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Research Article

Enhancing the nutritional value of forage *Zea mays* '370' through growth-promoting rhizobacteria: Insights from azotobacter and pseudomonas intervention

Karim Esazadeh[1], Mousa Arshad[2*] and Elnaz Farajzadeh[1]

[1] Department of Agronomy, Malekan Branch, Islamic Azad University, Malekan, Iran
[2] Department of Horticultural Science, Mahabad Branch, Islamic Azad University, Mahabad, Iran
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Abstract

A research study was conducted at the Agricultural and Natural Resources Research Station in Miandoab to assess the impact of growth promoters on the quality of maize cultivar 370. The study followed a factorial experiment design with two factors and was organized as randomized complete blocks in three replications. The first factor examined bacterial strains of *Azotobacter*, including three levels: non-inoculated (control), *Azotobacter* sp. strain 5, and *A. chroococcum* DSM 1691. The second factor involved the bacterium *Pseudomonas*, with four levels: non-inoculated (control), *Pseudomonas fluorescens* 93—strain R 168, *P. fluorescens* DSM 50090, and *P. putida* DSM. The study evaluated various traits such as oil percentage, protein percentage, nitrogen percentage, forage digestibility percentage, ash percentage, insoluble fiber percentage, and fodder raw energy. The highest nitrogen percentages were achieved by inoculating maize seeds with *A. chroococcum* DSM 1691 and *P. putida* DSM, yielding values of 4.96% and 4.91%, respectively. Conversely, the control plants exhibited the lowest nitrogen percentage. Furthermore, the quantities of seed protein and forage digestibility percentage exhibited a significant increase following the inoculation of *Rhizobacteria*. The statistical data analysis revealed that the combined inoculation of bacteria *A. chroococcum* DSM 1691 and *P. putida* DSM enhanced the quality characteristics of maize, notably increasing the protein percentage (8.9%) and forage digestibility percentage (69.04%). Taken together, the application of growth-promoting *Rhizobacteria* resulted in an enhancement of indicators associated with the nutritional value of maize, exemplifying a sustainable agricultural strategy.

Keywords: Fodder raw energy, Growth-promoting, *Rhizobacteria*, Maize, Quality traits

Introduction

Given the increasing demand for organic agricultural products and the critical importance of meticulous soil and environmental stewardship in facilitating optimal plant and arboreal growth, organic agriculture strives to maintain the balance of essential soil constituents necessary for nourishing plants (de Andrade *et al.*, 2023). It eschews the use of toxins and pesticides during cultivation, instead opting for natural fertilizers such as soil, leaves, algae, and biological fertilizers, in place of chemical alternatives (Timsina, 2018). Regarding pest management, this methodology leans on biological approaches, employing microorganisms, ladybirds, bees, bacteria, or pest-resistant cultivars, rather than relying on pesticides and chemical agents. Moreover, genetically modified and radiation-exposed seeds are not utilized, ensuring that the end product offered to consumers remains free from toxic residues, chemicals, and preservatives. Furthermore, high-quality food produced with bio-fertilizers not only meets consumers' expectations but also safeguards their physical health (Barea *et al*., 2002). According to Kizilkaya (2008), the influence of biological fertilizers containing *Azotobacter* on the grain yield of spring wheat is significant, resulting in an 84% increase compared to the control plants.

The researchers observed that maize seeds inoculated with the bacteria *Azotobacter* demonstrated accelerated germination. Furthermore, they noted an enhanced growth of maize seedling roots following inoculation with the bacteria *Azospirillum lipoferum* (Pereira *et al*., 2020). Glick et al. (1995) documented the enhancement of sweet maize seedling growth through inoculation with the bacteria *Pseudomonas*. Additionally, guerinot reported an augmentation in the plant dry weight (biomass) of maize seeds inoculated with the bacteria *Azotobacter*, an increase in fresh and dry weight, leaf surface area, and plant height for maize

*Corresponding Author, Email: mo_arshad2002@yahoo.com

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seeds inoculated with *Azospirillum*, and a rise in fresh weight of plants, number of leaves, and plant height for maize seeds inoculated with the bacterium *Pseudomonas fluorescens*. Furthermore, an increase in the yield of maize grains was observed when inoculated simultaneously with the bacteria *Azotobacter* and *Pseudomonas* (Guerinot, 1991).

