

Activity of the nitrate reductase enzyme in *Jatropha curcas* L. seedlings under different shade percentages

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Abstract

Objective: To determine the influence of different shade percentages on the activity of the nitrate reductase enzyme in leaves and roots of *Jatropha curcas* L. seedlings.

Materials and Methods: For such purpose, three shade levels (30, 50 and 70 %) and a treatment under full sunlight on leaf and root tissues were tested; and the total soluble protein content and activity of the nitrate reductase enzyme were determined. The data were processed through a simple variance and regression analysis. All this was done using the statistical program SPSS® version 22 for Microsoft Windows®.

Results: The values of the nitrate reductase activity were higher in the leaves than in the roots. Differences were observed in the enzyme activity in the leaves, with regards to the evaluated shade percentages. At 30 and 90 days of evaluation a linear relation was found, with $R^2=0,984$ and $R^2=0,945$, respectively, in which a decrease was observed of the enzymatic activity as the shade percentage increased.

Conclusions: The activity of the nitrate reductase enzyme and the soluble protein decreased linearly with the increase of the shade percentages, which indicates the importance of the high indexes of luminous radiation for the good functioning of nitrogen metabolism in *J. curcas*.

Keywords: nitrogen metabolism, radiations, plant tissues

Introduction

At present, biodiesel production plays an important role in the world economy. In this context there are different plants, such as *Jatropha curcas* L., *Moringa oleifera* Lam., *Ricinus communis* L. and *Reutealis trisperma* (= *Aleurites trisperma*) Airy Shaw, among others, which constitute a viable alternative for biofuel production, because they are crops that are not used in animal or human consumption.

One of these crops, physic nut (*J. curcas*), is differentiated from the others for being a native plant of Mesoamerica (Ureña-Padilla *et al.*, 2012), which shows a high content of oil in its seeds. In addition, it does not compete for the food production areas and shows drought and salinity resistance (Campuzano *et al.*, 2016).

On the other hand, the productivity of plant species is limited by the biotic and abiotic factors that cause stress on plants. To counteract the influence of these disturbances on productivity, an efficient use of water and of the photosynthetic capacity of plants under adverse conditions should be guaranteed.

In this sense, the nitrate reductase enzyme (NR EC 1.6.6.1) is a molybdoenzyme that catalyzes nitrate reduction (NO_3^-) to nitrite ($-\text{NO}_2^-$). Its function in higher plants is not very clear yet; although Raigón *et al.* (2006) stated that the abundant existence of nitrogen collaborates in the formation of chlorophyll, which increases the photosynthetic activity and, thus, plant development. A large quantity of nitrogen makes plant cells reach senescence later and continue being turgid. On the other hand, it delays tissue lignification and hardening. In general, nitrogen accelerates and maintains high development in the cultivated plant.

For such reasons, the objective of this study was to determine the influence of different shade percentages on the activity of the nitrate reductase enzyme in leaves and roots of *J. curcas* seedlings.

Materials and Methods

The study was conducted in the greenhouse of the Plant Physiology sector of the Biology department of the Universidade Federal de Lavras –UFLA– (Lavras, Minas Gerais, Brazil), which is located at 918 m of altitude, 21° 14' S of latitude and 45° 00' W of longitude.

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Three seeds of physic nut (*J. curcas* Cape Verde provenance) were sown in polyethylene bags, with capacity for three liters of substrate and with washed sand, without previous pregermination treatment. These seeds were obtained from dried fruits and were maintained in forced-air stove, at a temperature of 38 °C during 24 h.

The trial was irrigated daily by automatic spraying during three minutes, from 8 a.m. to 5 p.m., to prevent the hydric stress. Fifteen days after the seeds germinated, a thinning work was carried out to unify height and number of leaves, and only one plant was left per bag.

Forty milliliters were applied per plant through two Hoagland and Arnon (1938) nutrient solutions: one contained NO_3^- (solution 1) and the other did not (solution 2), twice per week, since 21 days after sowing.

