
**EFFECT OF AZOSPIRILLUM ISOLATES ON THE
GROWTH AND NITROGEN CONTENT OF TEF
[ERAGROSTIS TEF (ZUCC.) TROTTER]**

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ABSTRACT: Two diazotrophic bacteria, coded as A31 and A32, were isolated from the rhizosphere of two tef varieties DZ-01-354 and DZ-01-196, respectively. Different morphological, biochemical, and physiological comparisons with type cultures *Azospirillum lipoferum*, ATCC 29707, and *Azospirillum brasiliense*, ATCC 29145 showed that the isolates belong to *A. lipoferum*. Inoculation studies of isolates on pot-grown tef plants showed marked increases in height, grain yield, total shoot and root weight, root-shoot ratio and total grain nitrogen. An increase in grain yield up to 12% over uninoculated controls was observed. The bacterial isolate A32 was found to perform better than A31 in promoting growth and yield on both homologous and heterologous tef varieties. Of the two tef varieties, DZ-01-096 responded better than DZ-01-354 to inoculation. The study indicates a possible specific interaction between host cultivar and bacterial isolate that governs plant yield.

Key words/phrases: *Azospirillum lipoferum*, yield increase, *Eragrostis tef*

INTRODUCTION

Tef [*Eragrostis tef* (Zucc.) Trotter] is a staple cereal food crop in Ethiopia. The grain is used to prepare *injera*, a soft, thin and sour tasting pancake like local bread. It has high nutrient and mineral content, and accounts for about two third

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of the daily protein intake in the diet of the population (ENS, 1959 cited in Seifu Ketema, 1993). Tef is adapted to a wide range of environments and cultivated under diverse agroclimatic conditions (Seifu Ketema, 1987). The crop performs well in both water-logged and moisture-stressed soils (Hailu Tefera *et al.*, 1990). Although tef has several suitable characters, its low grain yield per unit area is one of the major limitations of its productivity (Seifu Ketema, 1993).

Higher grain yield of tef was recorded by applying inorganic fertilizers (Abate Bekele, 1993). However, chemical fertilizers are neither easily available nor affordable for the majority of subsistence farmers. Such economical considerations necessitate for an alternative less-expensive and environmentally-friendly agricultural technologies to improve production.

The manipulation of root-associated or rhizosphere microorganisms to improve yield is one of the alternative technologies. The association between grasses and bacteria of the genus *Azospirillum* living on the surface or inside of roots has been reported to fix nitrogen and provide for the host plant thereby reducing the demand for nitrogen fertilizers and increasing crop productivity (Doebereiner and Baldani, 1981). Several studies have shown that mechanisms other than nitrogen fixation are attributable to the increase in crop yield. These include phytohormone production for improving root growth that may increase the rate of water and mineral uptake and protection from diseases by antagonizing soil pathogens by their proliferation in the rhizosphere (Volpin and Kapulnik, 1994). Inoculation studies both in the field and in greenhouse for the last 20 years have shown increase in dry matter yield, total nitrogen content of grains, improved water status, and nutritive quality of crops such as millet, sorghum, maize, rice, wheat and barley (Boddey and Doebereiner, 1988). Plant responses to inoculation, however, was found to be diverse depending upon the plant genotypes, bacterial strains, and several environmental conditions (Boddey and Doebereiner, 1988; Bhattarai and Hess, 1993; Fallik and Okon, 1996).

In this study, attempt has been made to isolate *Azospirillum* strains from two tef growing areas to study their diversity, effect on grain yield, nitrogen content and plant productivity on two improved teff cultivars of Ethiopia.

MATERIAL AND METHODS

Media

Nitrogen-free basic (Nfb) medium

A modified semisolid agar medium of Doebereiner and Jay was used for the isolation of bacterial strains. One litre of the medium was prepared with: (Malic acid, 4; glucose, 1; $\text{Na}_2\text{MO}_4 \cdot 2\text{H}_2\text{O}$, 0.02; KOH, 0.7; Yeast extract, 0.21; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; NaCl, 0.2; Agar, 1.75 %, pH 6.5 (Doebereiner and Jay, 1976).

Congo red plate (RC)

This medium was used to select *Azospirillum* colony, and was prepared as described in Rodrigues-Caceres (1982). The pH was adjusted to 6.5.

