ΦYTON
REVISTA INTERNACIONAL DE BOTÁNICA EXPERIMENTAL
INTERNATIONAL JOURNAL OF EXPERIMENTAL BOTANY
FUNDACION ROMULO RAGGIO
Gaspar Campos 861, 1638 Vicente López (BA), Argentina
www.revistaphyton.fund-romuloraggio.org.ar

Boosting forage yield and quality of maize (*Zea mays* L.) with multi-species bacterial inoculation in Pakistan

Aumento del rendimiento y la calidad de forraje en maíz (*Zea mays* L.) con la inoculación de varias especies bacterianas en Pakistán

Iqbal A[1], MA Iqbal[1], A Iqbal[1], Z Aslam[1], M Maqsood[1], Z Ahmad[2], N Akbar[1], HZ Khan[1], RN Abbas[1], RD Khan[1], G Abbas[1], M Faisal[1]

Abstract. Seed inoculation with bacterial species has the potential to increase yield and agro-qualitative attributes of forage crops. This study determined the response of forage maize to three plant growth promoting rhizobacteria [PGPR1 (*Azotobacter chroococcum*), PGPR2 (*Pseudomonas flourescens*) and PGPR3 (*Bacillus megaterium*)] inoculated individually and in different combinations (PGPR1+2, PGPR1+3, PGPR2+3 and PGPR1+2+3). A non-inoculated treatment was kept as a control. We used a completely randomized block design with four replicates. The PGPR1+2+3 treatment showed an outstanding performance by improving yield attributes, green forage yield, dry matter biomass, crude protein and total ash. The same treatment gave the lowest crude fiber concentration. It was followed by PGPR2+3 which in turn was followed by PGPR1+2. Hence, our study suggests that employment of multi-species bacterial inoculation is an effective technique to improve economical production and agro-qualitative attributes of forage maize.

Keywords: Agro-biotechnology; Fodders; Ruminant nutrition; Rhizobacteria; Seed inoculation.

Resumen. La inoculación de semillas con especies bacterianas tiene el potencial para aumentar el rendimiento y agro-atributos cualitativos de los cultivos forrajeros. Este estudio determinó la respuesta de maíz forrajero a la inoculación con tres especies de bacterias de la rizosfera promotoras del crecimiento (PGPR) [PGPR1 (*Azotobacter chroococcum*), PGPR2 (*Pseudomonas flourescens*) y PGPR3 (*Bacillus megaterium*)] inoculadas individualmente y en diferentes combinaciones (PGPR1+2, PGPR1+3, PGPR2+3 y PGPR1+2+3). Un tratamiento no inoculado fue usado como control. Se utilizó un diseño en bloques completamente al azar (RCBD), con cuatro repeticiones. El tratamiento PGPR1+2+3 mostró un desempeño sobresaliente, mejorando los atributos de rendimiento, rendimiento de forraje verde, materia seca de la biomasa, proteína cruda y cenizas totales. El mismo tratamiento determinó la menor concentración de fibra cruda. Fue seguido por PGPR2+3, que a su vez fue seguido por PGPR1+2. Por lo tanto, este estudio sugiere que el empleo de la inoculación con varias especies bacterianas es una técnica eficaz para mejorar la producción económica y los atributos agro-cualitativos del maíz forrajero.

Palabras clave: Agrobiotecnología; Forrajes; Nutrición de rumiantes; Rizobacterias; Inoculación de semillas.

[1] Department of Agronomy, Faculty of Agriculture, University of Agriculture Faisalabad-38040, Pakistan.
[2] Cholistant Institute of Desert Studies, The Islamia University of Bahawalpur, Pakistan.
Address correspondence to: Muhammad Aamir Iqbal, Phone: +92-3143142187, *e-mail:* aamir1801@yahoo.com
Received 9.XI.2016. Accepted 2.II.2017.

