Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print) 2222-3045 (Online)
Vol. 5, No. 6, p. 179-183, 2014
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

Salt stress on solubilization of tricalcium phosphate by rhizobia nodulating horse gram [*Macrotylma uniflorum* (Lam.) Verdc.]

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Article published on December 14, 2014

Key words: salt tolerant rhizobia, salinity, phosphate solubilizing activity.

Abstract

Horse gram [*Macrotyloma uniflorum* (Lam.) Verdc. = *Dolichos biflorus* (Linn.)] is an important pulse crop and it is extensively cultivated on light red and gravel soils of peninsular India. It derives its importance from its adaptability to poor and adverse climatic conditions which are unsuitable for other pulse crops. Salt tolerant rhizobia are useful in improving the condition of disturbed and extreme climatic conditions. The rhizobia associated with horse gram were found to be highly salt tolerant and these rhizobia also showed tolerance to other salts like chlorides, sulphates and carbonates of sodium, potassium, manganese, calcium and magnesium. Therefore the present study was undertaken to investigate the influence of salt concentration on phosphate solubilization. Solubilization of tricalcium phosphate by salt tolerant strains of *Rhizobium* from horse gram was investigated. Out of 32 strains of *Rhizobium*, six strains were found to be efficient phosphate solubilizers at various salt concentrations. These strains showed maximum phosphate solubilization at 0.2M, 0.4M, and even at 1M salt concentration. These strains can be exploited for phosphate solubilization under salt stress.

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Introduction

Salinity has become an ever increasing problem in agriculture. The soil salinity problem is wide spread in the arid and semi arid areas and in the sub-humid and humid climates particularly in the coastal regions. Microbial aspects of salt affected soils have received very little attention. Interest in this field is now increasing because of its implied agricultural potential in the biological reclamation of salt affected soil. Soil microorganisms especially bacteria (Garg *et al.,* 1989 and Krishnaraj *et al.,* 1999) play an important role in the solubilization of bound phosphates and make available to higher plants. Increase in the incidence of phosphate solubilizing bacteria in neutral, alkaline and saline soils is essential to increase and maintain the supply of available phosphorus. Though much information is available on the role of soil microorganisms on phosphate solubilization, very little information is available on the effect of salt concentration on phosphate solubilization by bacteria. Salt tolerant rhizobia are useful in improving the condition of disturbed and extreme climatic conditions.

Horse gram [*Macrotyloma uniflorum* (Lam.) Verdc. = *Dolichos biflorus* (Linn.)] is an important pulse crop and it is extensively cultivated on light red and gravel soils of peninsular India. It derives its importance from its adaptability to poor and adverse climatic conditions which are unsuitable for other pulse crops. In India horse gram is cultivated in 1.1 million hectares during both kharif and rabi seasons. This grain legume is most extensively grown in the states viz., Tamil Nadu, Karnataka and Andhra Pradesh of South India. Being a legume it also fixes atmospheric nitrogen and improves soil fertility. It is widely cultivated as a grain legume and fodder crop in this country and constitutes an essential supplement for the cereal based balanced diet of low income populations (Virk *et al.,* 2006). Growth and yield of this legume crop were drastically increased when they were inoculated with nitrogen fixing rhizobia (Keshava *et al.,* 2007). The rhizobia associated with horse gram were found to be highly salt tolerant (Prabhavati and Mallaiah, 2007) and these rhizobia also showed tolerance to other salts like chlorides, sulphates and carbonates of sodium, potassium, manganese, calcium and magnesium (Prabhavati and Mallaiah, 2008). Therefore the present study was undertaken to investigate the influence of salt concentration on phosphate solubilization.

Materials and methods

Bacterial Strains

Root nodules were collected from the plants grown in soil samples collected from different regions in Andhra Pradesh, India. In the process, nodules were separated from the roots and washed with 0.01% mercuric chloride. The nodules were repeatedly washed with sterilized distilled water to get rid of the sterilizing agent. Following serial dilution agar plate technique (Somasegaran and Hoben, 1994) using yeast extract mannitol agar (YEMA) medium containing 0.0025% congo red dye (Vincent, 1970), bacterial isolations was carried out. After that these plates were incubated at 28±1^{o}C and observed daily. Bacterial colonies appeared after 2-3 days were picked up and streaked on YEMA plates. Pure cultures were obtained with one or more further sub culturing steps. Root nodulating ability of these isolates was determined by nodulation test (Weller and Cook, 1983). Furthermore, all the isolates were subjected to authentication test before performing all the experiments. They were designated as HGR-1 (Horse Gram Rhizobia) to HGR-32.