By producing plant growth-promoting hormones that enhance water and nutrient absorption, improve seedling germination and emergence, generate antimicrobial compounds, eradicate pathogens, and induce plant defense genes, PGPR bacteria can stimulate plant growth and mitigate root pathogens through competitive exclusion (Giles and Whitehead, 1977). Plant growth promoting *Rhizobacteria* are free-living soil microorganisms that inhabit the rhizosphere or plant roots during plant growth and development (Villa-Rodriguez *et al.,* 2019). One example of such microorganisms is *Rhizobium*, which possesses the potential for symbiotic nitrogen fixation alongside legumes. Additionally, non-symbiotic bacteria such as *Azotobacter* and *Azospirillum* can fix molecular nitrogen. While nitrogen fixation by non-symbiotic bacteria is comparatively less efficient than that of *Rhizobium*, these bacteria are ubiquitous in most soils and exhibit synergistic effects with *Rhizobium* (Cardoso and Kuyper, 2006). This category of bacteria can supply only a fraction of the nitrogen required by a plant. However, there are reports suggesting that this bacterium can fulfill all the nitrogen needs of a plant, obviating the necessity for nitrogen fertilizer, although the outcomes of inoculation with this bacterium can vary significantly (Douds Jr *et al*., 1993).

Materials and methods

This research was conducted at the Agricultural and Natural Resources Research Station in Miandoab to examine the impact of inoculating growth-promoting bacteria on the growth and development of maize. The location is positioned at a longitude of 45 degrees, 54 minutes, and 9 seconds and latitude of 37 degrees, 24 minutes, and 12 seconds; It is situated 1125 meters above sea level. This research was conducted as a factorial experiment involving two factors arranged within a randomized complete block design, with three replications. As depicted in Table 1, the bacterial strains utilized in this investigation were sourced from the Tehran Soil and Water Research Institute. Both of the bacteria (*Azotobacter* and *Pseudomonas*) utilized in this study are gram-negative and aerobic, characterized by a thin peptidoglycan wall enveloped by an outer membrane. While *Azotobacter* possesses the ability to fix nitrogen, *Pseudomonas* exhibits effectiveness across a broad spectrum of substrates. Notably, among the two bacteria investigated, *Pseudomonas* demonstrates greater adaptability to diverse environmental conditions (Silhavy *et al.,* 2010).

The maize cultivar utilized in this experiment is 370. To ensure that the seeds were not infected with contamination, they were repeatedly washed and disinfected. The surfaces of the seeds were sterilized with a 2% solution of sodium hypochlorite for 10 minutes to prevent a reduction in bacterial population. The minimum interval period between seed inoculation and planting was less than 24 hours. In terms of planting surface, a certain amount of seeds was measured and soaked in a 20% Arabic gum solution. Afterward, specified amounts of inoculants with a population of approximately 10^8 bacteria per ml were added and mixed thoroughly. Following the inoculation process, the seeds were dried in the shade and transferred to the farm for planting. Standard agricultural practices for land preparation, including plowing, clearing, and leveling, were performed. The distance between rows was set at 75 cm apart to allow for a spacing of 20 cm between seeds on the borders during manual planting in the rows on ridges at a depth of 3 to 5 cm. The first irrigation was carried out immediately after planting on June 28, and subsequent irrigation occurred at intervals of 7 to 10 days via leakage as needed. According to a density of 66,000 plants per hectare, wet planting of seeds was performed on ridges. To determine protein content, seeds were milled for 24 hrs. at an oven temperature of 75 °C. Subsequently, a mixture of copper sulfate, potassium sulfate, and selenium dioxide and 20 mL of 98% concentrated sulfuric acid were added to the milled samples. Protein percentage was calculated through titration using a magnetic stirring device and an automatic burette apparatus following the Kjeldahl method (Lynch and Barbano, 1999).