- Solution 1: $\text{Ca}(\text{NO}_3)_2 \cdot 2\text{H}_2\text{O}$ (50mM); KNO_3 (50mM); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (20mM); KH_2PO_4 (10mM); H_3BO_3 (0,46mM); $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0,091mM), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (7,65 μM); $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (3,2 μM); H_2MoO_4 (0,56 μM).
- Solution 2: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (2mM); KH_2PO_4 (1mM); $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ (2mM); K_2SO_4 (2mM); H_3BO_3 (0,46mM); $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0,091mM), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (7,65 μM); $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (3,2 μM); H_2MoO_4 (0,56 μM).

The two solutions were prepared with micronutrients and macronutrients, except iron, which was added at the moment of application, supplied by the solution Fe-EDTA (33,2 g L^{-1} $\text{Na}_2\text{-EDTA}$; 26 g L^{-1} $\text{Fe SO}_4 \cdot 7\text{H}_2\text{O}$; and 3,65 g L^{-1} NaOH).

Experimental design and treatments. A complete randomized design was used. The treatments consisted in three shade percentages (30, 50 and 70 %), provided by a polyethylene cloth, and one under full sunlight, for a total of 12 treatments with three repetitions.

Description of the evaluated variables. The determinations described below were carried out in the laboratory of plant physiology nutrition of the UFLA, with a frequency of 30, 60, 90 and 120 days after the beginning of the treatments; for that purpose four plants were used for each treatment, and the leaves and roots were taken into consideration. In the case of leaves, the first and second (true leaves) from the basis to the apex were sampled.

Quantification of soluble proteins. The soluble proteins were extracted according to the method described by Bielecki and Turner (1966). For 0,1 g of fresh material, 1 mL of MCW solution (60 mL

of methanol, 25 mL of chloroform and 15 mL of distilled H_2O) was added. The material (leaves and roots) was macerated at ambient temperature and centrifuged during 15 minutes at 3 000 rpm (in refrigerated centrifuge). After centrifugation and separation of the supernatant, 1 mL more of chloroform and 1,5 mL of distilled H_2O were added for every 4 mL of extract, which was left to stand during 24 h in the freezer. Afterwards, the precipitate was re-suspended in solution NaOH 0,1 g mol^{-1} , for a ratio of 10 mL of solution for each gram of fresh tissue, and it was centrifuged again during 15 minutes at 3 000 rpm; the supernatant was used for quantifying the proteins.

Determination of the total soluble protein content. The content of total soluble protein (TSP) was determined through the methodology proposed by Bradford (1976) and with the above-described extract. One hundred μL of root extract and 50 μL of leaf extract were used, diluted in 50 μL of NaOH 0,1 g mol^{-1} and 5 mL of reagent; it was prepared with 200 mg of Coomassie Brilliant Blue + 50 mL of 95 % ethanol, which was agitated for two hours, followed by the addition of 100 mL of 85 % orthophosphoric acid (H_3PO_4), and it was preserved during two more hours in agitation. After this time, the solution was left to stand for 24 h and was completed to 1 000 mL with deionized H_2O ; then it was filtered and stored in a dark flask at 4 °C.

The colorimetric reaction was read in the Genesys 20® Thermo Scientific® UV-visible spectrophotometer at 595 nm. The values were expressed in milligrams of protein per gram of fresh matter (FM). The pattern that was used for the protein was 10 to 100 mg of bovine serum albumin (BSA).

Determination of the nitrate reductase activity. For the determination of the activity of the nitrate reductase (NR) enzyme, an in vivo essay, described by Radin (1973), was used, in the leaves as well as the roots, with some modifications (regarding concentrations, due to the presence of secondary metabolites) for the studied species. The samples were collected at 7 a.m. on warm and sunny days, and were stored in polyethylene boxes with ice; they were later transported to the laboratory and washed with deionized H_2O .

An amount of 0,5 g of fresh leaves and 1 g of roots from four random plants was weighed, cut into pieces of approximately 5 x 5 mm, and inoculated in test tubes which contained 5 mL of phosphate buffer 0,1M + KNO_3 0,1M, pH 7,5. The test tubes were covered with aluminum foil, in order to avoid

light-caused oxidative damage. The samples were vacuum-infiltrated three times during two minutes, to facilitate and increase the penetration of the solution in the tissues, followed by air introduction. Afterwards, the essay was incubated in bain-marie at 30 °C during one hour.