INTRODUCTION

Production of good quality forage in large quantities is indispensable for a competent and prolific livestock industry (Iqbal & Iqbal, 2015). One of the reasons for low yield of fodder crops is the sub-optimal use of chemical fertilizers (Sarajuoghi et al., 2012). Now, we are also facing numerous hazards including soil, water and environmental contamination owing to fertilizer, pesticide and herbicide uses. This scenario demands for a biotechnological, economical, socially acceptable, easy to adopt and environment friendly solution for boosting crop productivity, including maize. Inoculation of plant beneficial bacteria has been coined by many researchers as a good strategy to improve plant growth and productivity (Iqbal et al., 2015).

Inoculation of bacterial species has proved its beneficial effects by increasing photosynthetic rate, root and shoot biomass, flag leaf area, water use efficiency and production of indole-acetic acid and gibberellins (Cleyet-Marcel et al., 2001). Single species inoculation is relatively un-reliable because root colonization by a single species of plant growth promoting rhizobacteria (PGPR) is lower (Raupach & Kloepper, 1998) than multi-species inoculation. Multi-species inoculation can perform better under heterogeneous soil conditions because they usually require diverse temperature, pH and soil moisture conditions for their activities (Zahir et al., 1996; Patten & Glick, 2003). Most of the earlier work has been done on single species inoculation of PGPR, and there are very few and contrasting reports for the combined inoculation of PGPR having different types of mechanisms and associations to improve plant growth and yield in field conditions.

The present study was conducted with three bacterial inoculants including *Azotobacter chroococcum, Pseudomonas flourescens* and *Bacillus megaterium* that were inoculated individually and in different combinations to evaluate their potential for improving growth, yield and quality of forage maize under irrigated field conditions.

MATERIALS AND METHODS

Experimental site. The experiment was conducted at Agronomic Research Area, University of Agriculture Faisalabad (Pakistan) during the spring seasons of 2014 and 2015. The experimental area is located at 30.35-31.47° N and 72.08-73.0° E. The general elevation of the land is about 184 m above sea level. Prior to sowing, experimental soil was analyzed for physico-chemical characteristics (Table 1). The soil was sandy clay loam with pH of 7.6 and 7.8 during 2014 and 2015, respectively, and was deficient in macro-nutrients as well as organic matter. The climate of Faisalabad is classified as semi-arid (BWh) in Köppen-Geiger classification with very hot and humid summers and dry, cool winters. Precipitation, and mean monthly temperatures and relative humidity were also taken from a meteorological center located in the close vicinity of the experimental site (Fig. 1).

Table 1. Pre-sowing physico-chemical analysis of experimental soil.
Tabla 1. Análisis físico-químico del suelo experimental antes de la siembra.

Soil characteristics	**Recorded values**	
Mechanical analysis	2014	2015
Sand (%)	55	56
Silt (%)	19	19.5
Clay (%)	26	24.5
Textural class	Sandy clay loam	Sandy clay loam
Chemical analysis	2014	2015
pH	7.6	7.8
EC (dS/m)	1.51	1.54
Organic matter (%)	0.69	0.65
Total nitrogen (mg/kg)	288.7	306.1
Available phosphorous (mg/kg)	6.9	6.2
Available potassium (mg/kg)	171	146

