Do multifunctional organisms and Chlorella sp. microalgae benefit the production of Hymenaea courbaril L. seedlings?

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Abstract

The use of *Azospirillum brasilense* and *Chlorella* sp. has gained popularity in agriculture and forestry due to the benefits they offer to plants. These two biological solutions have demonstrated potential to improve the performance and sustainability of crops and forest species, opening new perspectives for agriculture and the recovery of degraded areas. Given the importance of establishing management strategies for seedling production and that information for forest essences is insufficient, the aim of this study was to evaluate the effect of intervals of *Chlorella* sp. microalgae application and inoculation with *Azospirillum brasilense* doses on the growth and quality of *Hymenaea courbaril* L. seedlings. The experiment was conducted in a randomized block experimental design, and treatments were arranged in a 5 x 4 factorial scheme, with four replicates, testing five *A. brasilense* doses: 0, 3, 6, 9 and 12 mL/plant and four intervals of *Chlorella* sp microalgae application: single application and every 7, 14 and 21 after transplanting (DAT), for 120 days. Growth and photosynthetic parameters were evaluated. Inoculation with 6 and 9 mL of *Azospirillum brasiliense*, regardless of *Chlorella* sp. microalgae application favored the growth of *H. courbaril* seedlings. *Chlorella* sp. microalgae application at intervals of 14 and 21 days favored the responses of seedlings inoculated with *Azospirillum brasilenses*. The association between *Azospirillum brasiliense* and *Chlorella* sp. promoted gains in seedling production and quality.

Introduction

In the global context, forest ecosystems have been negatively affected by several factors, such as intensive and inadequate exploitation of renewable natural resources. Due to these events, there has been a growing global demand for alternatives to meet sustainable development objectives determined by the 2030 agenda (UNITED NATIONS, 2015).

Among native Brazilian species, Jatobá (*Hymenaea courbaril* L., Fabaceae), is a species with wide distribution throughout the American continent, presenting commercial interest in the timber, medicinal and ecological sectors, with rapid growth, being indicated for the recovery of degraded areas (Sousa et al. 2020).

With the aim of promoting the propagation and contributing to the growth of forest species, biofertilizers and multifunctional organisms emerge as a promising practice in the production of seedlings (Ambrosio et al. 2017). *Chlorella* sp. microalgae stand out, as they contribute to plant nutrition, and tolerance and resilience to various environmental stresses (Lima et al. 2020; Tombesi et al. 2021). However, there are no records in literature regarding the frequency of their application in the production of *H. courbaril* seedlings. Inoculation with *Azospirillum brasilense* also shows good results, favoring the synthesis of phytohormones and assimilation of nutrients, contributing to photosynthetic metabolism and plant growth (Buitrago et al. 2021).

Given the importance of establishing management strategies for seedling production and that information for forest essences is insufficient, the aim of this study was to evaluate the effect of intervals of *Chlorella* sp. microalgae application and inoculation with *Azospirillum brasilense* doses on the growth and quality of *Hymenaea courbaril* L. seedlings.

Material and methods

Ripe *H. courbaril* fruits were randomly collected from matrices in a remnant Cerrado area (Authorization for Access and Shipment of Genetic Heritage Component Samples No. 010220/2015-1 - CNPq/CGEN/MMA). Fruits were manually pulped and seeds were immersed in 1% sodium hypochlorite solution for 5 minutes for sanitation and washed in running water. Subsequently, mechanical scarification was carried out until reaching visible wear of the integument on the side opposite to the micropyle to overcome dormancy, and then, seeds were soaked for 24 hours in distilled water at room temperature (Azevedo et al. 2003).

Sowing was carried out in 280 cm^3 tubes filled with Bioplant® commercial substrate consisting of filter cake, pine bark, coconut fiber, rice husk, sphagnum peat, agricultural gypsum and limestone, maintaining the substrate daily at 70% of water holding capacity.

