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Sorghum vulgare Plus *Azospirillum lipoferum* and *Rhizobium tropici* with NH_4NO_3 at 50% Reduce N_2O Releasing

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Abstract

Sorghum vulgare, a gramineae of commercial value, requires NH_4NO_3 for healthy growth, that in excess causes loss of soil productivity and the release of N_2O, a greenhouse gas that contributes to global warming. An alternative solution to reduce and optimize NH_4NO_3 with: Azospirillum lipoferum and Rhizobium tropici. The objective of this work was: to analyze the effect of A. lipoferum and R. tropici 50%. For this, A. lipoferum and R. tropici were inoculated in S. vulgare in a hydroponic system in Leonard jars, under a randomized block experimental design with 5 treatments and 5 repetitions each. The response variables were percentage (%) of germination, aerial and root phenology; plant height and root length, biomass: aerial fresh weight and radical fresh weight, experimental data were analyzed by Tukey. The results showed 100% germination of S. vulgare with A. lipoferum and R. tropici statistically different numerical value compared to the 75% S. vulgare used as relative control (RC) with 100% NH_4NO_3 not inoculated. S. vulgare to seedling aerial dry weight (ADW) with A. lipoferum and R. tropici was 0.90g statistically different numerical value compared to 0.35g ADW of S. vulgare used as RC. At the flowering level, S. vulgare with A. lipoferum and R. tropici reached 3.17g of radical dry weight (RDW), a statistically different numerical value compared to the 1.59g of RDW of S. vulgare with 100% NH_4NO_3 not inoculated. It was evident that the integration of the metabolic capacity of A. lipoferum combined with the analogue of R. tropici, generated sufficient phytohormones to increase the capacity of S. vulgare for maximum uptake of NH_4NO_3, but non compromising its healthy growth, that avoiding lost soil fertility, contamination of surface water and aquifers, especially the release of N_2O greenhouse gas partly responsible for global warming. Concludes that A. lipoferum and R. tropici in S. vulgar, through a synergistic action, optimized NH_4NO to 50%.

Keywords: Beneficial endophytic bacteria, Grain, Nitrogen fertilizer, Phytohormone, Soil, Sustainable agriculture

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INTRODUCTION

The healthy growth of *Sorghum vulgare* typically requires NH4NO3 to be applied in relatively high doses. However, this does not necessarily increase

S. vulgare's capacity to uptake NH4NO3 [1–3]. A biological alternative to address this issue is the inoculation of *Azospirillum lipoferum*, which can enhance NH_4NO_3 uptake [4, 8], especially when the dose is reduced by up to 50%. While inconsistent responses have been observed in general [5, 6], there is a principle that higher plants and prokaryotes evolved simultaneously [7, 8] and can have positive interactions. Positive effects on gramineae phenology and biomass have been reported when *A. lipoferum* is mixed with different species of *Rhizobium* [9, 10]. Similarly, mixing a *Rhizobium* species with nitrogen-fixing bacteria such as *Azospirillum* [11, 12] has been shown to increase the number of effective nodules, benefiting legume growth and reducing soil fertility loss [5]. This approach also mitigates contamination of surface water and aquifers due to inadequate uptake by the plant root system [1, 12, 13]. Furthermore, NH_4NO_3, depending on environmental conditions, can be used as an oxygen source and can transform into N_2O, a greenhouse gas responsible for global warming [1–3].

Therefore, the objective of this work was to analyze the growth of *S. vulgare* when inoculated with *A. lipoferum* and *R. tropici* using 50% of the recommended NH_4NO_3 dose.

MATERIAL AND METHODS

Origin of *Azospirillum lipoferum* and *Rhizobium tropici*

A. lipoferum was isolated from the interior of *Zea mays* var. mexicana (teosinte) grown on the shores of Lake Cuitzeo, Michoacán, México using Day and Dobereiner agar. The roots were disinfected with 1% sodium hypochlorite and rinsed with sterile water six times. Then, the roots were disinfected with 70% alcohol for 5 minutes. Using a sterile scalpel, pieces 5 cm long were placed in a sterile dish with 20 ml of saline solution (0.85% NaCl and 0.1% Roma detergent). One milliliter of the crushed root material was sown using a Drigalski loop in Day and Dobereiner agar petri dishes, which were incubated for 3 days at 30°C. The isolated round mucoid translucent colonies were Gram-negative rods, and the axenic cultures were preserved in sterile soil.

