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**SOIL AND PLANT NUTRITION -** Article

# The influence of topdressing nitrogen on *Azospirillum spp*. inoculation in maize crops through meta-analysis

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**ABSTRACT:** The *Azospirillum* is considered one of the most studied plant growth promoter genus. These bacteria are capable of promoting plant growth through several factors. However, the effect of *Azospirillum spp*. associated to nitrogen fertilization on maize grain yield has brought about controversial results. Thus, the objective of this study was to verify the influence of topdressing nitrogen on the effect of *Azospirillum spp*. inoculation on maize crops through meta-analysis. Data were collected from articles published in scientific journals, obtained from the Web of Science®, Scopus® and Google Scholar® databases. The bibliographic review included only articles with direct comparisons between maize yield in the presence and absence of *Azospirillum spp*. under field conditions in Brazil. The meta-analysis of the random effects was carried out using all entries. Subgroup and meta-regression analysis were realized to verify the influence of nitrogen fertilization on the explanation of the possible heterogeneity among effect measures. Maize inoculation with *Azospirillum spp*. showed an average yield increase of 651.58 $kg \cdot ha^{-1}$, considering all trials, and of 1034.28 $kg \cdot ha^{-1}$ considering only the trials without topdressing nitrogen. There was no significant increase in grain yield when inoculation with bacteria from the *Azospirillum* genus was realized together with nitrogen fertilization, indicating that such technologies are non-additive.

**Key words:** *Zea mays* L., meta-regression, plant growth-promoting bacteria.

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# INTRODUCTION

Among the microorganisms that live in the rhizosphere, a heterogeneous group of bacterial species, also known as plant growth-promoting bacteria (PGPB), are capable of promoting plant growth (Bach et al. 2016). These bacteria can play an important role in nutrients acquisition by plants, acting as biofertilizers, phytostimulants and biotic and abiotic stress controllers (Lugtenberg and Kamilova 2009; Pii et al. 2015). Several PGPB genera show association with different species of agricultural importance, such as *Azospirillum, Arthobacter, Azobacter, Bacillus, Bradyrhizobium, Burkholderia, Clostridium, Gluconacetobacter, Herbaspirillum, Pseudomonas, Rhizobium* and *Streptomyces* (Steenhoudt and Vanderleyden 2000; Videira et al. 2012).

The *Azospirillum* (Beijerinck 1925) genus includes a group of diazotrophic bacteria that can be associated with the plant's rhizosphere, characterizing an external colonization, or associated endophytically when penetrating the roots intercellular spaces (Van Dommelen and Vanderleyden 2007). According to the List of Prokaryotic Names with Standing in Nomenclature (LPSN 2017), 19 species of *Azospirillum* were described, making it one of the most studied PGPB genera (Cassán and Díaz-Zorita 2016). Among the main species are the *A. brasilense*, *A. lipoferum*, *A. halopraeferens* and *A. oryzae*, widely used as biofertilizers, mainly for cereals. However, Pereg et al. (2016) verified that bacteria of the *Azospirillum* genus has affinities with more than 113 species of plants and 35 botanic families.

*Azospirillum*-genus bacteria, when associated with the plant's roots, are capable of promoting plant growth due to the production of aminoacids, indoleacetic acid, giberelines and other polyamines, according to the additive hypothesis (Bashan and Levanony 1990), which favors root system growth and, consequently, the absorption of water and nutrients by plants (Bashan and de-Bashan 2010; Doornbos et al. 2012; Tien et al. 2012). Such bacteria also have the capacity to fix atmospheric nitrogen and, for this reason, contribute directly in increasing nitrogen availability to several non-leguminous species (Hungria et al. 2010; de-Bashan et al. 2012; Ferreira et al. 2013). However, according to Van Dommelen and Vanderleyden (2007), the amount of ammonia released by these bacteria is limited and, therefore, their contribution has been questioned many times (Steenhoudt and Vanderleyden 2000; Bashan and de-Bashan 2010).