Oil concentration was determined using the Soxhlet technique as follows: Firstly, approximately 5 g of milled samples were poured into filter paper that had been prepared in an envelope-like manner. To remove any moisture from the samples, pockets containing them were placed in an oven at 55 °C for 24 hours. After this time, 2 g of the samples prepared for oil determination were inserted into thimbles used in the Soxhlet apparatus. N-hexane solvent was used to extract 100 ml of oil seeds as the sample in Soxhlet. The oil percentage was calculated by measuring the weight difference between the secondary and primary weights of the Soxhlet cups.

Fodder raw energy determination: The assessment of total energy content, commenced with the subdivision of samples into uniformly sized particles via a shredding process, followed by desiccation in an oven. Subsequently, the desiccated samples were introduced into the calorimeter bomb apparatus. This device, employing an electric current to incinerate the sample, facilitated the quantification of raw (total) energy, expressed in cal/g, with resultant measurements being meticulously recorded (Cherney and Hall, 1992).

To determine the nitrogen content, 10 g of seed samples from each plot were chopped and sent to a laboratory for analysis. Other quality indicators,

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Table 1. Combinations of *Azotobacter* and *Pseudomonas* treatments tested

Azotobacter		*Pseudomonas*		
Control-non inoculation	(0 A)	Control-non inoculation	(0P)	0A;0P
		P. fluorescens 93 – strain R 168	(1P)	0A;1P
		P. fluorescens DSM 50090	(2P)	0A;2P
		P. putida DSM	(3P)	0A;3P
A. sp. Strain 5	(1 A)	Control-non inoculation	(0P)	1A;0P
		P. fluorescens 93 – strain R 168	(1P)	1A;1P
		P. fluorescens DSM 50090	(2P)	1A;2P
		P. putida DSM	(3P)	1A;3P
A. chroococcum DSM 1691	(2 A)	Control-non incubation	(0P)	2A;0P
		P. *fluorescens* 93 – strain R 168	(1P)	2A;1P
		P. fluorescens DSM 50090	(2P)	2A;2P
		P. putida DSM	(3P)	2A;3P

including the percentage of raw fiber and ash, were determined, and the mean value of these parameters was calculated for each plot. Data analysis was conducted using SPSS software, and treatment means were compared utilizing the LSD test.

Results and discussion

Nitrogen percentage: The data from the first table, analyzed for variances, indicate that single inoculation of *Azotobacter* and *Pseudomonas* increases the percentage of nitrogen in maize compared to the control treatment, and statistically significant effects are observed at probability levels of 1% and 5%, respectively (Table 1). The highest percentages of nitrogen were obtained through the inoculation of maize seeds with *A. chroococcum* DSM 1691 and *P. putida* DSM, with values of 4.96% and 4.91%, respectively, while the lowest percentage of nitrogen is associated with the control (Table 2). Inoculating maize seeds with nitrogen-fixing bacteria such as *Azotobacter* and *Pseudomonas* constitutes a viable strategy to augment nitrogen availability to maize plants. Through the process of biological nitrogen fixation, these bacteria convert atmospheric nitrogen into forms readily assimilable by plants (Wang *et al.,* 2004), thus enhancing nitrogen uptake by maize. Additionally, *Azotobacter* and *Pseudomonas* promote root development and produce plant growth-promoting substances, fostering a more robust root system and overall plant vigor (Bhat *et al.,* 2023). This results in improved nutrient uptake efficiency, including nitrogen, and subsequently contributes to enhanced maize growth and yield. Therefore, the inoculation of maize with *Azotobacter* and *Pseudomonas* represents an effective approach for sustainable nitrogen management in maize cultivation, with implications for agricultural productivity and resource utilization optimization. Bethlenfalvay and Linderman (1992) conducted their experiments under greenhouse conditions and concluded that due to increased absorption of minerals from soil, growth-promoting bacteria raise nitrogen in maize and accelerate enzymes involved in food assimilation and photosynthesis.

Protein percentage: In line with the findings of Aghdam and Jalili (2023) and Pour-Aboughadareh *et al*. (2021), the use of growth-promoting bacteria has been shown to increase crude protein in maize grains and aerial organs. This observation is also applicable to the current study, as indicated by the results presented in the table of variance analysis, which reveal that the interactive effects of *Azotobacter* and *Pseudomonas* have significant effects on maize yield at a probability level of 1% (Table 1).