R. tropici was isolated from red nodules of *Leucaena leucocephala* grown in a wild area of the University City of UMSNH of Morelia, Michoacán, México. The nodules were disinfected with 1% sodium hypochlorite, rinsed with sterile water six times, and then disinfected with 70% alcohol for 10 minutes, followed by six rinses with sterile water. Approximately one gram of the nodules was crushed in a sterile mortar with 10 ml of detergent saline solution. From this, 0.1 ml was sown in tubes containing 5 ml of Congo red mannitol yeast extract broth, incubated for 3 days, and then cultivated on Congo red mannitol yeast extract agar for 3 days at 30°C. Round pink mucoid colonies, which were Gram-negative rods, were isolated and preserved in sterile soil. The isolates were identified by biochemical tests according to literature [4, 10, 11] and inoculated into seeds of S. vulgare.

Each of the isolates, *A. lipoferum* and *R. tropici*, was grown on Day and Dobereiner agar and Congo red mannitol yeast extract agar as previously described, incubated for 30 hours at 30°C. A suspension was prepared in detergent saline solution equivalent to 10 x 10^6 colony forming units (CFU)/ml, determined by viable plate count. This suspension was used to separately inoculate 10 grams of *S. vulgaris* seeds, which were sown in agricultural soil 5 km from the highway Morelia to Patzcuaro, Michoacán, México. The soil was poor in organic matter (less than 1.0%) and had total nitrogen around 9 mg/kg of soil, with a clay loam texture, pH 6.7, a real density of 2.0 g/cm^3, an apparent density of 1.08 g/cm^3, a porosity of 4.35%, moisture saturation of 46.9%, field capacity of 30.08%, and usable humidity of 13.2%. These physical and chemical tests were carried out according to the Mexican standard for soil, NOM-021-RECNAT-2002 [14, 15].

S. vulgaris was sown in this agricultural soil placed in Leonard's jars, as shown in Figure 1, and fed with a mineral solution containing 50% NH_4NO_3 (the recommended dose for *S. vulgare* in Michoacán, State). The chemical composition of the mineral solution was as follows (g/L): NH_4NO_3 5, K_2HPO_4 2.5,

KH_2PO_4 2.0, $MgSO_4$ 1.0, NaCl 0.1, $CaCl_2$ 0.1, $FeSO_4$ 0.01, and 10.0 ml of microelement solution (g/L): H_3BO_3 2.86, $ZnSO_4 \cdot 7H_2O$ 0.22, $MgCl_2 \cdot 7H_2O$ 1.81, pH 6.7.

The experimental design to analyze the effect of *Azospirillum lipoferum* and *Rhizobium tropici* on *Sorghum vulgare* at 50% NH_4NO_3 included 2 controls, 3 treatments, and 9 repetitions, as shown in Table 1. The response variables were percentage (%) of germination and days to emergence, phenology: plant height (PH) and root length (RL); biomass: aerial fresh/dry weight (AFW/RFW) and root weight (DRW) at the seedling level. All experimental values were analyzed by the ANOVA/Tukey HSD statistical test ($p<0.05$) [16–18].

Table 1. Experimental design to analyze the effect of *Azospirillum lipoferum* and *Rhizobium tropici* on *Sorghum vulgare* at 50% NH_4NO_3.

Treatment (T) *Sorghum vulgare*	*Azospirillum lipoferum*	*Rhizobium tropici*	*Sorghum vulgare*	water	NH_4NO_3
Irrigated only water (Absolute Control)	-	-	+	+	-
NH_4NO_3 at 100% (Relative Control) uninoculated	-	-	+	-	+100%
T1 *A. lipoferum* NH_4NO_3 at 50%	-	+	+	-	+50%
T2 *R. tropici* NH_4NO_3 at 50%	-	+	-	-	+50%
T3 A. lipoferum and *R. tropici* NH_4NO_3 at 50%	+	-	+	-	+50%

