



## The ability of rhizobacteria to solubilize phosphate and synthesize of indoleacetic acid in cowpea

### Capacidade de rizobactérias na solubilização de fosfato e síntese de ácido indol acético em feijão caupi

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**Abstract:** The Cerrado comprises a vast ecoregion in central Brazil where plants show both growth and nitrogen fixation deficiencies due to low soil fertility. Farmers may overcome such problem using species of microorganisms capable of improving soil fertility such as the Rhizobia bacteria. This work aimed to assess the ability of phosphate solubilization and synthesis of indoleacetic acid (IAA) of *Rhizobium* isolates obtained from Cerrado soils in the state of Tocantins, Brazil, evaluating their symbiotic efficiency in cowpea (*Vigna unguiculata* L. Walp.) plants. We used a total of 32 isolates (or strains) of *Rhizobium* and a reference species of *Bradyrhizobium*. The capacity of phosphate solubilization and synthesis of IAA was evaluated *in vitro*, while the symbiotic function of rhizobia isolates and the effect on cowpea biomass was assessed in a greenhouse. Only eight strains were able to solubilize calcium phosphate, while all isolates produced IAA. The rhizobia inoculation caused a significant increase in biomass and nodulation of cowpea. The isolates UFT R122 and UFT R124 stood out with the highest values for the studied parameters, showing rises above 33% of relative efficiency in comparison to the treatment with nitrogen fertilization. By associating the results of phosphate solubilization capacity, IAA synthesis, symbiotic ability, and nodulation, we conclude that the isolates that showed good performance are potential inoculants for cowpea in Cerrado soils.

**Keywords:** Indole acetic acid; *Bradyrhizobium*; *Vigna unguiculata* L. Walp.; Biomass; Plant growth.

**Resumo:** As espécies de rhizobia são conhecidas como microorganismos efetivos para melhorar a fertilidade do solo, como solos de Cerrado com deficiência de crescimento da planta e problemas de fixação de nitrogênio. O objetivo deste trabalho foi avaliar a capacidade de solubilização de fosfato e síntese de ácido indol acético de isolados de rizóbio obtidos de solos de Cerrado em Tocantins, avaliando a eficiência simbiótica dos mesmos em plantas de feijão caupi (*Vigna unguiculata* L. Walp.). Foram utilizados 32 isolados de rizóbio e uma espécie referência de *Bradyrhizobium*, onde foram avaliadas a capacidade de solubilização de fosfato e síntese de ácido indol acético (AIA), *in vitro*, e capacidade simbiótica dos isolados de rizóbio e o efeito na biomassa de feijão caupi em casa de vegetação. Dos 32 isolados e a estirpe referência, apenas oito obtiveram capacidade de solubilizar fosfato de cálcio, e todos os isolados produziram AIA. Houve aumento significativo para a biomassa e nodulação de feijão caupi com a inoculação de diferentes isolados de rizóbio, com os isolados UFT R122 e UFT R124 com os maiores valores para os parâmetros estudados, com aumentos acima de 33% para a eficiência relativa em relação ao tratamento com adubação nitrogenada. Associando-se os resultados de capacidade de solubilização de fosfato, síntese de AIA e eficiência simbiótica e nodulação, observou-se que os isolados que apresentaram bom desempenho são potenciais inoculantes para o feijão caupi em solos de Cerrado.

**Palavras-chave:** Ácido indol acético; *Bradyrhizobium*, *Vigna unguiculata* L. Walp., Biomassa; Plant growth.

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## INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp.) cultivation generates employment and maintenance for low-income rural populations in the north and northeast Brazil. Also, the technology adoption has allowed sowing cowpea in large areas (BASTOS et al., 2012). The ability to grow in low fertility soils arises among the main characteristics of this crop (FREIRE FILHO, 2011). However, poor availability of nutrients, especially phosphorus and nitrogen, comprises one of the most limiting factors to bean productivity in agricultural soils.