Nutrient medium (NM)

The medium was prepared with the following components g l^{-1} : Nutrient broth (Merck) 8, KCl, 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.12, $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$, 1 mg, pH adjusted to 6.5.

Soil and root samples

Soils and wheat root samples were collected from two sites, Holetta and Debrezeit, that are 50 km west and south of Addis Ababa, respectively. These sites are known cereal growing areas. The plots from where the samples were collected were used for the growth of tef for several years. Seventy to hundred kilo grams of soil from each site was taken and collected in plastic bags. The soil samples were used to grow tef seedlings and establish the diazotrophic bacteria in the rhizospheres using plant trap method, and for the controlled inoculation experiment of the isolated strains.

Establishments of Azospirillum from the soil

A mixture of 1.5 kg of soil from each of the Holetta and Debrezeit samples was separately mixed with 0.5 kg of washed and autoclaved sand, and each mixture was filled into six 3-litre capacity synthetic pots. Six to ten tef seeds of two improved varieties, DZ-01-196 and DZ-01-354 were surface-sterilized with 0.5% (w/v) HgCl_2 for two minutes and subsequently washed three times with

sterile water and sown on their respective plastic pots. The pots were then kept in the greenhouse and watered every other day. The seedlings were thinned to five plants per pot 10 days after emergence and allowed to grow for about one month.

Isolation of Azospirillum

Two seedlings from every pot were randomly dug out and cut at the soil line with a sterile razor blade. The root part from each seedling was then transferred to sterile petriplates and washed thoroughly with sterile distilled water. Washed roots were finally cut into 0.5 cm pieces and aseptically inoculated into 9 ml small test tubes containing 4 ml semisolid nitrogen free basic (NFB) and congo red (CR) medium. A total of 12 tubes were incubated at 35° C for 3–4 days. At the end of the inoculation period, tubes with typical subsurface pellicle under the surface of the semisolid NFB medium, red to scarlet colony formation on RC plates were selected and streaked on nutrient agar plates. Representative colonies from each plate were purified by successive streaking on the same medium, and maintained on Tryptone Soy Broth (TSB) agar slants and kept at 40° C as stock cultures.

Characterization and identification of isolates

Morphological characters; shape, size, cellular arrangement, and physiological tests; gram reaction, sodium hydroxide reaction, catalase activity, urease activity, aesculin hydrolysis, aerobic and anaerobic acidification of glucose and fructose, and H₂S formation were performed as described by (Collins and Lynne, 1976). The ability to utilize different sugars (glucose, mannitol, maltose, xylose, lactose, and galactose) as sole carbon source for growth in nitrogen free media was tested using the medium of Dobereiner and Jay (1976) with slight modification. The sugars were added to the basal medium after having been filter-sterilized to give a final concentration of 1% (Tarrand *et al.*, 1978). The cultures were incubated at 37° C for three days.

Pot Experiments

Preparation of seeds for germination

Soil samples from each site were mixed with washed sand in the ratio of 3 to 1 and autoclaved at 121° C and 15 lb sq² for 30 minutes. Aliquots of the

autoclaved soil were filled into thirty six, plastic pots (19 cm by 19 cm) rinsed with 95% alcohol. A handful of the two tef varieties (DZ-01-196 and DZ-01-354) were surface-sterilized as before. Six to eight treated seeds of each variety were then sown into each of the eighteen pots. The number of seedlings were thinned down to four 10 days of emergence.

Greenhouse experiment

Pure cultures of bacteria were grown into nutrient broth tubes in 250 ml Erlenmeyer flasks in a reciprocal shaker (120 rpm, 30° C) for 24 hr. Cells were pelleted and resuspended in sterile water at a concentration of 10^7 - 10^8 cells. Fifteen days after planting (DAP) 1.0 ml of bacterial suspension was injected near the root system of each plant with sterile disposable 1 ml syringes. Three control pots from each group were inoculated similarly with equal amount of sterile distilled water. Cross contamination was minimized by covering the surface of the pots with sterile coarse gravels. The pot experiment was carried out under glasshouse conditions at the Science Faculty Campus between November 1997 and February 1998 under natural illumination with minimum and maximum temperatures of 15° C and 33° C, respectively. Pots were laid down in randomized block design with three treatments and five replications. Pots were watered, two times a week with N-free Jensen's medium, and once in a fortnight with distilled water to avoid salt accumulation. The Jensen's N-free medium contained the following g l⁻¹: K₂HPO₄, 0.2; Ca₃(PO₄)₂, 1.0; MgSO₄.7H₂O, 0.2; NaCl, 0.2; FeCl₃, 0.14, pH 6.6 (Bergerson, 1980).

