Article

Phosphatase-alkaline activity and growth of rice with biological inoculation and micronutrients

Maura Isabel Díaz Lezcano[1§]
Carlos Fiori Fernández[2]
Líder Ayala Aguilera[1]
Fátima Yubero[3]
Roberto Martínez López[4]
Marcelo López[5]

[1]Faculty of Agrarian Sciences-National University of Asunción. San Lorenzo, Paraguay. (lider.ayala@agr.una.py). [2]Master of Science in Biotechnology-National University of Asunción. San Lorenzo, Paraguay. (fiorif88@gmail.com). [3]Faculty of Chemical Sciences-National University of Asunción. San Lorenzo. (fyubero@qui.una.py). [4]General Directorate for Scientific and Technological Research-National University of Asunción. San Lorenzo, Paraguay. (zoogen.py@gmail.com). [5]Faculty of Chemical Sciences-National University of Asunción. San Lorenzo. Paraguay. (marcelolopez3@hotmail.com).

[§]Corresponding author: maura.diaz@agr.una.py.

Abstract

Inoculation with growth promoting bacteria in crops is an alternative to reduce the use of nitrogen fertilizers and production costs. The objective of this research was to evaluate the effect of inoculating *Oryza sativa* L. rice seeds with the bacteria *Azospirillum brasilense* on the enzymatic activity of alkaline phosphatase and its growth. The research was carried out at the Faculty of Agrarian Sciences and the Faculty of Chemical Sciences of the National University of Asuncion, Paraguay. A completely randomized design with five treatments and five repetitions was used. Treatments consisted of T1 (Absolute Control): untreated rice seeds, T2 (Co+Mo): rice seeds treated with cobalt plus molybdenum, T3 (Az): rice seeds inoculated with *A. brasilense*, T4 (Az+1F): rice seeds inoculated with *A. brasilense* plus one foliar application of the inoculant and T5 (Az+2F): rice seeds inoculated plus two foliar applications. Anava and Tukey's test ($p<0.05$) were applied for the analysis of agronomic variables and the Kruskal-Wallis test ($p<0.05$) for enzymatic evaluations. The variables evaluated were root length (cm), dry mass of aerial parts and roots (g), and alkaline phosphatase enzyme activity (μg de PNP g^{-1} soil h^{-1}). No significant differences were observed between treatments with *Azospirillum brasilense* regarding dry mass. Phosphatase activity in treatments with *A. brasilense* ranged from 1 655.49 in T3 to 7 429.55 μg of PNP g^{-1} soil h^{-1} in T4. The use of cobalt and molybdenum-based micronutrients led to greater root growth.

Keywords: *Azospirillum brasilense*, *Oryza sativa* L., enzymatic activity, inoculation.

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Introduction

Paraguay experiences in recent years a growth in terms of cultivated area and irrigated rice yield, with more than 140 000 ha cultivated, it is accompanied by technological innovations, inputs, seeds, research and others focusing on exports.

Rice has remained within production standards, increasingly aiming to increase its production on an annual and continuous basis (MAG, 2018). Inoculation with growth-promoting bacteria in crops is an alternative to reduce the use of chemical compounds, minimizing environmental damage and production costs (Brito *et al*., 2018).

The generation of new knowledge through the use of bacteria such as those of the genus *Azospirillum brasilense* can contribute to improve yields and at the same time represent a sustainable alternative in relation to chemical fertilizers.

Inoculation with bacteria of the genus *Azospirillum* in plants leads to a significant increase in the root system, in addition to inducing resistance to pathogens and providing necessary elements such as nitrogen, inhibiting the proliferation of parasitic plants and producing hormones that stimulate plant growth, allowing a more economic and healthy development of crops (Bouillant *et al.*, 1997).

Microorganisms play a role in processes that affect the transformation of phosphorus in the soil and its availability to plants. In order to assess the bioavailability of the presence of phosphates in the culture, the measurement of the activity of the phosphatase-alkaline enzyme in the soils was used, in the rhizosphere area, a zone inhabited by these bacteria.

In particular, they can solubilize and mineralize organic and inorganic phosphorous forms; through mechanisms such as the release of organic acids and hydrolytic enzymes that increase the mobilization and availability of this element for plant nutrition (Fernandez-Rodríguez, 2005). The objective of this work was to evaluate the agronomic response of *O. sativa* L. rice whose seeds were inoculated with *A. brasilense*; through the measurement of phosphatase activity in the soil.