Seedlings were transplanted into pots with capacity of 7 dm^{-3} when they reached average height of 15 cm, which occurred 65 days after sowing. Pots were filled with Oxisols corresponding to Dystrophic Red Latosol (SANTOS et al. 2018), with clayey texture and the following chemical characteristics: pH in water = 5.05; P = 0.02 mg dm^3; Ca, Mg, K, Al, H + Al, SB and CEC of 0.69; 0.38; 0.11; 0.086; 6.57; 1.17 and 7.74 cmolc dm^3, respectively, and base saturation = 15.1%. Pots were placed in an agricultural screen-type nursery with top and side coverage with black nylon mesh with 50% shading and top protection with 150 µm plastic cover.

The experimental design used was randomized blocks, and treatments were arranged in a 5 x 4 factorial scheme, with four replicates, and the experimental unit consisted of pot with two plants each in a factorial scheme, testing five *A. brasilense* doses: 0, 3, 6, 9 and 12 mL/plant according to Aguirre-Medina et al. (2014) and four intervals of *Chlorella* sp. microalgae application: single application at 7 days, every 7, 14 and 21 up to 120 days after transplanting (DAT). Data collected were considered as subdivided plots over time.

Inoculation of *A. brasilense* strains AB-v5 and AB-v6 with density of 1.00 g cm^3 and 2 x 10^8 CFU/mL in the corresponding doses was carried out on the day of transplantation directly onto seedling roots using hypodermic syringe in the morning. After seven days, *C.* sp. application started, in which applications were carried out in the morning according to pre-established intervals, using dose of 1.5 L ha^{-1} with broth of 100 liters of water (100 mL per plant) and addition of 0.3% LI 700 adjuvant as indicated by the manufacturer, adding the solution to the soil around seedlings.

The inoculant is composed of water, dipotassium phosphate, yeast extract, magnesium sulfate, ammonium chloride, sodium chloride, manganese sulfate, sucrose and stabilizers according to the manufacturer. Microalgae have the following characteristics according to the manufacturer: density = 1.1

g L^{-1}, 29.3%; 0.2%; 0.9%; 0.1%; 0.1%; 0.1% of organic C, N, P, K, Ca, S, Mg, respectively, 10.19; 0.001; 113.7; 11.13; 4.57 mg kg^{-1} of Zn, Bo, Fe, Cu and Mn, respectively, pH = 6.3 and 2 x 10^7 of microalgae cells per mL.

Irrigation was periodically carried out according to the seedlings' needs to maintain 75% of the soil's water holding capacity. At 45 DAT, nitrogen was applied using urea (45% N) as source at dose of 10 g 10 L^{-1} of water, adding 50 mL per plant as recommended by Souza and Lobato (2004). Minimum and maximum relative temperature and humidity values during the experimental period are shown in Fig. 1.

Every 30 days, starting at 30 up to 120 days, plant height assessments were carried out using graduated ruler, using as standard the distance between the soil and the insertion of the last leaf. The stem diameter was measured with digital caliper inserted 1.0 cm above the soil, and the number of leaves was manually counted. The chlorophyll index was measured using Falker CFL 1030 portable chlorophyll meter in fully expanded leaves located in the middle third of the plant, between 08:00 and 10:00 am.

Initial (F_0) and maximum (F_m) chlorophyll *a* fluorescence and the photochemical potential quantum efficiency of photosystem II (F_v/M) were measured using portable OS p30 fluorometer (OPTI-SCIENCES Chlorophyll Fluorometer, Hudson, USA). For this, the same leaves used for chlorophyll index evaluation were submitted to dark conditions, with leaf clips for 30 minutes. From data obtained, the absorbed energy conversion efficiency (F_v/F_0) and maximum non-photochemical basal yield (F_0/F_v) were calculated.