Growth parameters

Plants were harvested sixteen weeks after planting. Fresh root mass and shoot weight, were immediately recorded. Dry weights of root, grain and straw of plants were determined by drying at 60° C for 48 hr in an oven. Nitrogen content of tef-grain and shoot was determined using the Kjeldhal method.

Statistical analysis

For each data, mean values of treatments were calculated in a one-way analysis of variance and comparison of means at 5% level was made by least significance difference (LSD). Statistical analysis was done using the statistical program package - Statistica.

RESULTS

Two strains of *Azospirillum*, A31, and A32, were isolated after having screened several diazotrophs from tef roots using plant trap method. Subsequent physiological and biochemical characterization categorized them into the species *Azospirillum lipoferum* since many of their characters are comparable to type strain *Azospirillum lipoferum* ATCC 29707 (German collection) (Tables 1, 2 and 3). A31 and A32 were recovered from the roots of tef varieties DZ-01-354 and DZ-01-196, grown on Debrezeit light soil, respectively. No *Azospirillum* strain was isolated from Holetta soil. The two isolates were motile, gram negative, slightly curved short rods (Table 2). The following characters were also shared by both isolates (Table 3): oxidase, urease and catalase positive, production of acid anaerobically from glucose and fructose in both peptone and yeast extract medium. They hydrolysed esculin, and were found to alkalize nitrogen free Bromothymol Blue medium. They were differentiated into their respective strain on the basis of their colony colour, size and denitrifying activity (Tables 1, 2 and 3).

Table 1. Cultural characteristics of the two bacterial isolates.

Isolate code	Cultural characteristics				
	Size	Colour	Form	Margin	Elevation
A31	1.5 mm	White	circular	entire	raised
A32	2.0 mm	pink	circular	entire	raised

Table 2. Morphological characteristics of the two bacterial isolates.

Isolate code	Morphological characteristics			Growth characteristics in Nfb medium		Alkalinization of Nitrogen Free Bromothymol blue media (NFB)
	Gram rxn	Shape	Motility	Pellicle	D.F.S	
A31	-	s.c.rod*	+	present	2-3 mm	+
A32	-	s.c.rod*	+	present	1 mm	+

*, s.c. rod-slightly curved rods; D.F.S., distance from the surface.

Table 3. Physiological and biochemical characteristics of isolate A31 and A32 compared to *Azospirillum* type cultures (*A. lipoferrum* ATCC 20707 and *A. brasilense* ATCC 29145).

Characteristics	Isolate A31	Isolate A32	<i>A. lipoferrum</i>	<i>A. brasilense</i>
Cytochrome oxidase	+(strong)	+(moderate)	+	+
Catalase	+(strong)	+(moderate)	+	+
Esculin hydrolysis	+	+	+	+
Acidification of glucose media				
yeast extract based broth	+	+	+	-
yeast extract based broth (with 2 x phosphate)	+	+	+	-
peptone based broth				
Acidification from glucose and fructose anaerobically				
glucose(yeast extract)	+	+	+	-
glucose (peptone)	+	+	+	-
fructose (yeast extract)	+	+	+	-
fructose(peptone)				
Sole carbon source				
glucose	+	+	+	-
mannitol	+	+	+	+
fructose	+	+	+	-
malate	+	+	+	-
Indole production	-	-	-	-
Hydrogen sulfide production	-	-	-	-
Starch hydrolysis	-	-	-	-
Acidification of sugars				
glucose	+	+	+	-
mannitol	+	+	+	-
maltose	-	-	-	-
xylose	+	+	+	-
fructose	+	+	+	+
lactose	-	-	-	-
galactose	+	+	+	+
Urease	+	+	+	+
Denitrification	+	+	-	-
Dissimilation of NO ₃ to NO ₂	+	+	+	+
Growth in 3% NaCl	-	-	-	-
Biotin requirement	+	+	+	-

+, positive for the test; -, negative for the test.

Greenhouse experiment

Height of both varieties was significantly increased by the inoculation of both strains (Fig. 1A). The increase in height was found to be twice as much with the inoculation of A32 as with A31 in both tef varieties compared to the uninoculated ones. Although isolate A32 produced a higher increment in tillering than A31 on both varieties, the difference between bacterial treatments was not significant (Fig. 1B).