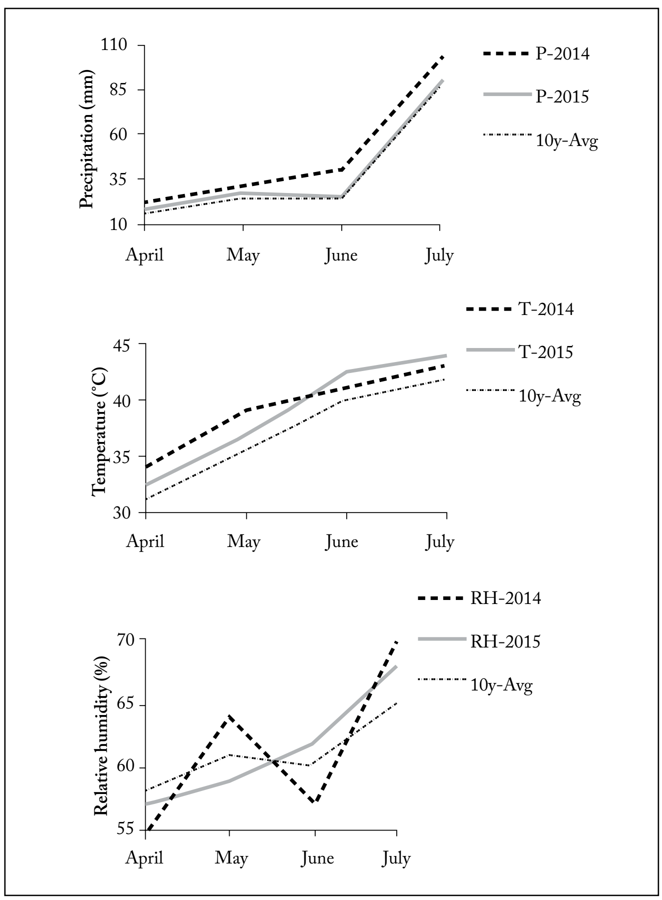

Fig. 1. Precipitation, temperature and relative humidity during the maize growing season.
Fig. 1. Precipitaciones, temperatura y humedad relativa durante la estación de crecimiento del maíz.

A completely randomized block design (RCBD) was used to make the experiment during both years with four replicates each. The size of the net plot was 1.8 m × 5.0 m. The seeds of forage maize (cv. Sargodha-2002) were inoculated with three pre-isolated (separated from mixed colonies) PGPR species including PGPR1 (*Azotobacter chroococcum),* PGPR2 *(Pseudomonas flourescens)* and PGPR3 (*Bacillus megaterium)* individually and in different combinations (PGPR1+2, PGPR1+3, PGPR2+3, PGPR1+2+3), while a non-inoculated treatment was used as a control.

Experimental protocol. Carrier (peat) based inoculants (5 mL for 1000 seeds with 10^7 alive bacteria) were mixed with a liter of water for preparing a slurry to which 100 mL of 2% sugar solution was added so that the inoculants may get energy for their prolonged survival. The slurry was poured over the seeds which were kept in a container. The seeds were mixed well with the slurry of inoculants by pouring the seeds and slurry into another container, which was properly shaken. Then, the treated seeds were dried in a cool and dry shady place, and sown immediately in the field. Sowing in 30 cm spaced rows was done manually method using a seeding rate of 100 kg/ha. Half dose of N in the form of urea along with full dose of P as di-ammonium phosphate (DAP) were applied by the broadcasting method at the time of sowing, while the remaining half dose of nitrogen was applied with a first irrigation 15 days after sowing. The remaining four irrigations were applied according to the crop requirements. Irrigation was applied directly to every plot from main and sub-water channels to avoid inoculant dispersal. No water flow was allowed among plots. Furadan (3-G) was applied to protect the crop from maize borer and shoot fly at 20 kg/ha. Seedling density was recorded at complete emergence 15 days after sowing, while the remaining experimental variables were recorded at the time of harvest. Plant height was recorded from the base to the tip of each of three plants with help of a measuring tape, after harvesting three plants from the middle row of each plot and then taking an average of n=3. A Vernier caliper was used to record the stem diameter from each of three plant parts (base, mid and top), and once again making an average of n=3. Fresh weight was recorded per plant with help of an electric balance, while dry weight was also recorded per plant by chopping and drying plant samples in an oven at 70 °C until a constant dry weight was obtained. Finally, green forage yield was recorded by harvesting all plants in a plot, weighing them with a spring balance, and expressing weight as tons per hectare. A chemical analysis of the preserved and dried samples (3 samples per plot) was carried out to determine the agro-qualitative attributes of forage maize following the procedures outlined in AOAC (2003).

Statistical analysis. ANOVA was employed for statistical analysis of experimental variables using Statistics 8.1 version. Whenever F tests were significant, treatment means were compared using the Fisher's LSD at α=0.05 (Steel et al., 1997).