Materials and methods

Location

The experiment was installed under greenhouse conditions at the Faculty of Agricultural Sciences, where the morpho-agronomic evaluations were carried out. The enzymatic analyzes of the soil were carried out in the Department of Physicochemistry of the Faculty of Chemical Sciences, both academic units belonging to the National University of Asunción located in San Lorenzo, Paraguay.

Vegetal material

One kilogram of commercial rice seeds was used (Table 1), they were divided into three batches of 500, 300 and 200 g. The 500 g batch received a chemical treatment with a mixture of fungicides consisting of Carbendazin plus Thiran at a rate of 2 mL and Imidacloprid plus Thiodicarb insecticides at a rate of 5 mL.

Table 1. Quality analysis of the batch of rice seeds *Oryza sativa* L., used for the experiment.

Sample weight (g)	Normal seedlings (%)	Abnormal seedlings (%)	Germination (%)	Physical purity (%)	Inert material (%)	Humidity (%)
700	84	11	84	99.6	0.4	12.7

The 300 g batch of seeds was used to inoculate them with commercial strains of *A. brasilense*, with a commercial formulation with a concentration of 1 x 10^9 viable bacteria per mL at the time of preparation at a rate of 6 mL. Of the total 200 g, 100 g that received treatment with cobalt plus molybdenum micronutrients at a rate of 0.3 mL were used, and the remaining 100 g that received no treatment were used as controls.

Planting rice seeds

For the sowing of the seeds, three kilograms of homogenized soil were placed in 25 pots of 24 cm in diameter and 22 cm in height, with a total capacity of seven liters, they were placed in greenhouse conditions. Approximately 40 seeds were sown per container at a centimeter depth evenly distributed.

After germination and emergence, a two-centimeter sheet of water was kept on the surface until the end of the experiment and morphoagronomic evaluations. After emergence and after having developed twelve true leaves, before the first bunch, the leaves were sprayed twice at ten-day intervals with the same product based on *A. brasilense* at a rate of 10 mL in 350 mL of sterile distilled water.

Substrate used

A soil sample was used as a substrate from a rice farm located in the Ype ka’e Company in the Villeta District, 70 km from the city of Asunción, Paraguay. It had a sandy texture and light brown coloration, classified within the *Typic Albaqualf* (Al) Subgroup of soils corresponding to the Order Alfisoles (López *et al*., 1995) and whose chemical characteristics are shown in Table 2.

Table 2. Chemical characteristics of the substrate used for planting rice.

Depth (cm)	pH	Organic material (%)	P (m kg^{-1})	Ca^{+2}	Mg^{+2}	K^+	Na^+	Al^{+3}+H^+
0-10	5	1.21	11.9	2.4	1.41	0.13	0.38	0.94

Enzymatic evaluation

At the same time, the enzymatic activity of the alkaline phosphatase was determined, for which soil samples were taken from the five containers per treatment, it was homogenized and leveled to a weight of 100 g, thus totaling five samples of 100 g each they were kept in sterile containers at a temperature of 7 ºC until the time of their analysis.

Determination of phosphatase activity

The procedure was as described by Becerra *et al*. (2011).

Variables evaluated

The evaluation of the agronomic variables was carried out after panning and filling of the grains, they consisted of: root length, expressed in centimeters with a millimeter ruler, the roots of a total of fifty plants were measured for each treatment and repetition. Dry mass of aerial parts was placed in an oven at 70 °C for three days and then weighed on an analytical balance. Dry mass of roots, the same methodology of dry mass of aerial parts was used.

Phosphatase enzymatic activity, micrograms of paranitrophenol per gram of soil per hour (µg of PNP g^{-1} soil h^{-1}) were measured by a colorimetric technique, using a spectrophotometer, determining the concentration of paranitrophenol resulting from the splitting of paranitrophenyl phosphate.

Treatments

The treatments consisted of: T1 (control)= seeds without any type of treatment, T2 (Co + Mo)= seeds with chemical treatment of cobalt plus molybdenum, T3 (Az) seeds inoculated with *A. brasilense*, T4 (Az + 1F)= seeds inoculated with *A. brasilense* plus a foliar application of the inoculant at a rate of 10 mL.