For the case of the leaves an aliquot of 100 µL was removed. The reaction medium was prepared with 2,9 mL of distilled water + 1 mL of mixture (v:v) of 0,02 % N-naphthylethylene dichloride, which stops the reaction; and 1 % sulfanilamide prepared in HCl 1,5 N, which reacts and provides color. For the roots an aliquot of 500 µL was removed and the reaction medium was prepared with 0,5 mL of distilled water + 1 mL of N-naphthylethylene and sulfanilamide mixture.

After 15 minutes (colorimetric reaction), the reading was performed in the Genesys 20® Thermo Scientific® UV-visible spectrophotometer at 540 nm, and the values of the NR activity were expressed in µmol NO₂·g FM⁻¹ h⁻¹, determined by the quantity of nitrite produced; a pattern curve of sodium nitrite solution was used (from 0 to 200 µmoles).

Statistical analysis. The data were subject to variance analysis, according to simple classification linear model, considering the shade levels. The means were compared through Duncan's test for 5 % of significance, after verifying that they fulfilled

the adjustment of normal distribution and variance homogeneity. A regression analysis was also carried out; in order to select the best fit equation the criteria expressed by Guerra *et al.* (2003) were taken into consideration, related to the significance level, determination coefficient R² higher than 0,70, residual variance V(e), residue analysis (e_i) and standard error of the estimated parameters SE (β_i). For this, the statistical program SPSS® version 22.0 for Microsoft Windows® was used.

Results and Discussion

Table 1 shows the activity of the nitrate reductase enzyme in the leaves and roots, with regards to the shade percentages at the four evaluated moments.

The NR activity was higher in the leaves than in the roots. According to Hernández-Cruz *et al.* (2015), the nitrate absorbed by the plants, mostly, can be assimilated by the roots or by the leaves. These same authors state that in many plants, when small nitrate quantities are received, their absorption in the roots is reduced; for such reason, as the nitrate contribution increases its absorption by this organ increases and, thus, higher proportion is transported to the stem and from there to the leaves, in which the activity of the above-mentioned enzyme is higher, especially when they are fully extended and physiologically mature. The

Table 1. Activity of the nitrate reductase enzyme with regards to the shade levels µmoles NO₂·g FM⁻¹ h⁻¹.

Shade, %	Evaluation moment, days			
	30	60	90	120
Leaves				
0	3,79 ^a	3,12 ^a	1,34	1,51
30	3,27 ^a	2,69 ^b	1,41	1,42
50	2,90 ^b	3,09 ^a	1,76	1,62
70	2,27 ^c	2,21 ^c	1,0	1,16
\bar{X}	3,06	2,6	1,42	1,46
SE ±	0,15 ^{**}	0,14 ^{**}	0,07 ^{ns}	0,07 ^{ns}
Roots				
0	0,05 ^a	0,06	0,05 ^a	0,16 ^a
30	0,06 ^a	0,06	0,06 ^a	0,16 ^a
50	0,04 ^b	0,07	0,05 ^a	0,11 ^b
70	0,04 ^b	0,05	0,04 ^b	0,09 ^b
\bar{X}	0,04	0,06	0,05	0,13
SE ±	0,0023 ^{**}	0,003	0,002 [*]	0,006 ^{**}

Different letters in the same column differ for p > 0,05

** p > 0,01; *p > 0,05

utilization of nitrate by the plant varies depending on the species, morphological differentiation, age or root growth and environmental conditions, as indicated by the NR activity in each of the organs.

Likewise, Taiz and Zeiger (2013) state that in some plants, such as *Xanthium strumarium* L., nitrate is absorbed in the leaves; while in the others, like *Lupinus albus* L., most of the nitrate is assimilated in the roots. In general, the native species from temperate regions make a better utilization of nitrate by the roots than the ones from tropical and subtropical regions.