The shoot dry matter of the variety DZ-01-196 was found to increase following inoculation with *Azospirillum* strains as compared to the uninoculated ones. However the differences between the two strains were not statistically significant (Fig. 1C). Inoculation on variety DZ-354 did not show any difference between inoculation and distilled water treatments. No significant difference was observed on root dry weight between the two varieties upon inoculation by both strains of bacteria although differences were recorded between uninoculated and inoculated treatments in each variety (Fig. 1D). The same trend was also recorded on shoot-root ratio between the two varieties with a marked difference on inoculated treatments over their respective controls (Fig. 2C).

Mean fresh weight of roots of both teff cultivars increased by 13.1% and 22.6% in DZ-01-196, and 6.13% and 11.2% in DZ-01-354 when inoculated with isolate A31 and A32, respectively. Inoculation of tef seedlings with *Azospirillum* isolates produced increase in mean grain yield and grain nitrogen contents over uninoculated ones (Figs 2A and B). The difference in mean grain nitrogen content, however, was significant between inoculated strains on variety DZ-01-96 but not that of variety DZ-01-354. The percentage grain yield was also found to be higher on variety DZ-01-96 with both A31 and A32 *Azospirillum* strains as compared to the variety DZ-01-354. The mean total grain nitrogen content of inoculated and uninoculated tef varieties also showed similar trends with increased values of mean grain nitrogen content on both varieties with higher effect on DZ-01-196 with both strains. The percentage increase in total nitrogen content by inoculated groups over their respective controls was found to fall between 3.4% and 5.8%.

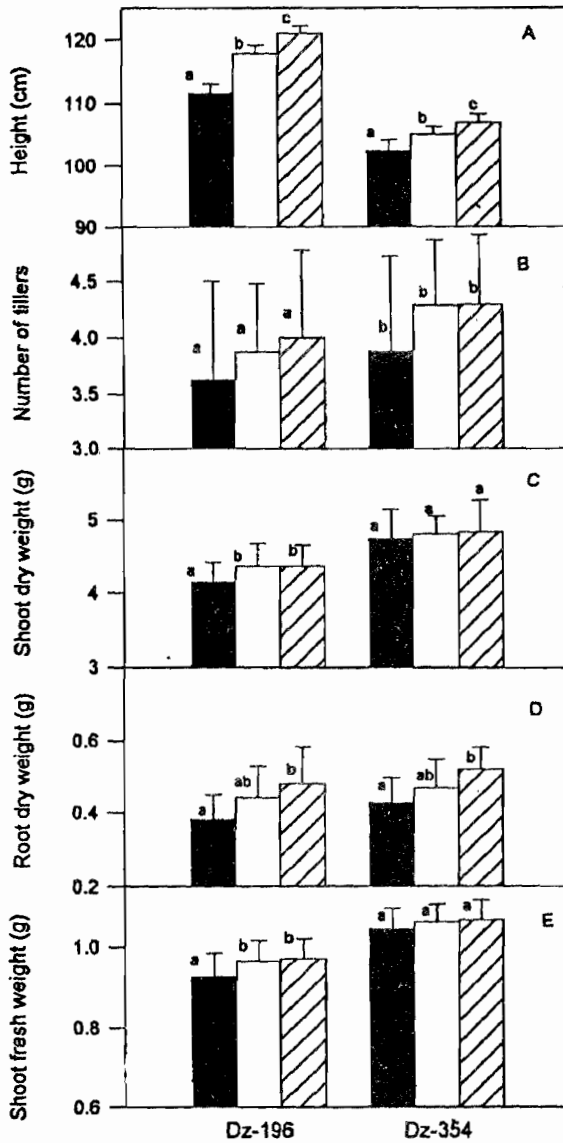


Fig. 1. Effect of inoculation of tef varieties Dz-01-196 and Dz-01-354 by *Azospirillum lipoferum* strains A31 and A32 on height (A), Number of tillers (B), Shoot dry weight (C), Root dry weight (D) and shoot fresh weight (E) of tef seedlings. Dark bars, controls; open bars, strain A31; and striped bars, strain A32.