Brazilian soils have low nitrogen content. They are insufficient to sustain the nitrogen demand of plants; thus, biological nitrogen fixation becomes fundamental (HUNGRIA; CAMPO, 2007). Theoretically, the amount of nitrogen fixed through symbiosis, added to the nitrogen that the cowpea extracts directly from the soil, make up enough supply for plant nitrogen needs. However, the amount of nitrogen fixed by the rhizobia in the plant can vary according to the amount of native rhizobia cells present in the soil. These native species compete against inoculated rhizobia. The competition relates to the affinity of the microorganisms to the host plant, which is affected by some factors, such as sensitivity and adaptability of the inoculated strains, climate, soil, and pH. Therefore, the technique of inoculation in the cowpea crop is still insufficient; although it is an economical and sustainable alternative, it still needs more studies under climate and savanna soil conditions.

The efficiency of biological nitrogen fixation may increase sharply with the use of native strains adapted to the edaphoclimatic conditions of the region. Introduction of exotic strains in higher inoculum rates than the native rhizobial population affects the density and competitiveness of native rhizobial (Krasova-Wade et al. 2006). Then, it is possible to obtain a competitive inoculant capable of rapidly adapting to soil and promote better symbiotic performance.

Efficient strains adapted to prevailing local conditions, such as acidic and low fertility Cerrado soils, should be competitive with the native population. They should aggregate highly important agronomic and cultural aspects, such as nodulation potential, N<sub>2</sub> fixation, and enhancement of host plant growth (ZILLI et al., 2006). Also, the rhizobia species present essential physiological characteristics such as the phosphate solubilization capacity and the production of phytonutrients such as the indoleacetic acid (IAA) (CHAGAS JUNIOR et al., 2009; CHAGAS JUNIOR et al., 2010a), which can help increase phosphate availability for plant growth. Therefore, rhizobia strains involved in phosphorus solubilization and IAA synthesis may increase plant growth by increasing the efficiency of biological nitrogen fixation (CHAGAS JUNIOR et al., 2010b). The microbial effect in the rhizosphere of plants, such as the increased ability to solubilize inorganic phosphates, may positively affect the physiology of host plants. Then, they became available to plants and to root growth as a function of the IAA synthesis.

This work aimed to evaluate the ability of phosphate solubilization and synthesis of indoleacetic acid by rhizobium isolates obtained from savanna soils in the state of Tocantins, Brazil, as well as to evaluate their symbiotic efficiency in cowpea plants.

## MATERIALS AND METHODS

### Rhizobia strains and phosphate solubilization analysis

The Microbiology Laboratory of the Federal University of Tocantins, Gurupi Campus, provided the 32 rhizobia isolates for our study. These rhizobia were isolated from soil samples collected in different regions of the state of Tocantins, using cowpea as bait plant. Each isolate was grown in specific media for solubilization of phosphates: 10 g of glucose, 2 g of yeast extract, and 15 g of agar. For the formation of the precipitated calcium phosphate, we added two solutions above medium: Solution A, containing 5 g of K<sub>2</sub>HPO<sub>4</sub> (50 mL of water); and solution B, containing 10 g of CaCl<sub>2</sub> (100 mL of water). The bromothymol blue dye was added to the medium to verify the alteration of the pH by the bacteria. The pH was adjusted to 6.5. The phosphate solubilization was quantified by the measurement of solubilization halos around the colony, characteristic of a translucent zone. The bacteria were classified based on the solubilization indexes: low (SI < 2), medium (2 ≤ SI < 4) and high solubilization (SI > 4).

### Synthesis indole-3-acetic acid

Colonies of rhizobia with 2-3 mm in diameter grown on YMA medium were transferred to Erlenmeyer flasks containing 25 mL YM broth in the presence of 100 mL.L<sup>-1</sup> of L-Tryptophan. Colonies were incubated in a shaker (100 rpm) at 26±2°C during four days, after which, the bacterial supernatant was separated by rotation (12.000 rpm) for 15 minutes (GORDON; WEBER, 1951). Two parts of the supernatant obtained from each bacterium were used for the colorimetric analyzes of indoleacetic acid. After the qualitative verification of the presence of IAA (pink color after 25 minutes of reaction at 26±2°C), the phytohormone was quantified in a spectrophotometer at 530 nm. The concentrations in µg mL<sup>-1</sup> were estimated using a standard curve made with the synthetic form of IAA.