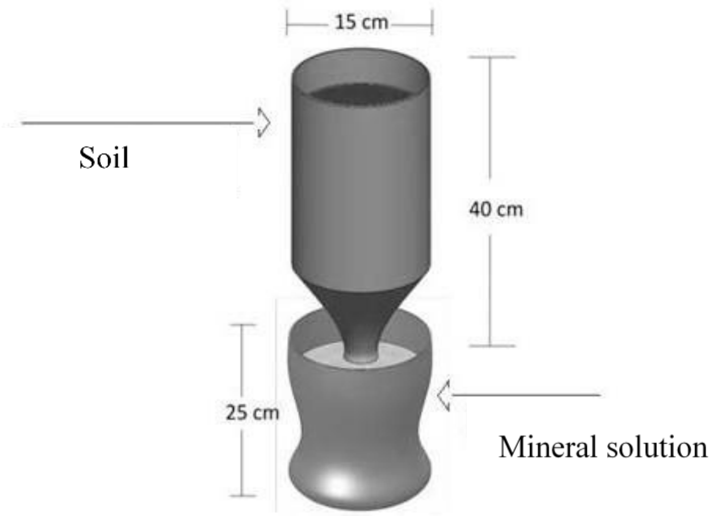

Figure 1. Design diagram of Leonard jar.

RESULTS AND DISCUSSION

Table 2 shows 100% germination of *S. vulgare* treated with *A. lipoferum* alone or mixed with *R. tropici*. These bacteria, when invading the interior of the germinating seed, transform organic germination compounds such as amino acids into phytohormones [4, 6, 7], reducing dormancy time and maximizing the number of seeds that emerge. This phenomenon, known as the spermosphere effect [19–21], involves the generation of metabolites derived from the catabolism of sugars and proteins [8]. *A. lipoferum*, in particular, has the biochemical ability to utilize the organic germination products of *S. vulgare* to induce better stem and root primordia emergence [12] compared to *R. tropici*.

While both *A. lipoferum* and *R. tropici* convert organic compounds into phytohormones to contribute to the formation of stem and root primordia [9, 17], *A. lipoferum* is more effective in this process. This demonstrates the importance of mixing these two genera and species of plant growth-promoting bacteria [6, 17] to ensure maximum germination and enhance positive effects within the Gramineae family [8, 10].

In comparison, 75% of the *S. vulgare* seeds used as the relative control (RC) with 100% non-inoculated NH_4NO_3 germinated due to their own phytohormones. However, these seeds produced a lower amount of phytohormones, resulting in a lower germination percentage compared to the *S. vulgare* seeds inoculated with *A. lipoferum* and *R. tropici* [10, 11].

Table 2. Effect of *Azospirillum lipoferum* and *Rhizobium tropici* on germination of *Sorghum vulgare* seeds seven days after sowing.

Treatment (T) *Sorghum vulgare	**Germination per cent (%) ***
Irrigated only water (Absolute control)	$65^{d\text{¨}**}$
Fed with NH_4NO_3 at 100 % (Relative control)	75^{c}
1. A. lipoferum + NH_4NO_3 at 50%	100^{a*}
2. *R. tropici* + NH_4NO_3 at 50%	90^{b}
3. A. lipoferum. tropici NH_4NO_3 at 50%	100^{a}

**All values are an average *n=50, **different letters indicated statically different at 0.05% according to ANOVA-Tukey.*

Table 3 shows the positive seedling response of *S. vulgare* to *A. lipoferum* in terms of aerial dry weight (ADW), which reached 0.61g, while *R. tropici* achieved an ADW of 0.90g. These values are statistically different compared to the uninoculated *S. vulgare* used as the relative control (RC) with 100% NH_4NO_3, which had an ADW of 0.16g. In contrast, *S. vulgare* inoculated with *A. lipoferum* reached a root dry weight (RDW) of 0.59g, while *S. vulgare* inoculated with both *A. lipoferum* and *R. tropici* had an RDW of 0.84g.

These results support that *A. lipoferum* and *R. tropici* transform compounds from the root metabolism of *S. vulgare* into phytohormones, enhancing the maximum uptake of NH_4NO_3 reduced by 50% [6, 8, 18]. By invading the interior of the roots of S. vulgare, both *A. lipoferum* and *R. tropici* [11, 13] combine their biochemical capabilities and convert compounds derived from photosynthesis and root metabolism of *S. vulgare* [10, 12] into various types of phytohormones, such as auxins and gibberellins [4, 8], to improve the growth of both the roots and aerial parts of S. vulgare. This has been reported in other Gramineae inoculated with genera and species of beneficial plant endophytic bacteria [1, 2, 6].

Table 3. Effect of *Azospirillum lipoferum* and *Rhizobium tropici* on the biomass of *Sorghum vulgare a*t seedling stage 35 days after sowing.