Per experimental unit and T5 (Az + 2F)= seeds inoculated with *A. brasilense* plus two foliar applications of the inoculant at a rate of 10 mL per experimental unit after ten days of the first application. Only T5 (Az + 2F) received two foliar applications.

Experimental design and data analysis

The experimental design used was completely randomized with five treatments for five repetitions. The means of the agronomic measurements were submitted to Anava and the variables that presented statistical significance were compared with the Tukey test ($p < 0.05$). The data from the biochemical analyzes were subjected to the Kruskal-Wallis test ($p < 0.05$), this test has been used since the data did not present normality.

Results and discussion

Root length

Figure 1 shows the data regarding the length of roots, the same sample as in treatment 2, corresponding to the group of seeds treated with cobalt plus molybdenum (Co + Mo), presented a significant difference compared to control and treatment 5 which make up inoculated seeds plus two foliar applications (Az + 2F) but not with treatments 3, consisting of inoculated seeds (Az) or with treatment 4, inoculated seeds plus a foliar application (Az + 1F).

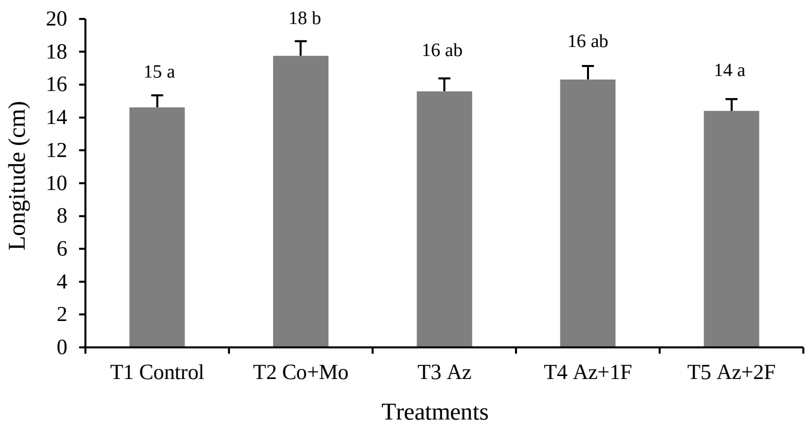

Figure 1. Root length of rice plants from seeds inoculated with *A. brasilense* and treated with cobalt plus molybdenum. T1= control (absolute control), seeds without any treatment; T2= Co + Mo, seeds treated with cobalt plus molybdenum; T3= Az, seeds inoculated with *A. brasilense*; T4= Az + 1F= seeds inoculated with *A.* baselines plus a foliar application; T5= Az + 2F= seeds inoculated with *A. brasilense* plus two foliar applications.

However, treatments 3, 4 and 5 do not differ significantly from the control, therefore, according to the evidence presented, apparently inoculation with Co + Mo would be more convenient than only with *A. brasilense* and even applying it by the foliar route, observing a greater efficiency in root length only with Co + Mo.

These results coincide with those reported by Santos *et al*. (2013) who observed a greater root length in rice plants applying doses of 4.5 mg L^{-1} of micronutrients. Likewise, experiments by Askary *et al*. (2009) demonstrate that cobalt and molybdenum in rice seeds with doses of 3 mg L^{-1} increase the germination rate if the same.

The null effect of the *A. brasilense* strains used for the inoculation of rice seeds could be due to survival problems in the soil, because it was not sterilized at the time of its use, the same observations were mentioned by Becquer *et al*. (2012) commenting that, in non-sterile soils, native microorganisms interfere with the colonization capacity of *A. brasilense*, so it is recommended for future research to use a sterilized substrate to thereby evaluate the effect of rhizobacteria on rice cultivation.

The mixed inoculation of *Azospirillum lipoferum* and the mycorrhizal fungus *Glomus intraradices* in sorghum plants, increased all the growth parameters of the plants, the concentration of phosphatases in roots, as well as the absorption of minerals, when compared to single inoculations (Veereswamy *et al*., 1992).