### Evaluation of the symbiotic capacity of Rhizobium isolates

The experiment was carried out in a greenhouse located in the experimental area at the Federal University of Tocantins, Gurupi Campus, Tocantins, located at 11°43'45"S and 49°04'07"W at 278 m of altitude. We put 2 kg of soil from a cultivation area at the experimental station in black plastic pots. The soil had the following characteristics: pH 5.1 in water; 2.3 mg dm<sup>-3</sup> P; 0.19 cmolc dm<sup>-3</sup> K; 1.8 cmolc dm<sup>-3</sup> Ca; 0.8 cmolc dm<sup>-3</sup> Mg; 0.00 cmolc dm<sup>-3</sup> Al; 1.0% organic matter; 72.3% sand; 8.2% silt; and 19.5% clay; in the 0-20 cm depth layer (EMBRAPA, 2009). The fertilization was carried out at planting with 0.098 g of K<sub>2</sub>O (KCl) and 0.4 g of P<sub>2</sub>O<sub>5</sub> (single super phosphate) per pot.

We applied the treatments as follows: (1) the 32 rhizobia isolates; (2) a standard *Bradyrhizobium* strain (INPA 03-11B); (3) a control treatment with 0.081 g of nitrogen (corresponding to 50 kg of urea per hectare); (4) a treatment without inoculation of rhizobia and nitrogen. The standard strain was obtained from the rhizobia collection of the Laboratory of Microbiology of the Federal University of Lavras. This strains was approved by RELARE (Rede de Laboratórios para Recomendação, Padronização e Difusão de Tecnologia de Inoculantes Microbianos de Interesse Agrícola) and recognized by MAPA (Ministério da Agricultura,

Pecuária e Abastecimento) through the SDA (Secretaria de Defesa Agropecuária), Normative Instruction No. 10 of March 21, 2006, becoming part of the list of authorized microorganisms for the production of commercial inoculants for cowpea crops in Brazil.

The isolates used were suspended individually in saline solution (0.2% MgSO<sub>4</sub>) after growth in YMA medium for five days, and each of these suspensions (averaged 1 x 10<sup>8</sup> CFU mL<sup>-1</sup>) were inoculated to the seeds at the time of planting.

After five days of germination, the thinning was done, leaving two plants per pot. Irrigation was done manually, providing water up to 80% of field capacity.

### Biomass analysis

The plants were harvested manually at the initial stage of flowering at 50 days after planting (DAP). After manual detachment, we counted the nodules, packed the shoots in paper bags, and take them to a forced circulation oven (65 to 70°C) to the constant weight. We estimated the following biomass variables: shoot dry mass (SDM), root dry mass (RDM), total dry mass (TDM), number of nodules (NN), and dry mass of nodules (DMN). The relative efficiency (RE) was calculated according to the formula: RE = (SDM inoculated /

SDM with N) x 100, where the inoculated SDM is the shoot dry mass with inoculation and SDM with N is the shoot dry mass with mineral N.

### Statistical analysis

Data were submitted to analysis of variance using the statistical software ASSISTAT version 7.6 beta (SILVA, 2008), and the means were compared by the Scott-Knott test at 5% probability.

## RESULTS AND DISCUSSION

From the 32 isolates, only eight were able to solubilize calcium phosphate, four of which showed a medium phosphate solubilization index (between 2 and 4) and four showed low indexes (less than 2) (Table 1). The standard strain was unable to solubilize phosphate. Visual detection and quantitative estimation of the ability of rhizobia strains to solubilize poorly soluble phosphates have been possible using Petri plate methods. Thus, the culture medium for solubilizers containing insoluble mineral phosphate as the only source of phosphorus is characterized by the appearance of a translucent zone around the solubilizing colony (CHAGAS JUNIOR et al., 2009).

