Caracterização bioquímica de sementes de trigo produzidas a partir da associação de *Azospirillum Brasiliense* com nitrogênio

Biochemical characterization of wheat seeds produced with the association of *Azospirillum Brasiliense* with nitrogen

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RESUMO
O objetivo com a pesquisa foi avaliar o efeito de diferentes estirpes da bactéria do gênero *Azospirillum* sp., associado com a aplicação de doses de nitrogênio em trigo, e seu reflexo nos caracteres bioquímicos de sementes produzidas. O experimento foi constituído por delineamento de blocos casualizados, em um esquema fatorial duplo, 4x5 com quatro repetições, totalizando 80 unidades experimentais. Os tratamentos foram constituídos por quatro combinações de duas estirpes (ABv5 e ABv6) da bactéria *Azospirillum brasiliense* e cinco doses de nitrogênio: testemunha (zero); 30; 60; 90 e 120 Kg de nitrogênio por hectare. Foram realizadas as seguintes análises bioquímicas: Índice de clorofila foliar total; Teor de nitrogênio total, aminoácidos, açúcar solúvel total e proteína e a atividade da enzima nitrato redutase. As estirpes da bactéria *Azospirillum brasiliense*, influenciaram positivamente o índice de clorofila foliar total, teor de nitrogênio na folha e teor de proteína quando aplicadas de forma isolada, no entanto, se obteve o melhor resultado quando as estirpes foram aplicadas de forma associada. Além disso, cada kg de N aplicado proporcionou incremento de 0,0223 no (ICFT) e 0,0286 g.Kg\(^{-1}\) de nitrogênio na folha e 0,024 mg.g\(^{-1}\)FW de proteína na folha. A inoculação com as duas estirpes (ABv5 + ABv6) promoveu maior acúmulo de açúcares na folha, em relação aos demais tratamentos. A atividade de nitrato redutase foi maior nas plantas inoculadas com a associação das estirpes (ABv5 + ABv6), e para cada Kg\(^{-1}\) de N aplicado incrementou 0,0181 nmol NO\(_2\)-.g\(^{-1}\)FW.h\(^{-1}\).

Palavras chave: Estirpes, *Triticum aestivum*, análise bioquímica.

ABSTRACT
The aim of the study was to evaluate the effect of different strains of the bacteria of the genus *Azospirillum* sp., associated with the application of doses of nitrogen in wheat, and the reflexes in the biochemical characteristics of the seeds produced. The experiment was performed under a randomized block design, in a double factorial scheme, 4x5 with four replications, totaling 80 experimental units. The treatments consisted of four combinations of two strains (ABv5 and ABv6) of the bacteria *Azospirillum brasiliense* and five doses of nitrogen: control (zero); 30; 60; 90 and 120 kg of nitrogen per hectare. The following biochemical analyzes were performed: Total leaf chlorophyll index, total nitrogen, amino acids, total soluble sugar and protein contents and the activity of the nitrate reductase enzyme. The strains of the bacteria *Azospirillum brasiliense*, positively influenced the total leaf chlorophyll index, leaf nitrogen content and protein content when applied individually, however, the best result was observed when the strains were applied in association. Furthermore, each kg of N applied provided an increase of 0.0223 in the total leaf chlorophyll index and of 0.0286 g Kg\(^{-1}\) for leaf nitrogen and 0.024 mg g\(^{-1}\)FW for leaf protein. The inoculation with the two strains (ABv5 + ABv6) promoted greater accumulation of sugar in the leaf, compared to other treatments. Nitrate reductase activity was higher in plants inoculated with the associated strains (ABv5 + ABv6), and for each kg of N applied increased by 0.0181 nmol NO\(_2\)-. g\(^{-1}\)FW.h\(^{-1}\).

Keywords: Strains, *Triticum aestivum*, biochemical analysis.


1 INTRODUCTION

Wheat is a self-pollinated plant, with an annual cycle, being cultivated during the winter and the spring (NCBI taxonomy, 2016). The seed of this species is denominated caryopsis, possessing an oval shape with rounded extremes (Gwirtz et al., 2014). According to the USDA (2017), wheat is the second most cultivated crop in the world, only surpassed by corn. Furthermore, the crop is also the second most consumed cereal in the world (CONAB, 2017a). Therefore, seed production in large quantities and with high quality is necessary to meet the demand for sowing. In addition, it is noted that nitrogen is an essential macronutrient for plant development, being the most absorbed and exported nutrient in wheat (CONAB, 2017b; Kutman et al., 2011). The average N exports corresponds to 29 kg t\(^{-1}\) for whole plants and of 23 kg t\(^{-1}\) considering only the grain (Fornasieri Filho, 2008).