At 120 DAT, CO_2 assimilation rate (photosynthesis) - *A*, stomatal conductance-gs, intercellular CO_2 concentration - C*i* and transpiration - *E* were evaluated in two fully expanded leaves located in the middle third using portable photosynthesis analyzer LCIPro- SD (IRGA, Modelo ACD BioScientific Ltda.). Subsequently, Rubisco carboxylation efficiency (*A/Ci*) and water use efficiency - WUE (A/E) were calculated. Assessments were carried out between 08:00 and 10:00 am, considering photosynthetically active radiation > 850 µmol photons m^{-2} s^{-1}.

After this process, seedlings were harvested from pots, washed to remove excess substrate, and separated into leaf, stem and root system. The leaf area was determined using area integrator (LI-COR, 3100 C - Area Meter), and root length was measured with graduated ruler. Shoots (leaves and stems) and roots were packed in Kraft paper bags, dried in an oven at 60 ± 2°C for 72 hours, and weighed on a precision scale. The Dickson quality index (DQI) of seedlings was calculated as described by Dickson et al. (1960).

Data obtained were submitted to analysis of variance, and when significant by the F test ($p \leq 0.05$); the average *A. brasilense* doses and the microalgae application intervals were submitted to regression analysis testing mathematical models ($p \leq 0.05$), and the interaction of factors were compared by regression for *A. brasilense* and for evaluation intervals ($p \leq 0.05$). Data obtained over time were submitted to regression analysis testing linear or quadratic models ($p \leq 0.05$).

Results

Growth, photosynthetic metabolism and quality of *H. courbaril* seedlings were favored by the inoculation of *Azospirillum brasilense* and *Chlorella* microalgae, with variations as a function of doses and application intervals, respectively, demonstrating the beneficial effect of these bio-inputs for the species.

Stem diameter, plant height and number of leaves were influenced by evaluation times, with the highest values being 5mm, 30 cm, 8, respectively, all at 120 DAT (Figs. 2A-2C). Regarding the effect of bio-inputs on plant height, it was observed that in general, except for plants without *A. brasilense* inoculation, seedlings had higher values with the application of *Chlorella* sp. microalgae at intervals of 21 days, especially those cultivated with 6 mL of *A. brasilense*, which increased compared to the others (Fig. 2D).

A, E, gs and *WUE* values in *H. courbaril* L. seedlings were influenced by the interaction between factors under study. For *A*, it was observed that values presented quadratic adjustments, except for those with 3 mL of *A. brasilense*, which did not fit mathematical models. The highest values (10 µmol CO_2 m^{-2} S^{-1} of *A* were observed in plants grown in 14 microalgae application intervals, associated with *A. brasilense* inoculation of 9 mL (Fig. 3A). Leaf transpiration was greater when seedlings were grown with 0 and 21 days of *Chlorella* sp. application, both with 12 mL of *A. brasilense* (Fig. 3B), with values of 4.8 and 4.6 mmol H_2O m^{-2} S^{-1}, respectively.

Water use efficiency (*WUE*) was influenced by the interaction between factors under study, and *A. brasilense* inoculation of 12 mL contributed to higher values (0.5 and 0.47 µmol CO_2 m^{-2} s^{-2} / mmol H_2O m^{-2} s^{-2} without application or when applied to *Chlorella* sp. at intervals of 21 days, respectively (Fig. 3D). *Ci* and *A/Ci* were influenced by *A. brasilense* doses, with the best values (232 µmol CO_2 Mol^{-1} and 0.038 (µmol CO_2 m^{-2} s^{-1} / µmol CO_2 m^{-2} s^{-1}) with 12 mL of *A. brasilense* (Fig. 3D and 3F).

The chlorophyll index and photochemical indicators were influenced only by evaluation periods, all with quadratic adjustments, and for the chlorophyll index, initial fluorescence (F0) and basal yield (F0Fm), the maximum values were 0.044 and 0.32 to 80 DAT, respectively (Figs. 4A, 4B and 4C). FvFm and FvF0 values were minimal (0.66 and 2.2) at 80 and 60 DAT, respectively, but increased and had higher value at 120 DAT (Figs. 4).