These strains were found to be highly salt tolerant. All the thirty two isolates were screened for their phosphate solubilizing (PS) activity on Pikovskaya's tricalcium phosphate (TCP) agar plates. The amount of phosphorus (P) liberated and pH of the broth cultures was determined. Six strains viz. HGR1, HGR18, HGR19, HGR20, HGR22, and HGR27 which showed high phosphate solubilization on TCP plates were selected to study the effect of salt concentration on phosphate solubilizing efficiency in broth cultures. For this, 50 ml of Pikovskaya's broth containing different salt concentrations of sodium chloride

(NaCl) from 0 (without salt), 0.2M, 0.4M, 0.6M, 0.8M, 1M, 1.2M, 1.4M, 1.6M, 1.8M and 2Molars (1M=58.44 grams NaCl dissolved in 1000 ml) were inoculated with 1ml of the inoculum of each isolate. They were incubated for 10 days on gyrorotary shaker at 28±2°C. Culture broth was centrifuged at 10,000g for 15 minuites. The pelleted bacterial cells were separated by filtration and the supernatant was used for the estimation of the amount of phosphate solubilized (Subba Rao, 1993). Uninoculated flask from each set was kept as control.

Results and discussion

The rhizobial cells were Gram negative non spore forming rods, the size of the cells was 2 to 2.3 µm long with 0.5- to 1 µm width. The sizes of the colonies were 6-8 mm in diameter after 72 h on YEMA medium at room temperature. The optimum pH was in the range of 7-7.5. These strains grow at a temperature between 10 to 40˚C. Lower concentrations of NaCl favoured growth of these rhizobia. All the isolates were resistant to ampicillin and rifampicin. The isolates HGR-11, 22 and 23 showed resistance to most of the antibiotics tested, where as the isolate HGR-4 showed susceptibility towards most of the antibiotics. All the isolates are positive for citrase, nitrate reductase, tryptophanase, asparginase, catalase and the production of ammonia. Four strains were selected for 16S rRNA sequence and were submitted to the NCBI GenBank under the accession numbers GQ483457, GQ483458, GQ483459 and GQ483460.

Six strains showed zone of phosphate dissolution on TCP plates. After six days the zone of phosphate solubilization on agar plates ranged from 10.0 mm to 12.0 mm. Seventeen strains showed PS activity in liquid medium (Table.1). The final pH of the medium ranged from 3.9 to 6.9. At pH 3.9 and 4.0 TCP solubilization was high. The amount of TCP solubilized was low at pH 6.0 and 6.9. It showed that low pH was ideal for better solubilization.

The amount of P released varied with various salt concentrations and the isolate involved (Fig.1). In control, the amount of P solubilized varied from 10.2µg/ml (HGR-27) to 50µg/ml (HGR-22).

Table 1. Phosphate solubilization by rhizobia nodulating Horse gram.

Isolate No.	Zone of P solubilized after 6d (mm)	P solubilized in liquid medium (µg/ml)	Final pH of the medium
HGR-1	12.0	25.0	4.8
HGR-2	--	14.6	5.8
HGR-3	--	4.4	5.8
HGR-5	--	14.6	5.7
HGR-6	--	4.0	5.9
HGR-8	--	3.0	6.9
HGR-9	--	13.2	5.8
HGR-16	--	6.0	5.8
HGR-17	--	8.6	5.6
HGR-18	10.0	14.6	5.6
HGR-19	12.0	36.6	3.9
HGR-20	12.0	23.4	5.0
HGR-22	14.0	50.0	4.0
HGR-25	--	1.8	6.0
HGR-27	10.0	10.2	5.0
HGR-28	--	4.0	5.9
HGR-29	--	3.0	6.0

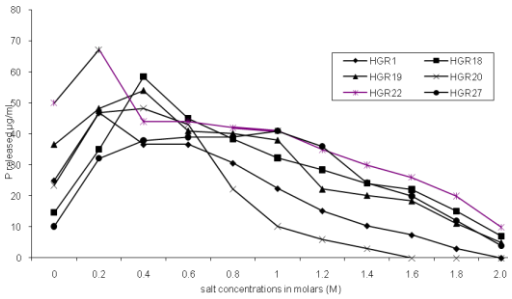

Fig. 1. Effect of salt concentration on phosphate solubilization.