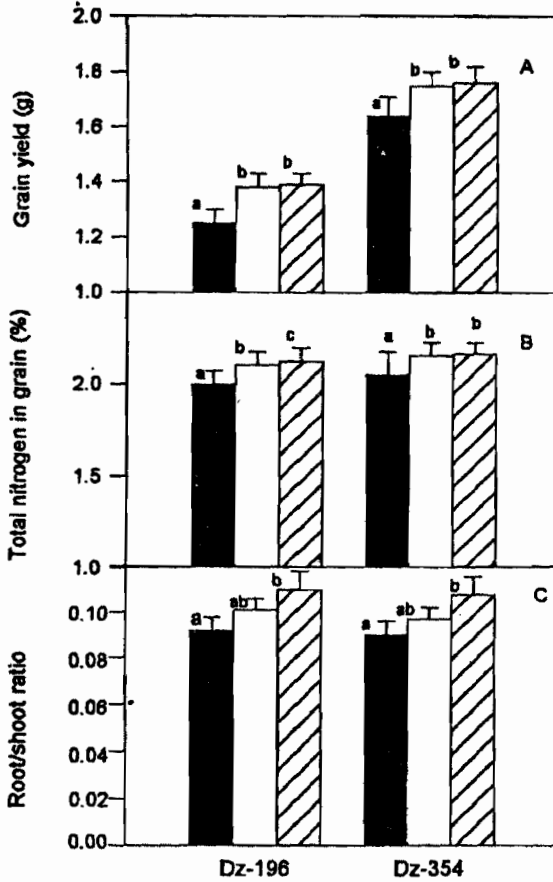


Fig. 2. Effect of inoculation of tef varieties Dz-01-196 and Dz 01-354 *Azospirillum lipoferum* strains A31 and A32 on grain yield (A), grain per cent nitrogen content (B); and root:shoot ratio (C) of tef. Dark bars, controls; open bars, strain A31; and striped bars, strain A32.

DISCUSSION

Within the limits of sampling sites, two bacterial strains of the genus *Azospirillum* were isolated from the root rhizoplane of tef, *Eragrostis tef*. Despite the fact that this nitrogen fixing diazotroph is abundant in most soils, attempt to isolate one from Holetta soil failed. The failure may be attributed to low

population number of these bacteria in the rhizoplane of the sampled soil due to domination by other diazotrophic bacteria. The characteristics of the two isolates closely resemble that of the type culture ATCC 29707 (German collection), and are identified as strains of *Azospirillum lipoferum*.

A marked and significant difference in height and a higher but non-significant difference in fertile tiller number was observed between inoculated and uninoculated controls. Although inoculation increased the mean height in both varieties, it was the variety DZ-01-196 that responded better to inoculation. The percentage increase due to inoculation for this variety was almost twice that of DZ-01-354. Several reports on close relationships between increase in plant height, tillering, grain yield by inoculated crops have been recorded (Kapulnik *et al.*, 1981a; 1981b; Millet *et al.*, 1984; Nuzziello *et al.*, 1987; Lee *et al.*, 1989). An increase in height and quantity is beneficial as animal feed and it can be mixed with clay for building of traditional houses. It has been reported that, tef straw, when used as animal feed is superior in weight gain to those of wheat and oat straw (Seifu Ketema, 1993).

Although significant differences on root dry weight between the two varieties upon inoculation by both strains of bacteria were not recorded, the difference in increase in weight over uninoculated control indicates that inoculation is beneficial in biomass accumulation. Similar trends were also observed on wheat, sorghum, and panicum (Kapulnik *et al.*, 1981a), corn (Lee *et al.*, 1989) and digit grass (Schank *et al.*, 1981).

A marked percentage increase in root fresh weight on both tef varieties by all inoculants over uninoculated controls suggests that water uptake was enhanced by inoculation. Sarig *et al.* (1988) reported a 15% significant increase in total soil moisture extraction of inoculated roots of sorghum over uninoculated controls. This and other previous reports showed that such a difference is due to the alteration of root morphology, and an increase in the number of root hairs, root length, and surface area as a result of the interaction of the root system of the plants and the *Azospirillum* species (Kapulnik *et al.*, 1985b; Okon and kapulnik, 1986). The alteration of the root system, apart from facilitating the uptake of more nutrients and speeding up the metabolic activities of the root and shoot systems, is said to enhance the water extracting ability of the roots to accumulate more water in their roots than uninoculated controls (Sarig *et al.*, 1988).