Nitrogen participates in several metabolic pathways in plants (SANGOI et al., 2008), composing the structure of proteins, enzymes, coenzymes, nucleic acids, phytochromes and chlorophyll. Thus, this nutrient is associated to various physiological processes, for example, photosynthesis is directly dependent of proteins such as rubisco, which is significatively affected by nitrogen starvation. A common practice currently used to reduce production costs while maintaining yield is seed inoculation with nitrogen-fixing and plant growth-promoting (PGP) bacteria, which is becoming fundamental for better plant nutrition and increased yields (GLICK, 2014). Therefore, the study of biological nitrogen fixation in grasses is highly relevant since is estimated that seed inoculation with nitrogen-fixing and PGP bacteria might generate savings of 1.2 billion dollars per year, considering only a partial replacement (50%) of the nitrogen fertilizer used in corn and wheat crops in Brazil (Hungria et al., 2010).

The evaluation of strains for inoculation in specific crops is indispensable for the development of technologies to produce inoculants with efficient strains of *Azospirillum* in cereals. Furthermore, the correct management of these bacteria will result in the decrease of nitrogen fertilizer usage and, consequently, in the production costs, besides promoting positive results on plant development and increased yields.

The aim of this study was to evaluate the effect of different strains of the bacteria of the genus *Azospirillum* sp. associated with doses of nitrogen on wheat and the reflexes on the biochemical characteristics of the seeds produced.
2 MATERIAL AND METHODS

The wheat seeds evaluated in the study were produced in an experimental area at the University of Passo Fundo in the 2016 growing season. The treatments consisted of four combinations of two strains (ABv5 and ABv6) of the bacteria *Azospirillum brasilense* and five doses of nitrogen: control (zero); 30; 60; 90 and 120 kg of nitrogen per hectare.

The total leaf chlorophyll index (TLCI) was measured in the reproductive growth stage (R1 – Bloom), in the superior, median and lower portions of the flag leaf of 10 plants per experimental unit, using a portable electronic chlorophyll meter, ClorofiLOG® CFL 1030 (Falker).

Total nitrogen (TLN) was determined from leaves randomly collected in each plot in the reproductive growth stage (R1 – Bloom), the leaves were dried at 65ºC using a kiln with forced air circulation until constant mass and were ground. The determination of total nitrogen was performed according to the methodology proposed by Tedesco et al. (1995). The analyzes were performed at the Soil Chemistry Laboratory, at the Federal University of Pelotas – Pelotas – RS – Brazil.

The biochemical composition was evaluated using flag leaves collected at the reproductive growth stage (R1–Bloom). The extraction was performed using MCW (Methanol:Chloroform:Water, at the ratios of 12:5:3). The extracts were obtained according to the methodology proposed by Bieleski e Turner (1966) with modifications. Briefly, 0.25g of wheat leaf samples were ground with liquid nitrogen and homogenized with 10 mL of the MCW extraction solution. After 24 hours, the extracts were centrifuged at 600g for 30 minutes. The supernatant containing the metabolites was collected and transferred to a water bath at 38ºC, held for 30 hours to eliminate chloroform residues and concentrate the samples for subsequent protein, amino acid and total soluble sugar analyzes.

Protein extraction and quantification (P): the precipitate obtained at the first centrifugation of the MCW, was resuspended in 10 mL of NaOH 0.1N and homogenized using a vortex. After 24 hours at rest, the extracts were centrifuged at 2000g for 30 minutes and the supernatant containing the protein portion was collected. The determination of total protein was performed according to the methodology described by Bradford (1976).

Amino acid quantification (AA): the extracts in a final volume of 1 mL, placed in test tubes, received 0.5 mL of citrate buffer 0.2 M with pH 5.0; 0.2 mL of ninhydrin reagent (5%) in methyl cellosolve and 1 mL of KCN 2% (v/v) in methyl cellosolve. The test tubes were agitated and transferred to a water bath at 100ºC for 20 minutes. Next, the tubes were placed in the dark until room temperature was reached and 1.3 mL of ethanol (60%) was added to complete a volume of 4 mL and tubes were agitated. The total soluble amino acid content was expressed in µmol g⁻¹ FW.
Total soluble sugars (TSS): performed according to Graham e Smydzuk (1965). 1 mL from each sample was collected and 3 mL of cold anthrone solution was added to each test tube which were immediately capped with glass marbles. After 15 minutes, the tubes were agitated and incubated in a water bath at 90ºC for 20 minutes. Next, the tubes were agitated and the optical density (D.O) of the standards was measured at 620 nm in a spectrophotometer (SP-22, Biospectro).