When inoculating wheat with *A. brasilense* and *Glomus* sp. the fresh and dry weight of sprouts and roots increased (Gori and Favilli, 1995). The double inoculation of *Glomus macrocarpum* and *A. brasilense* in the *Corchorus ollitorius* plant promoted its growth (Bali and Mukerji, 1991). The inoculation of *Azospirillum lipoferum* increased the contents of N, P and K due to the increase in the radical surface that allows a greater absorption of nutrients (Salvagiotti *et al*., 2014).

The assimilation of phosphorus for plants depends largely on the mineralization experienced by the different fractions of said phosphorous together with organic forms. Phosphatase enzymes are responsible for hydrolyzing phosphorus in organic form (phosphate esters) to inorganic forms, making it assimilable to plants. Phosphatase-alkaline is substrate inducible (Burns, 1982).

Dry mass of roots

The dry mass of roots of rice plants inoculated with *A. brasilense* and cobalt plus molybdenum micronutrients did not show significant differences between treatments as observed in Figure 2. They were evaluated at the end after panning and filling of grains.

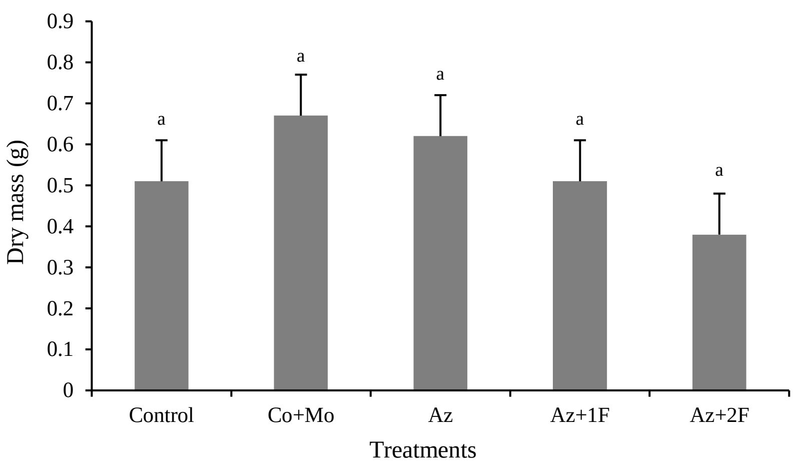

Figure 2. Dry mass of roots of rice plants from seeds inoculated with *A. brasilense* and treated with cobalt plus molybdenum. T1= control (absolute control), seeds without any treatment; T2= Co + Mo, seeds treated with cobalt plus molybdenum; T3= Az, seeds inoculated with *A. brasilense*; T4= Az + 1F= seeds inoculated with *A. brasilense* plus a foliar application; T5= Az + 2F= seeds inoculated with *A. brasilense* plus two foliar applications.

The ranges obtained in this variable range from 0.67 g for treatment 2 consisting of seeds treated with cobalt plus molybdenum (Co + Mo) and 0.38 g for treatment 5, consisting of inoculated seeds plus 2 foliar applications (Az + 2F). Cobalt (Co) is not an essential nutrient for plants; however, it is important in the metabolism of the bacteria that form the nodules.

It has been observed that in the absence of Co, bacteria reduce the production of proteins and leghemoglobin, a protein involved in maintaining the biological fixation of N (Sims, 1996). Molybdenum (Mo) is an essential nutrient that is directly involved in the N metabolism of soybean crops, as it is part of nitrogenase, the enzyme responsible for fixing atmospheric N and nitrate reductase. This enzyme is responsible for the reduction of nitrate to ammonium for its subsequent transformation to amino acids and proteins in plants (Marschner, 1995).

These results do not coincide with those of García *et al*. (2010) who report an increase in the dry mass of roots of rice plants grown in pots under greenhouse, highlighting the effectiveness of this genus of bacteria. Similarly, Díaz-Zorita *et al*. (2006).

They found higher dry matter yields of wheat roots (*Triticum aestivum*) using *A. brasilense* evidencing positive effects due to the presence of these microorganisms that allowed improvement in radical growth, increasing the soil's exploration capacity and efficient use of resources such as water and nutrients.

Dry mass of aerial parts

In the same way as the dry mass of roots, the weighing of the aerial parts of leaves of rice plants did not show significant differences between treatments (Figure 3), the ranges being 4.28 g for treatment 3 (Az) and 4 g observed in treatment 2 (Co + Mo).