RESULTS

Yield and yield components of forage maize. The highest values in all growth parameters were recorded in the PGPR1+2+3 treatment. The only exceptions were seedling density at complete emergence and plant population at harvest, whose values did not differ among treatments (Table 2). The PGPR2+3 treatment did not differ to PGPR1+2+3 in plant height, leaf number, leaf area per plant and green forage yield in 2014, while PGPR1+2 did not differ to PGPR1+2+3 in leaf number and dry weight during the same year. In addition, PGPR1+2 was found to be statistically similar to PGPR2+3 in terms of dry matter biomass production in the cropping season of 2014 (Table 3). On the other hand, the control values were lowest in all growth parameters, but they did not differ to treatments with only one PGPR species; this was the case in plant height and leaf number in the 2014 crop period. Moreover, PGPR3 treatment did not differ to control for leaf number and fresh weight in 2015.

Agro-qualitative attributes of forage maize. PGPR1+2+3 treatment showed the highest crude protein and total ash concentrations; it also recorded the lowest crude fiber concentration (Table 3). It did not differ to PGPR2+3 for crude fiber and total ash in the crop period of 2014. PGPR1+2 did not differ to PGPR1+3 neither in crude protein (crop period of 2014) nor in total ash (crop period of 2015). On the other hand, the control treatment recorded the lowest crude protein and total ash along with the highest crude fiber concentrations. Also, it did not differ to single species inoculation for crude protein and total ash concentrations during both years.

DISCUSSION

Multi-species bacterial inoculation (PGPR1+2+3) was effective in improving yield components and forage yield of maize. The increased vegetative growth might be attributed to the synthesis of the auxin hormone, which plays a significant role in cell division, cell expansion and differentiation of plant tissues which increased plant height, stem diameter, and fresh and dry weights per plant. These results are in line with previous studies of Tsavkelova et al. (2006), who reported that application of different species of PGPR initiated the synthesis of plant hormones which improved the plant's morphological characters of forage maize. Similar findings were also reported by Hernandez et al. (1995), who concluded that bacterial inoculation markedly increased the synthesis of numerous growth hormones, and suggested that their combined application might yield even better results. These results also agree with previous studies of Hamidi & Asgharzadeh (2006) and Bottini et al. (2004), who reported that seed inoculation with *Azotobacter chrococcum* and *Pseudomonas fluorescens* considerably increased the availability of nitrogen (fixed through BNF)

Table 2. Effect of multi-species bacterial inoculants on yield attributes of forage maize.
Tabla 2. Efecto de inoculantes bacterianos conteniendo una o más especies de bacterias sobre atributos de rendimiento de maíz forrajero.

Treatments	Seedling density at complete emergence (#/ m²)		Plant population at harvest (g/m²)		Plant height (cm)		Stem diameter (cm)		Number of leaves/ plant		Fresh weight/plant (g)		Dry weight/plant (g)	
	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015
Control	23.4	25.9	20.6	22.6	200.3 e	203.1e	10.50e	11.11e	12.03d	12.34f	316.93g	322.06f	67.40d	68.81e
PGPR1	24.7	26.0	22.1	22.8	212.9cd	216.7cd	10.86d	11.29d	12.76cd	13.12d	336.20de	340.57d	69.30bc	70.36c
PGPR2	24.1	25.9	21.3	23.2	207.2de	211.4d	10.91d	11.34d	12.50cd	13.0e	332.30ef	337.30e	69.12c	70.15c
PGPR3	23.2	25.0	21.1	23.1	206.0de	209.9d	11.11cd	11.40d	12.20d	12.30f	326.57f	331.92ef	68.88c	69.48d
PGPR1+2	24.0	24.8	22.2	23.4	222.6bc	227.1bc	11.21c	11.58c	14.20ab	14.34c	347.60bc	354.04c	70.16ab	72.30b
PGPR1+3	25.7	25.1	22.1	23.5	213.0cd	219.0c	11.34b	11.67c	13.40bc	14.51b	342.20cd	348.10cd	69.34bc	71.11c
PGPR2+3	24.2	24.7	22.2	23.0	224.6ab	232.3b	11.40b	11.84b	14.30ab	14.47b	350.17b	364.91b	70.55b	72.94b
PGPR1+2+3	24.9	25.3	22.7	23.6	235.7a	249.4a	11.57a	11.91a	14.90a	14.93a	360.57a	371.46a	73.08a	75.78a
LSD (0.05)	NS	NS	NS	NS	11.47	8.19	0.04	0.07	0.94	0.61	6.90	5.61	0.96	0.51

Means sharing the same letter do not differ significantly at 5% probability level. PGPR1 (*Azotobacter*), PGPR2 (*Pseudomonas*), PGPR3 (*Bacillus*).