Such observations have interesting implications when considering the role of *Azospirillum* inoculation in soils with low rainfall areas or when plants face occasional water stress conditions. Abuhay Takele (1997) reported a decrease in fresh and dry weight of shoots and number of productive tillers in tef varieties exposed to water stress. Moreover, the difference in shoot fresh weight of inoculated and uninoculated plants is important if growing conditions during grain filling are difficult, when stem reserves are translocated to the developing grain (Boyer and McPherson, 1975).

Similarly, root dry matter increases of tef plants can be associated with an extensive root development with a higher accumulation of nutrients in the root system (Lin *et al.*, 1983; Okon and Kapulnik, 1986). Although the root-shoot ratio of this work did not show significant differences among strains and varieties, the fact that inoculation gave better results than the uninoculated ones could imply that there is a better root development as a result of the positive interaction between the plants and associated diazotrophs (Fallik and Okon, 1996).

Inoculation of tef plants with *Azospirillum* isolates significantly increased grain yield. Higher yield responses were observed for the tef variety DZ-01-096 compared to DZ-01-354 by both isolates indicating the existence of some degree of specificity that affects plant productivity (Millet *et al.*, 1984; Dobereiner, 1988). Yield increases of 8–32% and 8.8–25.2% of wheat cultivars were obtained as a result of inoculation by single and multiple inoculation of *Azospirillum lipoferum* and *Azospirillum brasiliense* strains, respectively (Mertens and Hess, 1984; Bhattarai and Hess, 1993). Similar results were also recorded for corn by Arsac *et al.* (1990).

Increases in the mean percentage nitrogen content of both varieties was found to be higher than the control groups. However, DZ-01-196 responded better than DZ-01-354. A similar difference in total nitrogen between inoculated and control groups have been reported for various cereals including wheat, sorghum, and panicum (Kapulnik *et al.*, 1981b) in greenhouse and for pearl millet in field conditions (Wani *et al.*, 1985). Increase of nitrogen content between 3 and 5% of the different inoculated groups of the present work falls within the range of wheat varieties inoculated with *Azospirillum lipoferum* (Mertens and Hess, 1984; Bhattarai and Hess, 1993). The increase of total nitrogen of shoots together with the increase of root fresh weight (6–22%) and

root dry weight (9–21%) by inoculated tef varieties compared to uninoculated ones implies that the effect of inoculation enhances the mechanism of efficient nutrient uptake by altering the root system. The importance of this mechanism has been supported by different workers (Kapulnik *et al.*, 1985a; Okon and Kapulnick, 1986; Lin *et al.*, 1983; Jain and Patriquin, 1984). Although the role of nitrogen fixation was not measured in this work, its contribution cannot be ruled out in the total nitrogen uptake of tef. The findings of Bhattarai and Hess (1993) clearly showed the dual importance of nitrogen fixation as well as nutrient uptake in the increase of both parameters as a result of *Azospirillum* inoculation.

In general, the present study demonstrated that *Azospirillum* isolates were capable of improving the growth and different yield parameters of the two tef varieties. However, the two isolates displayed a marked difference in their effect on several features of growth and productivity of tef. The variation might be caused by differences in the degree of compatibility between the bacterial isolates and tef varieties.

In view of the importance of tef in the diet of the Ethiopian population, any improvement of its quality and quantity is very important. Moreover, the higher yield of tef due to inoculation in unfertilized sterile soil would show the potential of these isolates in supplementing fertilizer requirements. The pot experiments also revealed that the two tef varieties respond differently to inoculation with either of the bacterial isolates. The bacterial isolate A32 was found to perform better than A31 on both varieties, and the tef variety DZ-01-196 responded better to inoculation. This suggests the existence of some degree of specificity in the interaction between *Azospirillum* spp and different cereals (Boddey *et al.*, 1986), or different cultivar of the same species (Suikiman and New, 1990). Since effectiveness in yield depends on cultivar and bacterial diversity, a thorough study on the interaction of various tef cultivars with *Azospirillum* isolated from different agroclimatic regions of the country under field conditions is necessary for better results. As inoculation of tef by VAM fungi (Tekalign Mamo, 1987) and phosphate solubilizing fungi (Asfaw Haile Mariam, 1993) had shown to increase tef productivity, evaluation of mixed inoculation of these microorganisms and effective *Azospirillum* on tef may further improve yield.

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