The effect of Azospirillum on maize crop yield (*Zea mays* L.) has shown contrasting results (Hagh et al. 2010, Hungria et al. 2010, Repke et al. 2013; Quadros et al. 2014). Repke et al. (2013) found that the inoculation of *A. brasilense*, followed or not by nitrogen dosages, had no interference in maize yield. On the other hand, Hungria et al. (2010) evaluated the use of different strains of *A. brasilense* and *A. lipoferum* in maize and observed an average increase in gran yield by 27%. Hagh et al. (2010) reported a yield increase of 13% in maize inoculated with A*zospirillum lipoferum* in relation to the treatment without inoculation plus 70 kg·ha$^{-1}$ of N in topdress application. However, the same authors found no significant differences for the treatment with *A. lipoferum* when compared to treatments without inoculation plus 140 and 210 kg·ha$^{-1}$ of topdress N. Quadros et al. (2014) verified a significant interaction among three maize hybrids and the inoculation with *Azospirillum spp*., showing yield increase only for hybrids, with an average increase of 750 kg·ha$^{-1}$.

According to Castro-Sowinski et al. (2007), inoculation response may vary according to the plant genotype, bacterial strain, environment conditions, nitrogen fertilization management and the quality of the PGPB cells used as inoculant. In this sense, in the midst of all evidence inconsistencies on the contribution of the bacteria of the *Azospirillum* genera associated with nitrogen fertilization in maize grains yield, the statistical technique known as meta-analysis may be fundamental to indicate the effects of this technology (Veresoglou and Menexes 2010). Therefore, the objective of this work was to verify the influence of nitrogen fertilization under the effect of the *Azospirillum spp.* inoculation on maize crops through meta-analysis.

# MATERIAL AND METHODS

Data were collected from articles published in scientific journals, obtained through a bibliographic review, using the Web of Science®, Scopus® and Google Scholar® database. As search strategy, the following research terms were used: *Azospirillum* AND (maize OR corn) OR (yield OR productivity). The same terms were searched in the Portuguese language, with no restriction in regard to article publication dates. The search for the articles was done by two independent reviewers, in which only articles dealing with the direct comparison between maize yield in the presence and absence of bacteria of the *Azospirillum* genus, under field conditions, in Brazil, were included.

The following criteria were used for excluding articles:

I. Experiments without the following variability measures: environment variation coefficient, mean squared of the residue or standard error of the mean;
II. Experiments with an interactive effect with other biofertilizers;
III. Studies with results presented in graphs, preventing their tabulation.

Effect measures (Yi) were estimated using the grain yield variable ($kg{\cdot}ha^{-1}$) through the following equation, represented in Eq. 1:

$$Yi = \ln\,(\text{Inoculated treatment/Control without inoculation}) \quad (1)$$

The synthesis produced by the meta-analysis is weighed according to the studies weights, so that each one could contribute, independently, to the meta-analytical result. The inverse variance method was used to attribute weights, as shown in Eq. 2:

$$Wi = 1/Vi \qquad (2)$$

where Wi represents the weight attributed to the i$^{th}$ study and Vi the variance of the i$^{th}$ study.

Thus, the lowest the variability, the greater the study weight in the synthesis produced by the meta-analysis.

Heterogeneity among effect measures was verified through the Cochran Q test (Cochran 1954), at 1% of significance. The $I^2$ statistics of Higgins and Thompson (2002) was used to indicate the total variability percentage of a set of effect measures, due to the heterogeneity among the real effects, in which percentages of approximately 25, 50 and 75 would indicate low, average and high heterogeneity, respectively. Studies publication bias was verified through the funnel plot and the Egger significance test (Egger et al. 1997).

Effect measures normal distribution were verified by the Shapiro-Wilk test (Shapiro and Wilk 1965), at 5% of significance. The meta-analysis was realized by the random effect model to estimate the means and their respective 95% confidence intervals for each effect measure (Gurevitch and Hedges 1999; Borenstein et al. 2009).