Table 3. Effect of multi-species bacterial inoculation on yield and quality attributes of forage maize.
Tabla 3. Efecto de la inoculación bacteriana de múltiples especies en atributos de calidad y rendimiento de maíz forrajero.

Treatments	Leaf area/plant (cm²)		Green forage yield (t/ha)		Dry matter biomass (t/ha)		Crude protein (%)		Crude fiber (%)		Total ash (%)	
	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015
Control	2546.0e	2555.6e	58.11f	59.07f	9.56e	9.46f	8.14d	8.18f	24.85a	24.74a	6.91f	6.93e
PGPR1	2701.2cd	2788.3c	61.61cd	62.08d	11.51cd	11.25d	9.17bc	9.22d	24.35ab	24.29b	7.10d	7.12d
PGPR2	2676.3d	2665.4d	60.51de	60.91e	11.46cd	11.12d	8.84cd	8.91e	24.41ab	24.38c	7.05de	7.10d
PGPR3	2629.8de	2641.8d	59.01ef	60.12e	10.56de	10.69e	8.66cd	8.84e	24.66a	22.60d	6.98ef	6.98e
PGPR1+2	3133.8b	3188.9bc	62.92bc	63.33c	12.17bc	12.27c	9.61bc	9.50c	23.47cd	23.58e	7.43b	7.49c
PGPR1+3	2787.1c	2800.4c	62.04cd	63.07c	12.11bc	12.44c	9.20bc	9.27d	23.73bc	23.64e	7.30c	7.46c
PGPR2+3	3197.6ab	3244.2b	64.50ab	65.41b	13.08b	13.61b	9.76b	10.0b	22.66de	22.57f	7.54a	7.55b
PGPR1+2+3	3244.5a	3365.7a	66.03a	68.69a	13.74a	14.25a	10.70a	10.84a	22.59e	22.44g	7.61a	7.68a
LSD (0.05)	96.98	88.91	2.11	2.06	1.53	1.14	0.97	0.23	0.86	0.67	0.09	0.08

Means sharing the same letter do not differ significantly at 5% probability level. PGPR1 (*Azotobacter*), PGPR2 (*Pseudomonas*), PGPR3 (*Bacillus*).

and the uptake of phosphorous, by solubilization of persistent inorganic phosphates. As a result, significantly higher green forage yields and dry matter biomasses were recorded. In this study, inoculation with different bacterial species resulted in combining the individual benefits rendered by each bacterial species and ultimately a higher forage yield was recorded.

Crude protein and total ash concentrations are the most important agro-qualitative attributes of forage crops owing to their direct relationship with milk and meat production. The decrease in total crude fiber concentrations improves the fodder quality of maize by reducing bulkiness and increasing the feed intake. The remarkable increase in crude protein and total ash along with decrease in crude fiber of forage maize by PGPR1+2+3 might be due to fixing of nitrogen in the rhizosphere; this increased nitrogen availability might have improved the crude protein percentage (Hoflich et al., 1994). These results are also in line with previous findings of Singh et al. (2010), who concluded that different species of PGPR like *Azotobacter* and *Pseudomonas* were effective in increasing crude protein and reducing fiber concentrations. These results are also in agreement with previous findings of Kumar et al. (1999), who reported that seed inoculation with bacterial species either alone or in combination significantly decreased the crude fiber concentrations; it was concluded that application of PGPR favored the availability of nitrogen which enhanced the carbohydrate metabolism and subsequently increased the ash content.