**Table 1.** Solubilization of calcium phosphate and indoleacetic acid synthesis (IAA) by rhizobia isolates from cowpea.

ISOLATES	PSI <sup>1</sup>	IAA <sup>2</sup>	ISOLATES	PSI	IAA
UFT R02	-	++ <sup>(210)</sup>	UFT R 91	-	+ <sup>(10)</sup>
UFT R16	+ <sup>(1,17)</sup>	+++ <sup>(305)</sup>	UFT R 93	-	+ <sup>(70)</sup>
UFT R20	-	+ <sup>(21)</sup>	UFT R 97	-	++ <sup>(122)</sup>
UFT R27	-	++ <sup>(200)</sup>	UFT R 102	++ <sup>(2,4)</sup>	++ <sup>(120)</sup>
UFT R34	-	++ <sup>(194)</sup>	UFT R 109	+ <sup>(1,1)</sup>	++ <sup>(184)</sup>
UFT R35	+ <sup>(1,3)</sup>	+ <sup>(44)</sup>	UFT R 112	+ <sup>(1,3)</sup>	+++ <sup>(390)</sup>
UFT R44	++ <sup>(2,1)</sup>	++ <sup>(260)</sup>	UFT R 121	-	+ <sup>(50)</sup>
UFT R50	++ <sup>(2,3)</sup>	++ <sup>(122)</sup>	UFT R 122	-	+ <sup>(52)</sup>
UFT R57	-	+ <sup>(44)</sup>	UFT R 123	-	+ <sup>(65)</sup>
UFT R59	-	++ <sup>(110)</sup>	UFT R 124	-	+ <sup>(48)</sup>
UFT R63	-	++ <sup>(152)</sup>	UFT R 125	-	+ <sup>(25)</sup>
UFT R67	-	+ <sup>(24)</sup>	UFT R 126	-	+ <sup>(35)</sup>
UFT R76	-	+ <sup>(70)</sup>	UFT R 127	-	+ <sup>(50)</sup>
UFT R80	-	+ <sup>(72)</sup>	UFT R 128	-	+ <sup>(55)</sup>
UFT R82	-	+++ <sup>(330)</sup>	UFT R 129	-	+ <sup>(65)</sup>
UFT R83	++ <sup>(2,6)</sup>	+ <sup>(33)</sup>	INPA 03-11B <sup>3</sup>	-	+ <sup>(55)</sup>
UFT R87	-	++ <sup>(210)</sup>			

<sup>1</sup> Phosphate solubilization index (PSI): +, PSI <2 (low); ++, 2 <PSI <4 (medium); +++, PSI > 4 (high). <sup>2</sup> Indole acetic acid (IAA): +, IAA production below 100 µg mL<sup>-1</sup>; ++, above 100 µg mL<sup>-1</sup>; +++, above 300 µg mL<sup>-1</sup>. <sup>3</sup> NPA 03-11B, standard strain of *Bradyrhizobium*.

Dorozhkin (2002) described that microbial metabolism provides H<sup>+</sup> ions necessary to catalyze solubilization, and the rate of H<sup>+</sup> excretion is higher than its consumption by the reaction, leading to a reduction in pH. Then, this mechanism explains part of the solubilized P as a function of pH. In addition to the excretion of H<sup>+</sup>, the organic acids, organic binders, and extracellular polymeric substances are also involved in the dissolution of phosphorus (SILVA FILHO; VIDOR, 2001), which may vary in efficiency according to the composition of the medium (KUSS et al., 2007). On the other hand, the production of nitrogen-fixing bacteria inoculants is already a routine practice in soil microbiology laboratories. Bacteria are among the leading microorganisms responsible for the solubilization of phosphates (DOBBELAERE et al., 2003).

All isolates and the standard strain synthesized indoleacetic acid (IAA) even in small amounts. The UFT R112, UFT R82, and UFT R16 isolates produced the highest concentrations of IAA, above 300 µg mL<sup>-1</sup> (Table 1). The isolates UFT R02, UFT R27, UFT R34, UFT R44, UFT R50, UFT R59, UFT R63, UFT R87, UFT R97, UFT R102, and UFT R109 yielded IAA concentrations between 100 and 300 µg mL<sup>-1</sup>. The other isolates had IAA levels below 100 µg mL<sup>-1</sup>. Chagas Junior et al. (2010a, b), also verified that IAA production by the diazotrophic isolates, even when there was no addition of tryptophan to the culture medium. The yeast extract used in the culture medium is a rich source of amino acids including tryptophan. Bacteria produce IAA by various synthesis routes, and L-tryptophan is a precursor of IAA since its addition in culture media promotes increased synthesis, but there are independent routes of tryptophan. Although both

routes coexist in plants, the relative importance of each to the IAA content in plant tissues has not yet been clarified.