The extraction and quantification of the nitrate reductase activity was obtained using an in vivo essay. After preliminary tests, which evaluated the activity of the enzyme throughout the day, the time of 9 am was chosen for sample collection. Samples of 0.5 g of fresh leaves were placed into test tubes with 4 mL of potassium phosphate buffer K₂HPO₄/KH₂PO₄ (200 mM), pH 7.5, containing KNO₃ (100 mM) and n-propanol (1%) and vacuum infiltrated at 300 mm Hg for 1 minute, for three times. After infiltration, 1 mL was withdrawn from the reaction medium and placed into tubes with 4 mL of nitrite reagent (sulfanilamide 1% e N-1 naphthyl ethylenediamine hydrochloride 0.02%). From this mixture, samples were withdrawn at time zero and at fifteen minutes, when the samples were under incubation in a water bath. Samples removed from the water bath were held in the dark for approximately 15 minutes until measurement. The nitrite content was determined at 540 nm in a spectrophotometer (SP-22, Biospectro), comparing the values obtained with the values of the standard curve (0 to 500 nmol of NO₂⁻). The activity of the enzyme was expressed in µmol NO₂⁻ g⁻¹ FW h⁻¹.

The experiment was performed under a randomized block design, in a double factorial scheme, 4x5 with four replications, totaling 80 experimental units. Data were subjected to the analysis of variance, where the effect of the treatments was evaluated by the F test, when significant the averages were compared by the Tukey test at 5% probability of error and, when necessary, the regression analysis was performed for quantitative factors. The analyzes were performed with the aid of the statistical program RStudio Team (2016).

3 RESULTS AND DISCUSSION

The results presented on Table 1 demonstrate that the strains ABv5 and ABv6 of the bacteria *Azospirillum brasilense* positively affected the total leaf chlorophyll index (TLCI), total leaf nitrogen (TLN) and protein content (P) when applied individually, however, the best result was obtained when these strains were applied in association, for all doses of nitrogen. Even when there was no supplemental application of nitrogen, wheat plants cultivated with the association of the two strains presented high values, which were near those observed with the application of 120 kg of N hectare⁻¹ without inoculation.
Table 1 – Total leaf chlorophyll index (TLCI); Total leaf nitrogen (TLN) and leaf protein content (P) of wheat plants subjected to the inoculation with different strains of *Azospirillum brasilense* via seed treatment and doses of topdress nitrogen. 2016 Growing season. UFPel, 2018.

| Variable* | Strain  | Dose of Nitrogen (kg ha⁻¹) | Average | CV (%) |
|-----------|---------|-----------------------------|---------|--------|
| TLCI      | Control | 44.56 45.62 46.68 46.77 47.77 46.28c | 46.19** | 2.77   |
|           | ABv5    | 46.01 46.03 47.00 47.53 48.82 47.08bc | 47.08bc | 1.63   |
|           | ABv6    | 46.98 46.27 48.10 48.52 49.26 47.83ab | 47.83ab | 2.22   |
|           | ABv5 + ABv6 | 47.23 47.26 49.78 48.88 49.05 48.44a | 48.44a  | 2.97   |
| Average   |         | 46.19** 46.29 47.89 47.92 48.73 | 2.77   |
| TLN (g Kg⁻¹) | Control | 9.59 10.85 11.71 12.07 13.10 11.46c | 10.42** | 6.48   |
|           | ABv5    | 10.02 11.08 12.21 12.35 13.91 11.91bc | 11.91bc | 3.16   |
|           | ABv6    | 10.80 11.39 12.44 12.71 14.38 12.34ab | 12.34ab | 2.97   |
|           | ABv5 + ABv6 | 11.28 11.91 12.60 13.63 14.70 12.82a | 12.82a  | 1.97   |
| Average   |         | 10.42** 11.31 12.24 12.69 14.02 | 6.48   |
| P (mg g⁻¹MF) | Control | 11.30 12.26 13.75 12.72 13.91 12.79c | 12.79c  | 1.42   |
|           | ABv5    | 12.83 13.72 14.64 13.94 14.52 13.93bc | 13.93bc | 1.23   |
|           | ABv6    | 13.26 14.22 15.92 14.55 16.14 14.82b | 14.82b  | 1.14   |
|           | ABv5 + ABv6 | 14.27 14.87 17.75 16.63 20.11 16.72a | 16.72a  | 1.54   |
| Average   |         | 12.91** 13.77 15.51 14.46 16.17 | 11.57   |
| CV (%)    |         | 2.77   |

*Averages followed by the same letter in the column do not differ at the 5% probability level by the Tukey test (**Significant at the 5% probability level, 0.01=< p <0.05 by the F-Test; ns = not significant, p >=0.05 by the F-test).