At 0.2M salt concentration the highest amount of solubilized TCP was found to be 67.2µg/ml by the strain HGR-22. At this salt concentration the strains HGR-20 and HGR-1 showed similar (46.8µg/ml) PS activity. The amount of TCP solubilized by the strains HGR-19 and HGR-18 was 48.2µg/ml and 35.0µg/ml. The strain HGR-27 solubilized low amount of TCP (32.2µg/ml).

At 0.4M salt concentration the strains HGR-18, HGR-19 and HGR-20 showed high PS activity. The strain HGR-22 showed equal PS activity at 0.4M and 0.6M salt concentrations. The amount of TCP solubilized

by the strain HGR-1 was also similar (36.6µg/ml) at these two salt concentrations.

At 0.6M salt concentration the strain HGR-18 solubilized high amount (45.0µg/ml) of TCP followed by HGR-20 and HGR-19. At 0.6M and 0.8M salt concentrations the strain HGR-27 showed similar PS activity. At 0.8M salt concentration the strain HGR-22 showed high TCP solubilization followed by HGR-19, HGR-18, HGR-1 and HGR-20.

At 1M salt concentration the strains HGR-27 and HGR-22 showed high (41.0µg/ml) phosphate solubilizing activity than other strains. At 1M and 1.2M salt concentrations the strain HGR-20 showed low PS activity than other strains. At 1.2M salt concentrations the strain HGR-27 showed high PS activity followed by HGR-22, HGR-18, HGR-19 and HGR-1.

At 1.4M salt concentration the strain HGR-22 showed high (30.0µg/ml) PS activity. The strains HGR-18 and HGR-27 showed almost similar (24.0µg/ml and 24.2µg/ml) TCP solubilization followed by HGR-19 and HGR-1. The amount of TCP solubilized by the strain HGR-20 was very low (3.0µg/ml).

At 1.6M salt concentration high amount of TCP solubilized by HGR-22 followed by HGR-18, HGR-27, HGR-19 and HGR-1. At this concentration, the strain HGR-20 unable to solubilized TCP. At 1.8M salt concentration the amount of TCP solubilized by HGR-22 was 20.0µg/ml followed by HGR-18, HGR-27 and HGR-19. The strain HGR-1 showed very low TCP solubilization.

At 2M salt concentration four strains HGR-22, HGR-18, HGR-19, and HGR-27 able to slubilize TCP. Statistical analysis using **ANOVA** test showed that the variation in the amount of TCP solubilized at different salt concentrations by different *Rhizobium* strains is highly significant at 5% level.

From the above study, it is clear that these salt tolerant *Rhizobium* strains solubilized maximum TCP at 0.2 M (1.1688%) concentrations by the strains HGR-22, HGR-20 and HGR-1. Two strains (HGR-18 and HGR-19) showed high TCP solubilization at 0.4M (2.3376%) salt concentration. The strain HGR-27 able to show high PS activity even at 1M (5.844%) salt concentration. Among the bacteria Sunita and Gaur (1999) reported that *Bacillus* species showed better solubilization of TCP up to 0.05% salt concentration and at 3.5% salt concentration phosphate solubilization was completely inhibited. Nautiyal *et al.,* (2000) reported that among the four phosphate solubilizing bacterial strains from rhizosphere of Chick pea and alkaline soils, only one strain could solubilize phosphate in the presence of 10% salt.

Since *Rhizobium* strains from horse gram can withstand high salt concentration and bringabout phosphate solubilization, they can be used as bioinoculants for reclamation of saline soils.

Acknowledgement

One of the authors (E.Prabhavati) is grateful to University Grants Commission, New Delhi for financial assistance in the form of Research Fellowship.

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