The LSD test for comparing means reveals that dual inoculation with *Azotobacter chroococcum* DSM 1691 and *Pseudomonas putida* DSM leads to a maize protein level of 8.9%, which falls into the same statistical group as the dual inoculation of *A.* sp. Strain 5 and *P. putida* DSM. The lowest protein level is observed in the control group, with a value of 6.7% (Table 2). Inoculation of maize seeds with *Azotobacter* and *Pseudomonas* can potentially increase protein content in maize through their symbiotic relationship with the plant. These beneficial bacteria have the ability to fix atmospheric nitrogen into forms that are readily available for plant uptake, such as ammonium and nitrate (ref). By facilitating nitrogen fixation, *Azotobacter* and *Pseudomonas* enhance the plant's nitrogen assimilation capabilities, thereby promoting its overall growth and development. As a result, the increased availability of nitrogen can contribute to the synthesis of proteins within the maize plants. Khalili has also reported significant effects of the bacterium *Azotobacter* on total protein in maize kernels (Aghdam and Jalili, 2023), and *Pseudomonas putida* application has significantly increased the protein percentage in forage sorghum (Bala *et al*., 2010).

Digestibility percentage of forage: The results of the variance analysis indicate that the interactions between growth-promoting bacteria, specifically *Azotobacter* and *Pseudomonas*, have significant effects on the digestibility of dry forage in maize (Table 1). The dual inoculation of *A. chroococcum* DSM 1691 and *P. putida* DSM at a rate of 4.69% resulted in the highest percentage of dry forage digestibility, while the control treatment had the lowest percentage (Table 2). The percentage increase in digestibility is a crucial trait in determining forage quality (Ahemad and Kibret, 2014). Research on one-year-old summer forage crops by Vessey (2003) found that digestible dry matter is

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Table 2. The analysis of variance for the effect of a single inoculation of *Azotobacter* and *Pseudomonas* on the percentage of nitrogen, oil, protein, digestible forage, insoluble fiber, ash, and fodder raw energy in maize leaves.

Source of variance	df	MS						
		Nitrogen	Oil	Proteine	Digestible of forage	Insoluble fiber	Ash	Fodder raw energy
Block (R)	2	0.47 ^{ns}	0.79 ^{ns}	3.25 ^{ns}	1.89 ^{ns}	1.44 ^{ns}	1.81 ^{ns}	0.98
Azotobacter (A)	2	6.43**	0.96 ^{ns}	7.46**	20.12*	49.21**	16.01**	12.01**
Pseudomonas (P)	3	1.43*	86 ^{ns}	8.44**	21.86*	52.44**	3.24*	8.98*
A × *P*	6	0.66 ^{ns}	0.91 ^{ns}	5.99**	22.94**	48.16**	1.02 ^{ns}	7.94*
Error	10	0.25	0.39	0.58	4.01	3.24	0.99	0.58
CV (%)		10.89	11.67	9.56	3.06	8.91	2.34	16.62

****, * and n.s, is denote statistical significance at the 1%, 5%, and non-significant levels, respectively**

Table 3. The comparison of means for the effects of a single inoculation of *Azotobacter* and *Pseudomonas* on the percentage of nitrogen, oil, protein, digestible forage, insoluble fiber, ash, and metabolism energy in maize leaves