These results can be attributed to the availability of nitrogen provided to plants by the bacteria of the genus *Azospirillum*, since with greater nitrogen availability, leaf chlorophyll content increased, which allows a greater production of photoassimilates, improving shoot and root development and thus, reducing costs and increasing yields. Accordingly, Quadros et al. (2014), verified a positive effect in the TLCI due to the inoculation with *Azospirillum brasilense*, compared to treatments without inoculation. Likewise, Kappes et al. (2013), observed positive effects in the chlorophyll index on plants inoculated with the bacteria. Moreover, the increase in protein content occurred due to a greater chlorophyll and nitrogen content in the leaves, allowing plants to synthesize more proteins. In accordance, Martuscello et al. (2016) reported that the availability of nitrogen promoted a positive effect in the crude protein content of leaf blades and stalks of Napier grass.

Besides the positive results promoted by the bacteria, a significant and linear increase was also observed for TLCI, TLN and P with the increase in the dose of nitrogen (Figure 1). It is worth to highlight that each Kg of nitrogen applied via topdressing resulted in an increase of 0.0223 for TLCI (A), 0.0286 g Kg⁻¹ for TLN (B) and 0.024 mg Kg⁻¹FW for P (C), respectively. The results obtained are consistent with those of Theago et al (2014), which reported that the increase in the doses of nitrogen applied resulted in a linear increase in the concentrations of nitrogen and
chlorophyll in wheat leaves. Yet, Teixeira Filho et al. (2008), observed the increase of nitrogen in the flag leaf on wheat, as a response of the increase in nitrogen doses. Furthermore, Santos et al. (2010), reported that nitrogen fertilization resulted in a linear increase of 38% in the crude protein content of signal grass.

There was interaction between factors for the variable amino acids (AA) (Table 2), in the absence of nitrogen fertilization the seeds treated with the association of the strains ABv5 + ABv6 of the bacteria *Azospirillum brasilense*, were superior when compared to the inoculation of the strains applied individually, and from the control (no inoculation). Furthermore, at dose zero the...
strains ABv5 and Abv6 applied individually were superior to the control. At doses 30 and 60 kg of N ha\(^{-1}\), the results demonstrate that seed inoculation with the strains individually (ABv5 or ABv6) or combined (ABv5 + ABv6), were equally superior when compared with the control. Furthermore, at the dose of 90 Kg of N ha\(^{-1}\), only the inoculation with the association of strains stood out, increasing the concentration of AA (17.37 µmol g\(^{-1}\)FW) when compared to other treatments. At the dose of 120 Kg of N ha\(^{-1}\), the inoculation with the strain ABv6 individually or associated with ABv5 (ABv5 + ABv6) were superior compared to the inoculation with ABv5 and the control treatment (no inoculation) (Table 2).

Table 2 – Amino acids (AA), total soluble sugars (TSS) and activity of the nitrate reductase (ANR) enzyme of wheat plants subjected to the inoculation with different strains of *Azospirillum brasilense* via seed treatment and doses of topdress nitrogen. 2016 Growing season. UFPel, 2018.