Treatment (T) **Sorghum vulgare*	**Total aerial dry weight (g)**	**Total dry radical weight (g)**
Irrigated only water (Absolute Control)	0.18^{e**}	0.19^{e}
Fed NH_4NO_3 at 100% (Relative Control) uninoculated	0.35^{d}	0.41^{c}
T1 *A. lipoferum* NH_4NO_3 at 50%	0.61^{b}	0.59^{b}
T2 *R. tropici* NH_4NO_3 at 50%	0.41^{c}	0.22^{d}
T3 *A. lipoferum* + *R. tropici* + NH_4NO_3 at 50%	0.90^{a*}	0.84^{a}

**All values are an average *n= 25 **Values with different letters are sadistically different at 0.05% according to ANOVA-Tukey.*

Table 4. Effect of *Azospirillum lipoferum* and *Rhizobium tropici* on the biomass of *Sorghum vulgare* at flowering stage 70 days after sowing.

Treatment (T) **Sorghum vulgare*	**Aerial dry weight (g)**	**Radical dry weight (g)**
Irrigated only water (Absolute Control)	0.52^{e**}	0.32^{e}
Fed NH_4NO_3 at 100% (Relative Control) Uninoculated	1.59^{c}	0.53^{d}
T1 *A. lipoferum* + NH_4NO_3 at 50%	1.79^{b}	1.10^{b}
T2 *R. tropici* + NH_4NO_3 at 50%	1.07^{d}	0.79^{c}
T3 *A.lipoferum* + *R. tropici* + NH_4NO_3 at 50%	3.17^{a}	1.62^{a}

**All values are an average *n= 25 **values with different letters are statistically different (P<0.05) according to ANOVA-Tukey.*

Table 4 shows the effect of *A. lipoferum* on *S. vulgare* at flowering, resulting in an aerial dry weight (ADW) of 1.79g. This indicates that *A. lipoferum*, by invading the root system of the Gramineae, can reproduce within this tissue (data not shown) and use amino acids like tryptophan to produce auxin [12, 21]. In the case of *S. vulgare* inoculated with a mixture of *A. lipoferum* (isolated from teosinte) and *R. tropici* (isolated from *L. leucocephala*), it is assumed and supported that there was an integration of the biochemical capabilities of each plant growth-promoting bacterium [11-13]. This integration converts organic compounds derived from root metabolism and photosynthesis into phytohormones, contributing to increased plant height and maximum production of biomass, resulting in an ADW of 3.17g. These values are statistically different from the ADW of 1.58g in *S. vulgare* treated with 100% NH_4NO_3 (non-inoculated control), where not all the applied NH_4NO_3 is absorbed. Consequently, *S. vulgare* does not reach a higher dry weight in the aerial biomass compared to when inoculated with *A. lipoferum* and/or *R. tropici* [5, 6]. This surplus NH_4NO_3 can lead to loss of soil fertility, contamination of surface water and aquifers, and the possible release of N2O, contributing to global warming [1, 2, 6].

In contrast, the positive response of *S. vulgare* to *A. lipoferum* allowed it to reach a root dry weight (RDW) of 1.10g, while *S. vulgare* inoculated with both *A. lipoferum* and *R. tropici* had an RDW of 1.62g. These results support that *A. lipoferum* and *R. tropici* transform organic compounds from *S. vulgare* into phytohormones, enhancing the radical uptake capacity of NH_4NO_3 to 50%. This prevents the loss of organic matter, reduces NO_3- problems in water, and minimizes N_2O generation [16, 17], promoting sustainable agriculture and leveraging the potential of *R. tropici* in the production of *S. vulgare* [18, 20].

CONCLUSION

It was evident that *S. vulgare* can grow healthily with a lower dose of NH_4NO_3 than recommended if it is inoculated with *A. lipoferum* isolated from another grass. This genus and species of plant growth-promoting bacteria have a synergistic action in the synthesis of phytohormones, which not only contribute to increased germination but also to the healthy growth of *S. vulgare*. By reducing and optimizing NH_4NO_3, soil fertility is preserved, and water pollution, a critical resource at risk today, is prevented. Additionally, this practice helps prevent the generation of N_2O, a gas associated with global warming that has negative consequences for life on Earth.

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Conflicts of Interest

The authors declare no conflicts of interest.

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