In this sense, herbaceous species, which include crops of economic interest, reduce nitrate concentrations in the roots as well as the leaves; in this regard, Rodríguez-Larramendi *et al.* (2016) stated that its assimilation depends on two factors: the NR activity and nitrate (NO_3^-) availability in the environment.

A higher NR activity in the leaves is due to the assimilation of nitrate, which occurs in higher quantity in this tissue, contributing the necessary energy (ATP) and reducer agents (electron providers) for such process. In addition, in the leaves there is higher photosynthetic activity. In this sense, the quantity of chlorophyll is related to a higher NR action (Taiz and Zeiger, 2013).

Similar behavior as the one in this study was reported by Hernández-Cruz *et al.* (2015) in cotton (*Gossypium hirsutum* L.); however, in other species like sunflower (*Helianthus annuus* L.), radish (*Raphanus raphanistrum* subsp. sativus) and rubber tree (*Hevea* spp.), this last one from the same family as *J. curcas* (Euphorbiaceae), the roots showed higher NR activity than the leaves (Hiraki, 2011).

In the leaves, the means of the NR activity decreased gradually in each evaluated period. This

behavior was described in *G. hirsutum* (Hernández-Cruz *et al.*, 2015) and in other studies conducted by Min *et al.* (2014) and Palacios (2017); which can be ascribed to the fertilization that was performed at the beginning of the period and to irrigation during the experimental stage.

For such reason, the reduction in the general averages of the NR activity in the *J. curcas* leaves could be explained by the natural senescence of tissues, because the sampled leaves were always in the same position on the plants (first and second true leaves from the basis to the apex).

In this sense, Duursma *et al.* (2014) referred that in the leaves of *Pueraria phaseoloides* (Roxb.) Benth. a reduction occurred of the activity of the above-mentioned enzyme in the apex-basis sense; similar results were also verified in soybean (*Glycine max* L.). According to these authors, the highest activity of the enzyme in young leaves is due to the higher nitrate and photoassimilate concentration and the higher capacity of protein synthesis in those leaves; it was also because of the self-shading of the leaves from the basal layer, caused by the increase in the mean number of leaves.

Differences were observed in the NR activity in the leaves with regards to the shade percentages (table 1). In the periods of 30 and 60 days (fig. 1), the model that explained this relation with higher goodness of fit ($R^2 = 0,984$ and $R^2 = 0,945$, respectively) was the linear one, where a decrease of the enzymatic activity was observed as the amount of shade percentage increased.

In the case of roots difference was found in the activity at 30, 60 and 90 days, but not at 120 days (table 1). The model that explained this relation ($R^2 = 0,953$, $R^2 = 0,939$ and $R^2 = 0,911$, respectively)

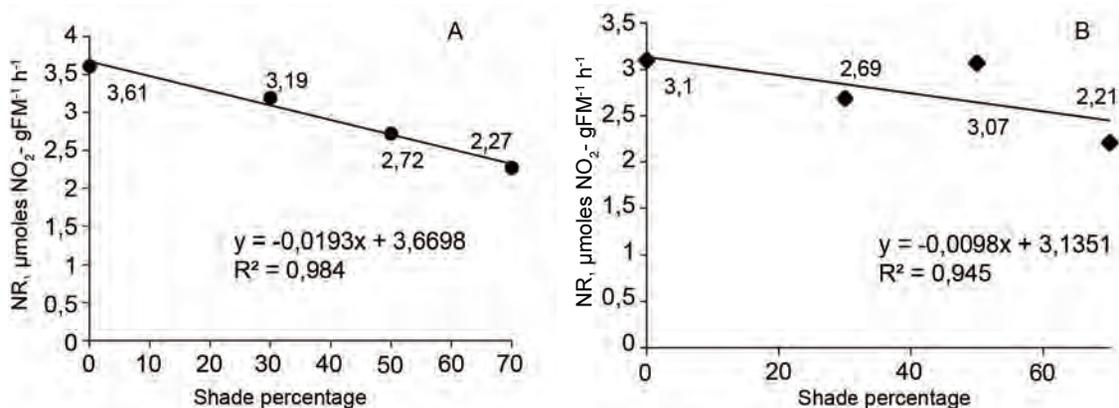


Figure 1. Activity of the nitrate reductase (NR) enzyme in the leaves at 30 (A) and 60 (B) days.

with higher goodness of fit was the quadratic one (fig. 2), where a slight increase of activity was observed with 30 % of shade and a reduction after that value. At 120 days, the NR activity decreased linearly with the increase of the shade percentage (fig. 2C), as occurred in the leaves.