Several plant-associated bacteria synthesize phytohormones, especially IAA production, which stimulates cell elongation, division, and differentiation in plants (HAMEED et al., 2004). In experiments with and without addition of L-tryptophan in YMA medium, Chagas Junior et al. (2009, 2010a, 2010b) found isolates of Amazon soils with IAA production. Similar results were found by Hameed et al. (2004) with *Rhizobium* and *Bradyrhizobium* isolates in culture medium supplemented with L-tryptophan. In a four-day incubation period in culture medium supplemented with 500 mg L<sup>-1</sup> of L-tryptophan, Bano and Musarrat (2003) recorded IAA levels below 100 µg mL<sup>-1</sup>.

Some isolates stood out both in phosphate solubilization and IAA production (Table 1), which suggests that these

isolates are more efficient than the others analyzed because of their double capacity. Therefore, prospects of this research in other study conditions becomes more interesting. The ability to solubilize phosphate and synthesize IAA by our best rhizobia isolates highlight their potential use as root growth promoters in crops. Similar results were reported for different rhizobia isolates (CHAGAS JUNIOR et al., 2010a, 2010b).

Regarding the vegetal growth of cowpea in the greenhouse, the shoot dry mass (SDM) differed significantly among the treatments. The isolates UFT R122, UFT R124, and the reference strain provided the highest SDM values compared to the other isolates (p < 0.05), followed by isolates UFT R123, UFT R126, and UFT R129, where the SDM did not differ related to the control treatment with fertilization (Table 2).

**Table 2.** Biomass evaluation parameters in cowpea inoculated with rhizobia isolates in the greenhouse at 50 days after planting<sup>1</sup>

Treatments	SDM (g)	RDM (g)	TDM (g)	NN	DMN (mg)	RE (%)
UFT R02	1.52 c	1.75 d	3.27 d	48.0 b	40 d	43.7 d
UFT R16	1.13 d	1.39 e	2.70 e	62.7 a	40 d	32.5 e
UFT R20	1.19 d	1.66 d	2.84 e	63.0 a	41 d	34.2 e
UFT R27	1.27 d	1.43 e	2.70 e	41.3 b	22 e	36.5 e
UFT R34	1.20 d	1.18 e	2.38 e	57.7 a	34 e	34.5 e
UFT R35	1.27 d	1.45 e	2.72 e	54.7 b	19 e	36.5 e
UFT R44	1.32 d	1.52 d	2.67 e	45.0 b	21 e	37.9 e
UFT R50	1.14 d	1.60 d	2.75 e	51.6 b	32 e	32.8 e
UFT R57	1.22 d	1.56 d	2.77 e	50.3 b	34 e	35.1 e
UFT R59	1.37 d	1.64 d	3.01 d	58.7 a	33 e	39.4 e
UFT R63	1.21 d	1.58 d	2.78 e	62.7 a	71 c	34.8 e
UFT R67	1.13 d	1.61 d	2.75 e	26.7 b	18 e	32.5 e
UFT R76	1.24 d	1.42 e	2.67 e	61.3 c	33 e	35.6 e
UFT R80	1.42 d	1.43 e	2.85 e	72.3 a	35 e	40.8 d
UFT R82	1.53 c	1.72 d	3.25 d	66.3 a	32 e	43.9 d
UFT R83	1.27 d	1.58 d	2.85 e	69.7 a	35 e	36.5 e
UFT R87	1.58 c	1.55 d	3.13 d	43.3 b	23 e	45.4 d
UFT R91	1.29 d	1.50 d	2.63 e	36.7 b	28 e	37.1 e
UFT R93	1.34 d	1.37 e	2.71 e	65.0 a	38 d	38.5 e
UFT R97	1.26 d	1.31 e	2.57 e	39.7 b	31 d	36.2 e
UFT R102	1.08 d	1.61 d	2.68 e	38.3 b	23 e	31.0 e
UFT R109	0.97 e	1.48 e	2.46 e	20.0 b	14 f	27.9 e
UFT R112	1.18 d	1.64 d	2.82 e	26.7 b	24 e	33.9 e
UFT R121	1.26 d	1.66 d	2.92 d	42.0 b	36 d	36.2 e
UFT R122	4.64 a	2.17 b	6.80 b	33.3 b	80 c	133.3 a
UFT R123	3.67 b	2.26 b	5.93 b	31.3 b	104 b	105.5 c
UFT R124	4.81 a	3.02 a	7.84 a	57.7 a	171 a	138.2 a
UFT R125	1.98 c	1.10 e	3.47 d	19.0 c	93 b	56.9 d
UFT R126	3.56 b	1.80 c	5.53 c	27.7 b	35 d	102.3 c
UFT R127	1.81 c	1.43 e	3.24 d	8.7 c	22 e	52.0 d
UFT R128	4.12 a	1.98 c	6.11 b	27.3 c	73 c	118.4 b
UFT R129	3.31 b	1.88 c	5.19 c	33.3 b	67 c	95.1 c
INPA 0311B	4.92 a	2.31 b	7.24 a	73.7 a	48 d	141.4 a
Fertilizer control	3.48 b	2.38 b	5.86 b	23.3 c	46 d	100.0 c
Control not fertilized	1.45 d	1.74 d	3.18 d	52.7 a	13 f	41.7 d
CV (%)	14.3	16.1	14.1	19.3	19.8	14.3