On the basis of the results obtained in this study, it might be concluded that multi-species inoculation of plant growth promoting rhizobacteria (PGPR) effectively promoted growth, yield and agro-qualitative attributes of forage maize in comparison to the control treatment under the agro-ecological conditions of Faisalabad. Hence, multi-species inoculants may be suggested after site-specific field investigations under diverse agro-climatic conditions. However, there is a need to test more PGPR species applied solely and in different combinations to find out their appropriateness for increasing crop productivity.

REFERENCES

AOAC (2003). Official methods of Analysis (17th Ed.). Association of official analytical chemistry, Washington, DC USA. 227 p.

Bottini, R., F. Cassan & P. Piccoli (2004). Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase. *Applied Microbiology and Biotechnology* 65: 497-503.

Cleyet-Marcel, J.C., M. Larcher, H. Bertrand, S. Rapior & X. Pinochet (2001). Plant growth enhancement by rhizobacteria. In: Morot-Gaudry, J.F. (ed). Nitrogen assimilation by plants: physiological, biochemical and molecular aspects. Science Publishers, Plymouth. 185 p.

Hamidi, A. & A. Asgharzadeh (2006). Study of the effect of plant growth promoting rhizobacteria in maize cropping. *Iranian Journal of Soil and Water* 20: 133-134.

Hernandez, A.N., A. Hernandez & M. Heydrich (1995). Selection of rhizobacteria for use in maize cultivation. *Cultivos Tropicales* 6: 5-8.

Hoflich, G., W. Wiehe & G. Kohn (1994). Plant growth stimulation by inoculation with symbiotic and associative rhizosphere microorganisms. *Experientia* 50: 897-905.

Iqbal, M.A. & A. Iqbal (2015). Overviewing forage shortage for dairy animals and suitability of forage sorghum for ensiling. *Global Veterinaria* 14: 173-177.

Iqbal, M.A., A. Iqbal, N. Akbar, H.Z. Khan & R.N. Abbas (2015). A study on feed stuffs role in enhancing the productivity of milch animals in Pakistan- Existing scenario and future prospect. *Global Veterinaria* 14: 23-33.

Kumar, V., S.S. Punia, K. Lakshminarayana & N. Narula (1999). Effect of phosphate solubilizing analogue resistant mutants of *Azotobacter chroococcum* on sorghum. *Indian Journal of Agricultural Sciences* 69: 198-200.

Patten, C. & B.R. Glick (2003). Methods for isolating and characterized ACC-deaminas containing plant growth promoters rhizobacteria. *Physiologia Plantarum* 118: 10-15.

Raupach, G.S. & J.W. Kloepper (1998). Mixtures of plant growth promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathology* 88: 1158-1163.

Sarajuoghi, M., M.R. Ardakani, G. Nurmohammadi, A. Kashani, F. Rejali & S. Mafakheri (2012). Response of yield and yield components of maize (*Zea mays* L.) to different bio-fertilizers and chemical fertilizers. *American-Eurasian Journal of Agricultural & Environmental Sciences* 12: 315-320.

Singh, G., D.R. Biswas & T.S. Marwaha (2010). Mobilization of potassium from waste mica by plant growth promoting rhizobacteria and its assimilation by maize (*Zea mays*) and wheat (*Triticum aestivum* L.): a hydroponics study under phytotron growth chamber. *Journal of Plant Nutrition* 33: 1236-1251.

Steel, D.G.D., J.H. Torrie & D.A. Dicky (1997). A biometrical approach. 3rd Ed. McGraw Hill, Inc. Book co. N.Y. USA. 352 p.

Tsavkelova, E.A., S.Y. Klimova, T.A. Cherdyntseva & A.I. Netrusov (2006). Microbial producers of plant growth stimulators and their practical use: a review. *Applied Biochemistry and Microbiology* 42: 117-126.

Zahir, Z.A., M. Arshad & A. Hussain (1996). Response of wheat (*Triticum aestivum* L.) to *Azotobacter* inoculation under fertilized. *Pakistan Journal of Agricultural Sciences* 12: 133-136.