The influence of light on the NR activity was observed in cocoa seedlings (*Theobroma cacao* L.) by Acheampong *et al.* (2013), and in oil palms by Rivera *et al.* (2013). These authors referred that the different irradiation regimes (20, 50 and 100 % of sunlight) influenced the enzyme partition between the leaves and roots, and that with high irradiation and nitrate levels, the reduction of the latter occurs more in the leaves than in the roots and vice versa.

According to references by Taiz and Zeiger (2013), under optimum growth conditions the nitrate reduction capacity is, approximately, twice the need of the plant. The NR activity varies during the day and shows low activity in the dark for most species. Under normal activation conditions and in the presence of light, its action would be in the range from 70 to 90 %, with a reduction from 10 to 30 % in the dark. However, light is not a direct signal for the activation of that enzyme, because even under intense and continuous luminosity it is inactive when carbonic gas is missing; this indicates that photosynthesis is required for its activation and that, probably, the exported photoassimilates out of

the chloroplast are indicators for that (Arboleda y Piña, 2010).

It is valid to mention that the quantity of carbohydrates present, available luminosity and other environmental factors, such as water availability, activate phosphatase, enzyme responsible for the dephosphorylation of several serine residues in the NR protein, favoring its activity; while the absence of light and magnesium stimulate phosphorylation of serine residues which interact with an inhibiting protein that acts on the inactivation of nitrate reductase (Farfán-Valencia y Mestre-Mestre, 2004).

Difference was observed in the contents of total soluble protein, for the leaves as well as for the roots of *J. curcas*, with regards to the treatments. In the same way as it was reported for the leaves in *Hevea brasiliensis* by Lemos *et al.* (1999), the averages of total soluble protein were always higher than the root means.

In each of the analyzed periods, the increase in the shade percentage caused linear decreases in the contents of leaf soluble protein (fig. 3), behavior accompanied by the activity of the NR enzyme in the same tissues; this suggests that there is a close relation between the activity of this enzyme and protein production in the tissues. In the case of the roots, the model that explained with higher goodness of fit at 30 and 120 days was the linear one, and at 60 days, the quadratic model (fig. 4).