<sup>1</sup> Mean followed by the same letter in the column do not differ from each other by the Scott-Knott test at 5%. Shoot dry mass (SDM), Root dry mass (RDM), Total dry mass (TDM), Number of nodules (NN), Dry mass of nodules (DMN), Relative Efficiency (RE).

The UFT R124 isolate obtained the highest results for root dry mass (RDM) (p < 0.05), followed by the isolates UFT R122, UFT R123, and standard strain INPA 03-11B, which did not differ from the control fertilized with nitrogen

(Table 2). The UFT R124 isolate and the standard strain INPA 03-11B yielded the highest total dry matter (TDM) (p < 0.01), followed by the isolates UFT R122, UFT R123, and

UFT R128, which were similar to the control fertilized with nitrogen (Table 2).

On the other hand, ten isolates and the standard strain presented higher averages in the number of nodules ( $p < 0.05$ ) (NN) (Table 2). However, the isolate UFT R124 was superior ( $p < 0.05$ ) to the other treatments for the nodules dry mass (DMN), followed by the UFT R123 isolate. These results show that the NN is uncorrelated with DMN, which may mean that some nodules are not fully active in the biological fixation of nitrogen. According to Zilli et al. (2009a), a mass of nodules higher than 0.56 g per plant and the NN higher than 49 per plant seems to guarantee the sufficiency of biological nitrogen fixation for this crop. Regarding the NN, 14 isolates and the reference strain reached averages above the values cited by Zilli et al. (2009a). In contrast to DMN, only seven isolates produced nodules greater than 0.56 g per plant. Thus, the most efficient were UFT R124, UFT R123, UFT R125, and UFT R122.

Regarding the relative efficiency, which relates the biomass of the shoots inoculated by isolates with the nitrogen fertilized control, the isolates UFT R122, UFT R124, and the reference strain reached the superior results (Table 2). Thus, the tested isolates showed excellent efficiency in the biological fixation of nitrogen, which evidences their efficacy in cowpea plants. Zilli et al. (2009b) and Chagas Júnior et al. (2010a, b) found a similar behavior regarding the biomass of cowpea inoculated by rhizobium isolates under greenhouse conditions.

The isolates UFT R122 and UFT R124 showed the highest values for the studied parameters and were equal to or higher than the values found for the reference strains and the control treatment. Similar results were reported in other studies by, Soares et al. (2006), Zilli et al. (2006), and Chagas Junior et al. (2009, 2010a, b), who observed a lower symbiotic efficiency of the reference strains related to strains isolated from cowpea nodules. The results of symbiotic efficiency and nodulation showed that the isolates that presented good performance are potential inoculants for cowpea beans in Cerrado soils. However, there is a need to assess the contribution of these isolates to nitrogen fixation and cowpea productivity through agronomic tests under field conditions.

Finally, the results of phosphate solubilization, IAA synthesis, symbiotic efficiency, and nodulation were correlated. Finally, the isolates with superior performances are potential inoculants for cowpea in Cerrado soils.

## CONCLUSION

Some rhizobia isolates showed the ability to solubilize calcium phosphate, while all the isolates produced indoleacetic acid (IAA). There was a significant increase in biomass and nodulation of cowpea with inoculation of the different rhizobia isolates. The isolates UFT R122 and UFT R124 showed the highest values for the studied parameters.

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