11 medium that showed it can fix molecular nitrogen and use it as nitrogen sourse. The capable isolate was belong to the rizosphere of maize field. Sekar and Subramanian [18] showed that cyanobacteria have the ability to growth on medium without N-source and have the nitrogen fixation ability.

P solubilizing

All of isolate could grow on the BG-11 medium supplemented with tri-calcium phosphate. That Showed isolate can utilize p source in media and grow normally. Cyanobacteria have different strategies to solubilize mineral phosphorous. Phosphate-solubilizing microorganisms produce extracellular enzymes like phosphatases along with organic acids for phosphate solubilization.Phosphatases are a group of extracellular hydrolytic enzymes that have an important ecological significance because they solubilize the insoluble phosphates in the wetland ecosystem. Cyanobacteria secrete alkaline phosphatase (APase) into surrounding medium under phosphate-starved conditions [19]. Under conditions of phosphate starvation cyanobacteria are known to show increased levels of intracellular and cell surface alkaline phosphatase activity to solubilize polyphosphates [20] Endogenous polyphosphate depleted *Anabaena* ARM310, solubilized extracellular tricalcium phosphate through increased phosphatase activity. [21]. Mahesh et all [22] defined that in general AR (acetylene reduction) activity in presence of MRP (Mussorie rock phosphate) was always less than that in presence of TCP (tricalcium phosphate) at all concentrations. Among the two cyanobacteria A. variabilis was best in terms of P-solubilization and nitrogen fixation and TCP (20 mg P l-1) was the best source of insoluble P rather than MRP or K2HPO4.

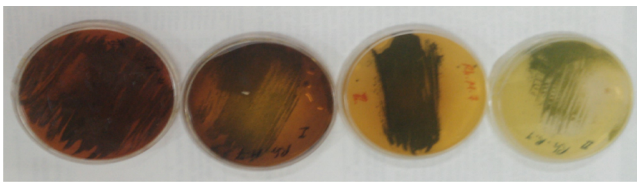

Fig.1. Cyanobacteria growth on BG-11 medium with tri-calcium phosphate

HCN production

Results showed that only one of isolates have the HCN-producing ability. MR7 that was a diazotrophic isolate indicated have a high ability to produce hydrogen cyanide. Diazotrophic cyanobacteria could produce hydrogen cyanide. Ahmad et al. [23] stated that some cyanobacteria strains by HCN production could protect plant from pathogenic factors. Reports available about cyanobacteria as biocontrol agents show that these organisms are effective against plant pathogens [24]. However, observations on the effects of cyanobacteria and their metabolites on other plant pathogens under field conditions are scarce.

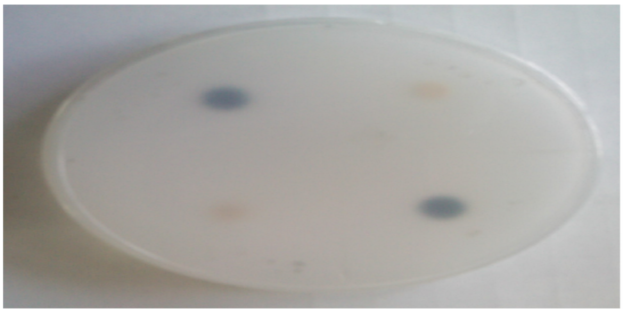

Fig.2. hcn production in cyanobacteria isolate

Table 3: Growth promoting traits of cyanobacteria strains.

Isolates	**Nitrogen fixation**	**Phosphate solubilization**	**Hydrogen cyanide production**
MS2	-	+	-
MS3	-	+	-
MR3	-	+	-
MR4	-	+	-
MR5	-	+	-
MS7	-	+	-
MR7	+	+	+
MS9	-	+	-
MR9	-	+	-
MS10	-	+	-
MR10	-	+	-

IV. Conclusion

Recently, cyanobacteria with nitrogen fixation ability were used as bio fertilizer and for soil remediation. Thus, determination of their habitat, distribution and taxonomical uses would be very beneficial. Present study was conducted with the aim of isolation and purification of heterocyst having cyanobacteria from five region of Gorgan, Golestan province, maize fields (at 0-5 and 5-10cm depths). After isolation and purification, isolates were subjected to PGP tests. From isolated cyanobacteria, all of them grown on BG-11 medium with tri-calcium phosphate as P source but they can't produce halo zone around the colony that indicate ability to solve insoluble phosphate in the medium by producing phosphatases or other mechanisms. MR7 isolate grown on medium without N source and produce hydrogen cyanide .Overall results showed this cyanobacteria that isolated from maize soil have plant growth promoting traits and could be used as biofertilizer for increasing growth and yield of crops after isolation and purification. Fixed nitrogen is a limiting nutrient in most environments, with the main reserve of nitrogen in the biosphere being molecular nitrogen from the atmosphere. Molecular nitrogen cannot be directly assimilated by plants, but it becomes available through the biological nitrogen fixation process that only prokaryotic cells have developed.

Detail field investigations regarding development of efficient high quality cyanobacterial soil inocula and their application in specific regions such as saline and drought-proven regions are also needed. This biofertilizer technique is still limited in use and therefore, it is important to introduce cyanobacterial application under field conditions for sustainable agriculture.

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