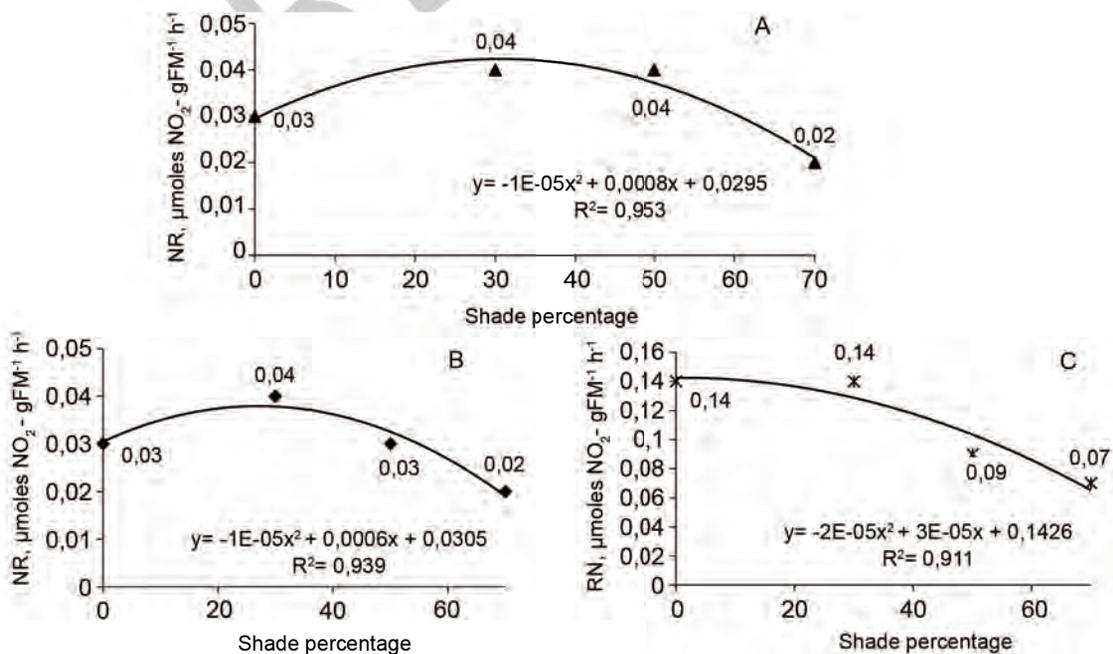


Figure 2. Activity of the nitrate reductase (NR) enzyme in the roots at 30 (A), 90 (B) and 120 (C) days.

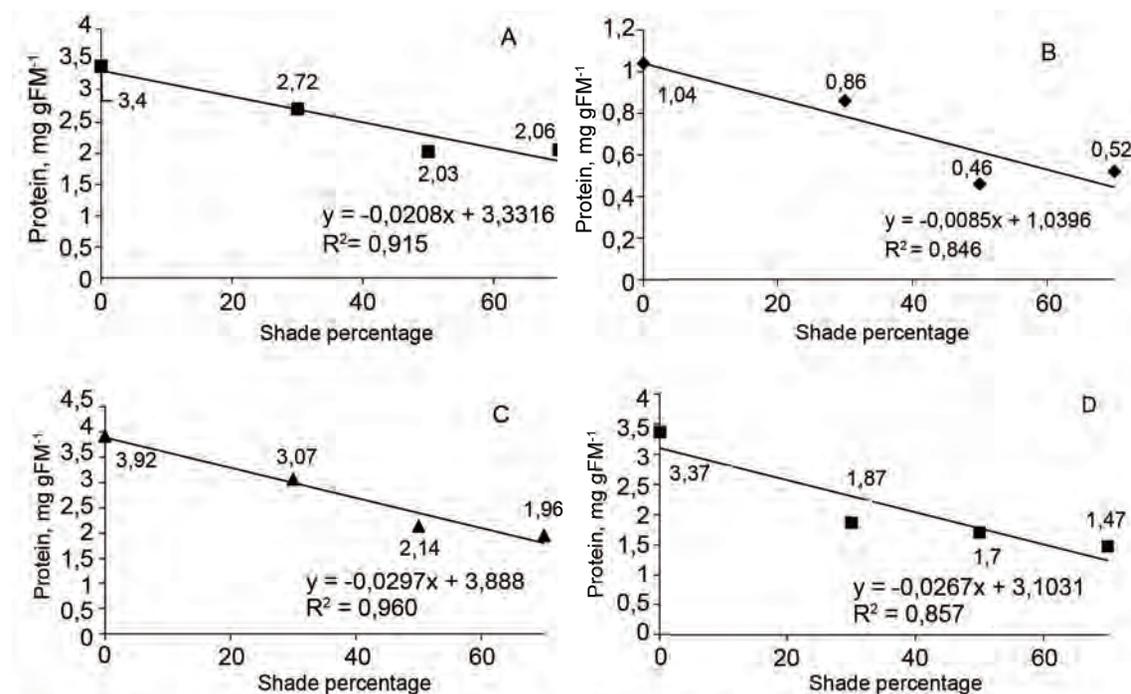


Figure 3. Content of total soluble protein in the leaves of *J. curcas* seedlings at 30 (A), 60 (B), 90 (C) and 120 (D) days.