Subgroup and meta-regression analyses are models that incorporate one or more moderators (study-specific categorical or continuous co-variables) which, in turn, can explain part of the heterogeneity found among real effect measures (Borenstein et al. 2009). In this sense, the objective of using these models in the present study was to verify the influence of nitrogen fertilization levels on the explanation of the possible heterogeneity among effect measures. To do so, the hypotheses were tested through mixed effects models, being the studies considered of random effect and the moderating variable (nitrogen fertilization level) of fixed effect. For the subgroups analysis, the studies were categorized according to the following topdressing nitrogen: absence of fertilization ($0\ kg{\cdot}ha^{-1}$ of N), low ($> 0$ and $\leq 50\ kg{\cdot}ha^{-1}$ of N), moderate ($> 50$ and $\leq 100\ kg{\cdot}ha^{-1}$ of N) and high ($> 100\ kg{\cdot}ha^{-1}$ of N).

Statistical analyses were carried out with the help of the software R (R Corel Team, 2016), using the meta (Schwarzer and Schwarzer 2017) and metafor (Viechtbauer 2010) packages.

## RESULTS AND DISCUSSION

Initially, the study surveyed 72 scientific articles; however, after the exclusion criteria, 14 articles, forming an aggregate of 26 trials, were considered for the meta-analysis study (Table 1).

The Cochran Q test observed heterogeneity among effect measures ($Q = 732.71$, $DF = 171$, $p < 0.001$). The statistical $I^2$ test also detected the existence of heterogeneity with the

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**Table 1.** References and trials number of articles used by the meta-analysis.

| References | Trials number |
|---|---|
| Cavallet et al. (2000) | 1 |
| Braccini et al. (2012) | 1 |
| Ferreira et al. (2013) | 1 |
| Martins et al. (2012) | 1 |
| Kappes et al. (2013) | 1 |
| Mazzuchelli et al. (2014) | 1 |
| Vogt et al. (2014) | 4 |
| Pandolfo et al. (2015) | 4 |
| Matsumura et al. (2015) | 1 |
| Sangoi et al. (2015) | 2 |
| Galindo et al. (2016) | 4 |
| Morais et al. (2016) | 1 |
| Portugal et al. (2016) | 2 |
| Spolaor et al. (2016) | 2 |

magnitude of 85.40%, inferring a high heterogeneity among effect measures. According to Higgins and Thompson (2002), the quantification of the heterogeneity extension among the studies is of extreme importance, since it can influence the meta-analysis conclusions. According to Borenstein et al. (2009), whenever this heterogeneity is identified, it can be incorporated in the statistical model through the random effect meta-analysis or explained, at least partially, by the subgroups and meta-regression analyses.

The funnel plot of the relationship between the mean standard deviation and the residual values of the effect measures used by the meta-analysis is shown in Fig. 1. In general, data dispersion behavior was relatively symmetrical, suggesting a weak publication bias evidence, also detected by the Egger method ($p = 0.89$). According to the Shapiro-Wilk test, the effect measures showed a normal distribution ($p > 0.05$). Therefore, all effect measures were considered during the meta-analysis.

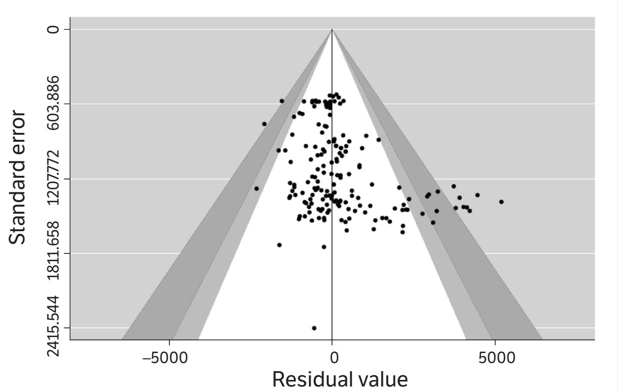

**Figure 1.** Funnel plot with confidence pseudo-limits of 90, 95 and 99% form the relationship between the mean standard deviation and the residual value of the effect measures used by the meta-analysis.