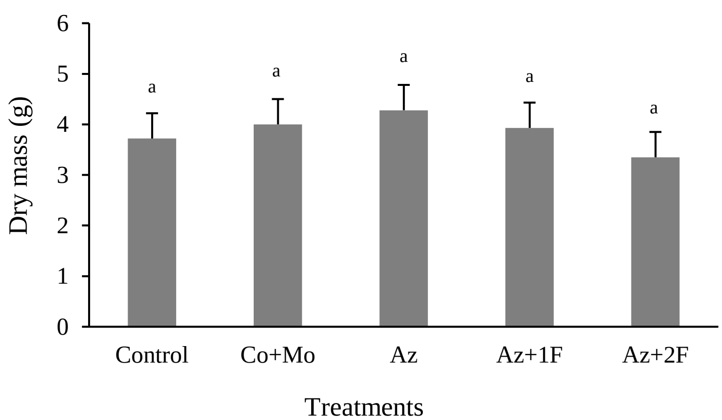

Figure 3. Dry mass of aerial parts of rice plants from seeds inoculated with *A. brasilense* and treated with cobalt plus molybdenum. T1= control (absolute control), seeds without any treatment; T2= Co + Mo, seeds treated with cobalt plus molybdenum; T3= Az, seeds inoculated with *A. brasilense*; T4= Az + 1F= seeds inoculated with *A. brasilense* plus a foliar application; T5= Az + 2F= seeds inoculated with *A. brasilense* plus two foliar applications.

Vogel *et al*. (2014) mention that different doses of *A. brasilense* practiced in different forage species of pulses help to increase the dry mass of only some of them, while Dartora *et al*. (2013) indicate that the effectiveness of the use of *A. brasilense* also depends on the level of nitrogen in the soil, which can reduce its effectiveness in poor concentrations as well as in high concentrations.

Results reported by Diaz and Ortegon (2006) evidenced an increase in the dry biomass of aerial parts of five samples of canola (*Brassica napus*) in full development, indicating occasional inconsistencies in the response of the plant to inoculations with rhizobacteria and it is not possible to generalize its effectiveness.

Kussell *et al*. (2005) mention that bacteria develop mechanisms to maintain cell viability during starvation and resume growth when nutrients are available, these include, among others, a phase variation that has been proposed as an important mechanism by which microorganisms adapt to environmental changes such as those in the soil rhizosphere (Van den Broek *et al*., 2005).

Bioavailability study of soluble phosphate in soil: enzymatic activity of soil phosphatase

Figure 4 shows that treatment 3 consisting of seeds inoculated with *A. brasilense* (Az) yielded a total of 1 655.49 µg of PNP g^{-1} soil h^{-1}, 590.75 times more than the control (control absolute) and 1 488.69 times greater than treatment 2, consisting of seeds treated with cobalt plus molybdenum which yielded a value of 168.8 µg PNP g^{-1} soil h^{-1}.

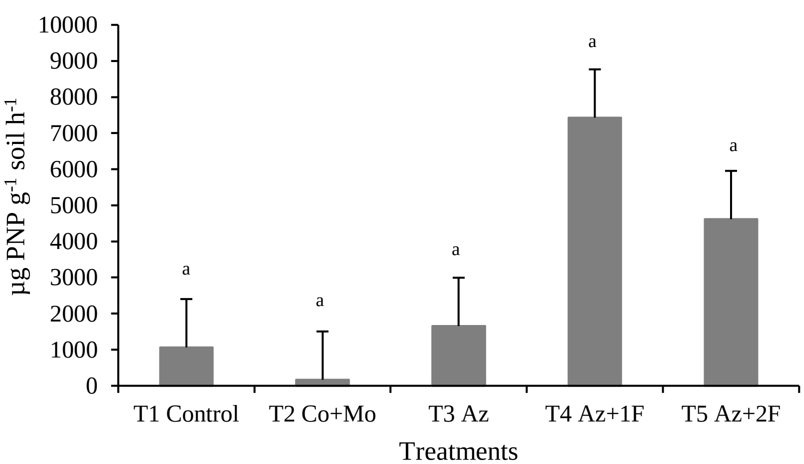

Figure 4. Enzymatic activity phosphatase of soil samples used for rice cultivation in the different treatments with *A. brasilense* and chemically treated. T1= control (absolute control), seeds without any treatment; T2= Co + Mo, seeds treated with cobalt plus molybdenum; T3= Az, seeds inoculated with *A. brasilense*; T4= Az + 1F= seeds inoculated with *A. brasilense* plus a foliar application; T5= Az + 2F= seeds inoculated with *A. brasilense* plus two foliar applications.