According to the studies conducted by Duursma *et al.* (2014) and Felicito-Alberca (2016), the photosynthetic capacity of plants depends on the nitrate supply. A considerable fraction of that element is found in the leaves, in the proteins involved in the photosynthetic process. Likewise, it could be proven that the total soluble protein content decreased with the increase of the shade percentage.

The results showed that the activity of the NR enzyme and the soluble protein content decreased linearly with the increase of the shade levels, which indicates the importance of the high luminous radiation indexes for nitrate assimilation in *J. curcas*.

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Conflicts of interests

The authors declare that there are no conflicts of interests.

Authors' contribution

- Hilda Beatriz Wencomo-Cárdenas. Carried out the conception, design, data acquisition, analysis and interpretation, as well the manuscript writing and revision.

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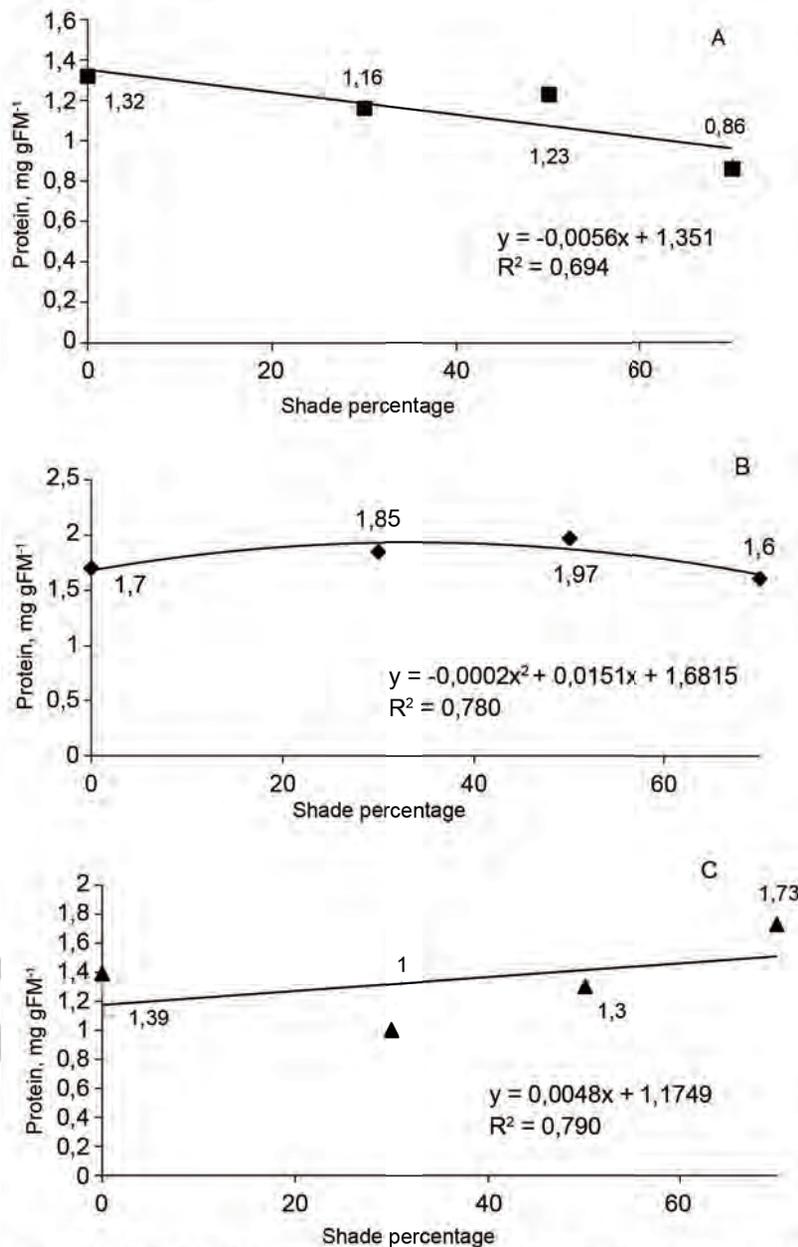


Figure 4. Content of total soluble protein in the roots of *J. curcas* seedlings at 30 (A), 60 (B), 90 (C) and 120 (D) days.

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