Random effect meta-analysis showed that maize inoculation with bacteria of the *Azospirillum* genus showed significant yield increase estimate ($p < 0.001$), with an average increase of 651.58 kg·ha$^{-1}$ (95% CI = 510.89 – 792.27 kg·ha$^{-1}$) in inoculated treatments in relation to non-inoculated treatments. Veresoglou and Menexes (2010), based on the meta-analysis of studies on the effect of *Azospirillum spp.* inoculation wheat grain yield observed an average yield increase of 8.9% in inoculated treatments in relation to non-inoculated under field conditions. In this same context, Díaz-Zorita et al. (2015) analyzed 47 articles published in scientific journals and described 347 cases of grain yield response of different species to inoculation with bacteria of the *Azospirillum* genus. Among the crops reported, the average grain yield response to inoculation with *Azospirillum* was 10%, as the highest responses were observed in winter cereals (14%), summer cereals (9.5%) and legumes (6.6%).

In addition to making nitrogen available and offering greater control of abiotic and abiotic stresses (Lugtenberg and Kamilova 2009; Hungria et al. 2010; De-Bashan et al. 2012; Pii et al. 2015), the bacteria of the *Azospirillum* genus also promotes plant growth through the production of aminoacids, indoleacetic acid, giberelines and other polyamines. This favors root system growth and, consequently, greater water and nutrients absorption by plants (Bashan and De-Bashan 2010; Doornbos et al. 2012; Tien et al. 2012). Although many mechanisms have been described to explain the plant growth promotion by *Azospirillum spp.*, in this context, a single mechanism, most of the times, is not responsible for the total effect (Cassán and Díaz-Zorita 2016). Thus, the action mode of the *Azospirillum spp*. could be better explained by the additive hypothesis, that considers multiple mechanisms in the successful association of *Azospirillum spp*. with plants (Bashan and Levanony 1990).

The incorporation of the nitrogen fertilization moderating variable in both meta-analytical models was significant ($p < 0.001$), inferring that the different levels of nitrogen fertilization can influence the response of *Azospirillum spp.* on maize crops grain yield. Veresoglou and Menexes (2010) reported that nitrogen fertilization is considered a key factor in determining the efficiency of *Azospirillum* inoculation in wheat crops.

Matsumura et al. (2015) evaluated the impact of nitrogen fertilization on the diversity of endophytic bacteria on maize plants inoculated with *A. brasilense.* The authors found that nitrogen fertilization management affects dominant populations from a metabolically active bacterial community. In addition, under nitrogen regular level, the libraries of the genes 16S rRNA indicate a smaller diversity of such populations in relation to libraries of plants with low nitrogen. The same authors still report that the combination of treatments with nitrogen regular levels and plant inoculation had no additive effect. On the contrary, it shows a tendency to reduce yield.

Through the adjusted meta-regression model (Fig 2), a decreasing linear behavior within nitrogen fertilization levels was detected. Thus, the average yield increase of 651.58 kg·ha$^{-1}$ (95% CI = 510.89 – 792.27 kg·ha$^{-1}$) attributed to *Azospirillum* tends to diminish by 4.36 kg·ha$^{-1}$ (95% CI = 1.81 – 7.21 kg·ha$^{-1}$)

in response to the addition of 1 kg·ha$^{-1}$ topdressing nitrogen. According to Hartmann (1988), the presence of high concentrations of nitrogen reduces or even inhibits the efficiency of the nitrogen biological fixation by bacteria of the *Azospirillum* genus, inhibiting the nitrogenase activity by these bacteria, enzymes responsible for converting atmospheric nitrogen ($N_2$) into ammonium ($NH_4^+$), which are highly digestible by plants.