Treatment 4 containing the group of seeds inoculated with *A. brasilense* plus a foliar application of the same product (Az + 1F) was the one that presented the maximum value, being the same of 7 429.55 µg of PNP g^{-1} soil h^{-1}. Finally, it is observed that the activity was lower in treatment 5, made up of the group of inoculated seeds whose foliage of the plants was sprayed twice with the inoculant (Az + 2F).

A value of 4 616.19 µg of PNP g^{-1} soil h^{-1} was recorded, the latter being 3 551.45 times greater than the control (absolute control) and 4 449.39 times than treatment 2, as well as phosphatase activity in the soils that contained seeds treated with cobalt and molybdenum also did not show significance with respect to the control ($p > 0.05$).

However, these values are slightly higher in relation to those reported by Paz-Ferreiro (2007), starting from 55.6 to 4017 µg of PNP g^{-1} soil h^{-1} and those obtained by Paul and Clark (2007) being from 12.51 to 56 295 µg of PNP g^{-1} soil h^{-1}. In this regard, Henríquez *et al*. (2014) mentions that phosphatase activity is correlated with the percentage of carbon in the soil.

Studies carried out by Dalurzo *et al*. (2000) mention that phosphatase activity tends to decrease in those soils under conventional tillage. The reduction of phosphatase activity in cultivated areas, both under conventional tillage and direct seeding, is related to the inhibitory effect of the use of rapidly soluble phosphorous sources, which is what happens in acidic soils (Rodríguez *et al*., 2012).

Another type of activities that promote plant growth, associated with the availability of P in the soil, is the acid solubilization of inorganic P and the mineralization of organic P, mediated by the action of acid and alkaline phosphatases, occurring as a function of soil pH. (Nash, 2007). The low content of organic matter and phosphorus could have been compensated by promoting this enzyme due to the application of *A. brasilense* since they are promoters or enhancers of enzymes (Martínez-Gallegos *et al*., 2007).

Apparently, the occurrence of symbiosis of *A. brasilense* with some other fungal microorganisms naturally present in the soil is possible, thus allowing efficiency in the transport of phosphorus in the plant, as demonstrated in their results by Ibarra-Puon *et al*. (2014).

Work done by Salgado *et al*. (2010) mention the importance of using phosphate-solubilizing bacteria from the rhizosphere to improve the solubilization of phosphorus fixed to the soil so that it is available to the plant, resulting in higher yield.

The production of the phosphatase enzyme is controlled by complex regulatory mechanisms, so that their activity is detectable only under specific environmental conditions and the interest in phosphatase enzymes has been increasing during the last decade due to its great application in biotechnology. Although the knowledge regarding their properties, regulation and role they play is still scarce (Fernández and Rodríguez, 2005).

Thus, in low pH systems, this transport occurs naturally, so that measurable phosphatase activity could decrease if the system is supplemented with *A. brasilense*. Phosphatase is an enzyme that is inhibited by its own substrate; therefore, it presents a limit of substrate where its activity increases, then decreases. This is demonstrated in figure 4, in treatment 5, consisting of seeds treated with *A. brasilense* plus foliar applications (Az + 1F).

Therefore, Henríquez *et al*. (2014) report an inverse relationship between phosphatase activity and available P content, suggesting that this inhibition trend occurs when the available P contents in the soil are very high and could occur near the rhizosphere under conditions of recent phosphoric fertilization.

Conclusions

The results of the experiment allowed concluding that the inoculation of rice seeds with *Azospirillum brasilense* strains and their foliar application did not improve the root length, root dry mass and aerial parts observed at the end of the crop development period. The application of cobalt plus molybdenum in the treatment of rice seeds caused a greater root length in the rice plants with respect to the treatment that integrated seeds inoculated with *A. brasilense* plus two foliar applications of the same.

The phosphatase enzyme activity presented higher values in the treatments that contained seeds inoculated with *A. brasilense* compared to those that lacked it, such as the control (absolute control) and treatment two (T2) with cobalt plus molybdenum.

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