Treatments		Nitrogen	Oil	Protein	Dry Digestible of forage	Insoluble fiber	Ash	Fodder raw Energy (Cal/g)
		(%)						
Azotobacter	Control (A0)	4.01 ^{c}	5.30 ^{a}	6.81 ^{b}	60.01 ^{b}	23.14 ^{a}	4.58 ^{a}	7.25 ^{c}
	A. sp. Strain 5 (A1)	4.89 ^{b}	5.51 ^{a}	8.54 ^{ab}	68.26 ^{a}	19.12 ^{b}	4.25 ^{b}	9.58 ^{b}
	A. chroococcum DSM 1691 (A2)	4.96 ^{a}	5.49 ^{a}	8.84 ^{a}	69.58 ^{a}	18.47 ^{b}	4.01 ^{c}	10.89 ^{a}
Pseudomona	Control (P0)	4.05 ^{c}	5.09 ^{a}	6.65 ^{c}	61.11 ^{c}	23.24 ^{c}	4.62 ^{a}	6.87 ^{c}
	P. fluorescens 93– Strain R 168 (P1)	4.50 ^{b}	5.57 ^{a}	7.46 ^{b}	64.41 ^{b}	19.45 ^{a}	4.21 ^{b}	8.94 ^{b}
	P. fluorescens DSM 50090 (P2)	4.84 ^{b}	5.45 ^{a}	8.45 ^{a}	65.71 ^{b}	19.74 ^{a}	4.12 ^{bc}	9.67 ^{a}
	P. putida DSM (P3)	4.91 ^{a}	5.10 ^{a}	8.96 ^{a}	68.49 ^{a}	18.14 ^{b}	4.08 ^{c}	9.01 ^{ab}
Azotobacter × *Pseudomona*	$P_0 \times A_0$	4.25 ^{a}	5.29 ^{a}	6.73 ^{e}	60.56 ^{e}	23.19 ^{a}	4.60 ^{a}	7.06 ^{d}
	$P_1 \times A_0$	4.32 ^{a}	5.31 ^{a}	7.14 ^{d}	62.21 ^{d}	21.30 ^{b}	4.40 ^{a}	8.10 ^{c}
	$P_2 \times A_0$	4.36 ^{a}	5.40 ^{a}	7.53 ^{c}	62.86 ^{d}	21.44 ^{b}	4.35 ^{a}	8.46 ^{bc}
	$P_3 \times A_0$	4.34 ^{a}	5.35 ^{a}	7.89 ^{b}	64.25 ^{c}	20.64 ^{bc}	4.33 ^{a}	8.13 ^{c}
	$P_0 \times A_1$	4.37 ^{a}	5.39 ^{a}	7.60 ^{c}	64.69 ^{c}	21.18 ^{b}	4.44 ^{a}	8.23 ^{c}
	$P_1 \times A_1$	4.41 ^{a}	5.42 ^{a}	8.00 ^{b}	66.34 ^{b}	19.29 ^{c}	4.23 ^{a}	8.26 ^{b}
	$P_2 \times A_1$	4.22 ^{a}	5.52 ^{a}	8.40 ^{ab}	66.69 ^{b}	19.43 ^{c}	4.19 ^{a}	9.63 ^{ab}
	$P_3 \times A_1$	4.26 ^{a}	5.47 ^{a}	8.75 ^{a}	68.38 ^{a}	18.63 ^{cd}	4.17 ^{a}	9.30 ^{b}
	$P_0 \times A_2$	4.29 ^{a}	5.46 ^{a}	7.75 ^{bc}	65.35 ^{bc}	20.86 ^{bc}	4.32 ^{a}	8.88 ^{bc}
	$P_1 \times A_2$	4.34 ^{a}	5.36 ^{a}	8.15 ^{b}	67.00 ^{b}	18.96 ^{cd}	4.11 ^{a}	9.92 ^{ab}
	$P_2 \times A_2$	4.32 ^{a}	5.48 ^{a}	8.55 ^{ab}	67.65 ^{b}	19.11 ^{c}	4.07 ^{a}	10.28 ^{a}
	$P_3 \times A_2$	4.33 ^{a}	5.47 ^{a}	8.90 ^{a}	69.04 ^{a}	18.31 ^{d}	4.05 ^{a}	9.95 ^{ab}

The different letters in each column indicated significant difference of treatments at 5% on the basis of LSD

negatively correlated with the percentage of crude protein and the percentage of fibers unsolved in acid detergent and ash. Environmental factors such as temperature, moisture tension, shade, soil texture, and others also affect digestibility (Zahir *et al*., 2003). However, some studies suggest that dry forage digestibility is not affected by drought.