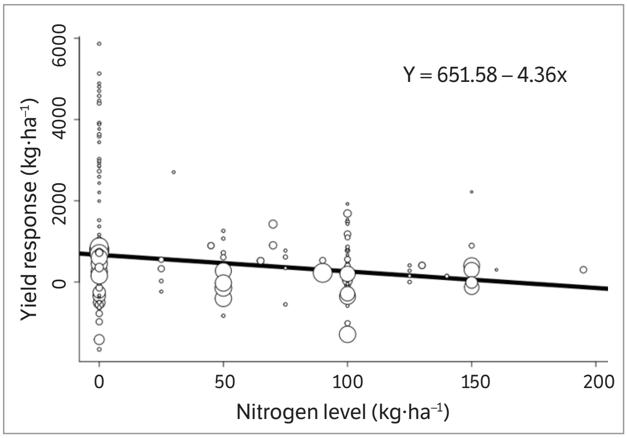

**Figure 2.** Bubble-plot of the linear relationship between measures of effect and topdressing nitrogen fertilization. Each bubble area is proportional to the study weight in the meta-analysis.

In addition to the process of controlling and regulating the nitrogenase complex, there are evidences that nitrogen contents in the soil can also regulate bacterial colonization (Carvalho et al. 2014). Coelho et al. (2009), for instance, quantified the presence of diazotrophic bacteria through the gene *nif*H in a rhizosphere and non-rhizosphere soil of two sorghum genotypes (*Sorghum bicolor* L.) under different nitrogen fertilization dosages. The authors reported a significant reduction in diazothrophic bacteria abundance in the rhizosphere soil for both genotypes, under high dosages of nitrogen (Coelho et al. 2009). On the other hand, there was no reduction in diazotrophic bacteria in the non-rhizosphere soil, where these bacteria remained constant, independent from nitrogen dosages.

Based on the subgroups analyses (Table 2), only the Without N subgroup showed a significant grain yield increase ($p < 0.001$), with an average increase of 1034.28 kg·ha$^{-1}$ (95% CI = 772.94 – 1295.80 kg·ha$^{-1}$) in relation to control treatments. Thus, the addition of topdressing nitrogen fertilizers does not promote significant yield increases in the inoculated treatments, suggesting that such technologies are non-additive (Spolaor et al. 2016).

According to Zhu et al. (2016), the structure of the microbial communities in the rhizosphere is the result of complex interactions between maize and nitrogen fertilization. Furthermore, nitrogen has the capacity to modify the composition and abundance of root exudates and, posteriorly, affect the microbial communities from the rhizosphere. In this sense, the increase in nitrogen fertilization levels tends to recruit greater number of microorganisms for the rhizosphere and, consequently, increasing competition among them, lowering the efficiency of the inoculation process.

Currently, more than 100 biological products with *Azospirillum spp.* are commercially available only in South America (Cassán and Diaz-Zorita 2016). In general, maize inoculation with these bacteria is recommended additionally to topdressing nitrogen fertilization, which is in disagreement with this study's results. Therefore, the use of inoculants with bacteria of the *Azospirillum* genus can be considered a promising practice, mainly under limiting cultivation conditions.

## CONCLUSION

Maize inoculation with *Azospirillum spp*. showed an average yield increase of 651.58 kg·ha$^{-1}$, considering all trials, and of 1034.28 kg·ha$^{-1}$ considering only trials without topdressing nitrogen fertilization. Topdressing nitrogen fertilization is considered a determining factor in maize inoculation with *Azospirillum spp*., showing no significant increase in grain yield when realized jointly.

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**Table 2.** Effect of nitrogen fertilization levels on maize yield in response to inoculation with bacteria of the Azospirillum genus.

| Subgroup[1] | Estimate | Standard error | 95% CI | | p-value |
|---|---|---|---|---|---|
| | | | Upper limit | Lower limit | |
| Without N | 1034.28 | 133.45 | 772.94 | 1295.80 | < 0.001 |
| Low N | 398.46 | 280.87 | -151.79 | 949.47 | 0.186 |
| Moderate N | 270.56 | 171.94 | -66.43 | 607.57 | 0.278 |
| Hight N | 287.07 | 291.87 | -284.98 | 859.13 | 0.312 |

[1] Without N (0 kg·ha$^{-1}$ of N); low N (> 0 and ≤ 50 kg·ha$^{-1}$ of N), moderate N (> 50 and ≤ 100 kg·ha$^{-1}$ of N) and high N (> 100 kg·ha$^{-1}$ of N)

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