| Variable* | Strain       | Dose of Nitrogen (kg ha\(^{-1}\)) | Average | CV (%) |
|-----------|--------------|----------------------------------|---------|--------|
|           |              | 0      | 30     | 60     | 90     | 120    |         |
| AA (µmol g\(^{-1}\)FW) | Control      | 7.53c  | 10.90b | 11.52b | 12.99b | 12.84b | 11.16   |
|           | ABv5         | 11.69b | 13.59a | 15.06a | 13.34b | 13.16b | 13.37   |
|           | ABv6         | 12.46b | 13.96a | 15.32a | 14.83b | 15.88a | 14.49   |
|           | ABv5 + ABv6  | 14.44a | 15.31a | 16.79a | 17.37a | 16.81a | 16.14   |
| Average   |              | 11.53  | 13.44  | 15.55  | 14.63  | 14.67  |         |
| CV (%)    |              | 8.03   |        |        |        |        |         |
| TSS (mg g\(^{-1}\)FW) | Control      | 28.76  | 31.32  | 35.85  | 38.28  | 36.31  | 34.10c  |
|           | ABv5         | 36.12  | 37.26  | 38.71  | 39.24  | 44.02  | 39.07bc |
|           | ABv6         | 40.26  | 40.59  | 39.03  | 44.61  | 45.60  | 42.02ab |
|           | ABv5 + ABv6  | 46.11  | 50.40  | 49.97  | 46.62  | 44.63  | 47.55a  |
| Average   |              | 37.81ns| 39.89  | 40.89  | 42.19  | 42.64  |         |
| CV (%)    |              | 17.95  |        |        |        |        |         |
| ANR (nmol NO\(_2\)-g\(^{-1}\)FW h\(^{-1}\)) | Control      | 2.29   | 3.94   | 3.65   | 3.68   | 4.93   | 3.70c   |
|           | ABv5         | 2.98   | 4.19   | 3.81   | 4.55   | 6.03   | 4.31b   |
|           | ABv6         | 3.62   | 4.19   | 4.39   | 5.69   | 6.22   | 4.82b   |
|           | ABv5 + ABv6  | 5.30   | 5.10   | 4.53   | 6.07   | 6.60   | 5.52a   |
| Average   |              | 3.55*  | 4.36   | 4.10   | 5.00   | 5.95   |         |
| CV (%)    |              | 15.32  |        |        |        |        |         |

*Averages followed by the same letter in the column do not differ at the 5% probability level by the Tukey test (**Significant at the 5% probability level, 0.01< p <0.05 by the F-Test; ns = not significant, p >=0.05 by the F-test).

For total soluble sugars (TSS) (Table 2), plants derived from seeds inoculated with both strains (ABv5 + ABv6) accumulated more sugars in the leaves, compared to the other treatments. On the other hand, plants derived from seeds which did not receive the inoculant accumulated less sugars in the leaves for all doses applied. Additionally, there was no statistical difference among the doses of nitrogen applied via topdressing for this variable.
The nitrate reductase enzyme presented greater activity on plants derived from seeds inoculated with the association of the strains (Table 2). On the other hand, plants derived from untreated seeds presented the lowest activity of the nitrate reductase enzyme. Furthermore, plants derived from seeds inoculated with the strains ABv5 or ABv6 presented an intermediate activity of the enzyme.

On Figure 2A, it is possible to observe that the control (no inoculation) and seed inoculation with ABv5, fitted in a positive quadratic polynomial wherein the doses which provided the greatest accumulation of amino acids on leaves were of 101 and 65.6 kg of N ha⁻¹. The inoculation with the strain ABv6 and the association of the strains (ABv5 + ABv6), presented a positive linear tendency, with an increase of 0.0257 and 0.0226 µmol of AA g⁻¹FW. The results presented on Figure 2B demonstrate that the activity of the nitrate reductase was positively affected by the increase of nitrogen doses applied, presenting a linear increase of the enzymatic activity, wherein each Kg of N applied resulted in an increase of 0.0181 nmol NO₂⁻ g⁻¹FW h⁻¹.

Figure 2 - (A) = Amino acids (AA) and (B) = Activity of the nitrate reductase (ANR) of wheat plants subjected to inoculation with different strains of *Azospirillum brasilense* via seed treatment and doses of topdress nitrogen. 2016 Growing season. UFPel. 2018.
Nitrogen metabolism is fundamental for amino acid and protein formation. In this sense, the bacteria *Azospirillum brasilense* can generate several stimuli for plant growth, such as performing biological nitrogen fixation, producing growth hormones (Fukami et al., 2016), increasing chlorophyll content (Hungria, 2011) and modifying plant photosynthetic activity (Gordillo Delgado et al., 2016). Yet, when these bacteria grow inside a plant, the activity of the nitrate reductase enzyme can be increased (Hungria, 2011). Furthermore, Lavres Junior; Monteiro (2006) observed that the activity of the nitrate reductase was increased due to nitrogen fertilization.

4 CONCLUSIONS

The addition of the strains ABv5 and ABv6 via seed inoculation, increases total leaf chlorophyll index; total leaf nitrogen content; protein content; amino acid content; total soluble sugars and the activity of nitrate reductase enzyme.

The application of nitrogen increases total leaf chlorophyll index; total leaf nitrogen; protein content; amino acid content and the activity of the nitrate reductase enzyme.
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