Insoluble fiber percentage: The results from the variance analysis table indicate that the interactions between growth-promoting bacteria of *Azotobacter* and *Pseudomonas* have significant effects at a level of 1% on the percentage of insoluble fiber. The highest percentage of insoluble fiber, at 23.19%, was observed in the control treatment, while the lowest percentage, at 18.31%, was observed in the bacterial dual inoculation of *A. chroococcum* DSM 1691 and *P. putida* DSM with maize seed (Table 2). An increase in the percentage of insoluble fiber leads to a decrease in forage digestibility and reduces its quality. The study by Al-Karaki and Hammad (2001) suggests that growth-promoting bacteria of *Azotobacter*, *Azospirillum*, and *Pseudomonas* reduce the synthesis of insoluble fiber as they enhance the plants' tolerance by creating colonized tissue in the root rhizosphere under drought and heat stress conditions. The reduction in insoluble fiber percentage observed subsequent to the inoculation of maize with *Azotobacter* and *Pseudomonas* may be ascribed to various factors. Primarily, these *Rhizobacteria* have been documented to augment nutrient availability in the soil (Etesami and Adl, 2020), thereby potentially fostering the synthesis of more readily digestible components within plant tissues, consequently leading to a decline in insoluble fiber content. Additionally, the stimulation of plant growth and development by *Azotobacter* and *Pseudomonas* may engender heightened metabolic activity and enhanced nutrient uptake by the plant, as evidenced by elevated nitrogen content (Table 3), conceivably resulting in

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alterations to the composition of plant tissues, including an increase in protein content (Table 3) alongside a decrease in insoluble fiber content. Nonetheless, the precise mechanisms and intricate interactions involved necessitate further investigation.

Ash percentage: The study also indicates that the strains of growth-promoting bacteria, specifically *Azotobacter*, have a significant and positive impact on reducing the ash content in forage. The results show that the highest percentage of ash is observed in the control treatment, with a value of 4.6%. On the other hand, seed inoculation with the strains of *A. chroococcum* DSM 1691 leads to a significant reduction in ash content, with a value of 4.01%. This reduction in ash content results in the production of more palatable forage with higher digestibility (Table 2). The decline in plant ash percentage observed in maize inoculated with *Rhizobacteria* may be attributed to enhanced nitrogen assimilation in these plants compared to control specimens (without inoculation). Indeed, the aforementioned bacteria have been demonstrated to facilitate elevated nitrogen fixation, thereby promoting increased nitrogen uptake in maize plants. Consequently, this results in a proportional elevation in organic compounds, notably proteins (as depicted in Table 3), relative to mineral constituents. This phenomenon underscores the intricate interplay between microbial inoculation and plant physiology, highlighting the symbiotic interactions between rhizobacteria and host plants that contribute to modifications in nutrient dynamics. Ultimately, these alterations influence the biochemical composition of plant tissues. The findings of this study align with prior research outcomes (Kapulnik *et al.,* 1981; Timofeeva *et al.,* 2023) concerning the diminishment of ash content percentage attributed to the influence of rhizobacteria.

Fodder raw energy: The study found that the use of growth-promoting bacteria significantly affects the fodder raw energy of plants, with statistical significance at a 5% probability level (Table 1). The results showed that the forage with seeds inoculated with *A. chroococcum* DSM 1691 and *P. fluorescens* DSM 50090 had the highest fodder raw energy (10.28%), while the control treatment had the lowest rate. This suggests that using growth-promoting bacteria can result in more nutritious and high-energy forage for farmers, as these bacteria provide food resources around the plant roots, improving their growing conditions and resulting in better quality forage. As stated by Bashan *et al*. (2014), this type of bacterium does not have any negative effects on the plants, making it an ideal solution for farmers.

Conclusions

The study found that inoculating maize seeds with the bacteria *Azotobacter* and *Pseudomonas* leads to an improvement in the quality of the maize yield. This suggests that using growth-promoting *Rhizobacteria* bacteria can have statistically significant effects on the quality of maize cultivars, specifically the cultivar 370 in this study. Moreover, the dual inoculation of *A. chroococcum* DSM 1691 and *P. putida* DSM bacteria significantly enhances the protein content (8.90%) and improves the digestibility of dry forage (69.04%) in maize. The study recommends repeating measurements on the effects of bio-fertilizers on maize cultivars in different years and suggests using other growth-promoting bacteria, such as mycorrhizal and other bacteria, in bio-fertilizers.

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