Leaf area was influenced by the interaction between factors under study and plants inoculated with *A. brasilienses* dose of 6 mL showed quadratic adjustment with the highest value (522 cm²) with the application of microalgae at interval of 21 days. On the other hand, seedlings grown without microalgae application and inoculated with 9 mL of *A. brasilense* showed adjustment with similar value (544 cm²) (Fig. 5A).

The roots of seedlings were influenced by the interaction between factors under study, and those cultivated with microalgae at interval of 7 days, associated with *A. brasilienses* doses of 6 and 12 mL showed higher values (57 and 54 cm, respectively) (Fig. 3). It was observed that inoculation with *A.*

brasilienses contributed to the length and volume of the root system of *H. courbaril* seedlings, in addition to the greater number of adventitious roots (Fig. 5B).

DIQ (Fig. 5C) also showed interaction between factors under study. *Azospirillum* dose of 6 mL and *Chlorella* sp. application with interval of 21 days contributed to higher values. Evaluating *A. brasilense* within application intervals at doses of 3 and 9 mL, regression adjustments were observed, with the highest values being observed when *Chlorella* sp. was applied in the 14-day interval, being an ideal application interval when associated with *A. brasilense* inoculation.

In general, plants presented different visual appearance (Fig. 6) according to the different treatments. It was observed that when there was no *A. brasiliense* inoculation according to the interval of *Chlorella* sp. application, seedlings showed greater growth of adventitious roots.

For *A. brasiliense* doses of 3, 6 and 9 ml, the largest increases were with the 7 and 21 days of *Chlorella* sp. application. *A. brasiliense* dose of 12 ml, on the other hand, showed better results for the 7 and 14 days of *Chlorella* sp. application.

Thus, considering the gains obtained with the use of *A. brasilienses* and *Chlorella* sp., the use of bio-inputs in the production of seedlings of native species as growth promoters is recommended.

Discussion

The improvement of the growth characteristics is understood by the beneficial relationship between microalgae and *A. brasilense*, as they act in the production of bioactive compounds such as amino acids, lipids, flavonoids, which in turn are released into the soil and improve its chemical attributes and plant nutrition (Castiglione et al. 2021). Furthermore, these compounds associated with bio-inputs induce the synthesis of phytohormones in plants and stimulate greater cell division, consequently increasing shoot growth and contributing to greater plant height (Rouphael and Colla, 2020).

It should be highlighted that *H. courbaril* seedlings reached ideal parameters for subsequent transplantation to the field, given that the average nursery time for native tree species is approximately 5 months, and can reach up to 12 months (Oliveira et al. 2016). According to Freitas et al. (2021), seedlings with 20 to 30 cm in height and with stem diameter greater than 3 mm present ideal allometric indicators of seedling quality, and can be transplanted to the field. However, in our study, seedlings showed good characteristics 90 days after transplanting, demonstrating efficiency with the use of *A. brasilienses* and *Chlorella* sp. and the beneficial effect of using these bio-inputs.

As for photochemical indicators, the reduction between 60 and 90 DAT may be associated with a change in temperature and relative humidity during this period, but with subsequent recovery in values, indicating that there was no limitation in the process of absorption and transfer of energy at PSII level, since values within the ideal range from 0.6 to 0.85 (Schock et al. 2014) (Silva et al. 2015) were observed. Corroborating our results, Shezi et al. (2019) reported that the physiological gas exchange assessment, associated with chlorophyll *a* fluorescence values, offers answers about the photosynthetic and biochemical performance of plants, as it determines the effect of growth conditions on photosynthetic metabolisms and defines the ideal indices in a non-invasive way to determine seedling development.

The best gas exchange results correspond to the hormonal regulation promoted by *A. brasilense*, since these multifunctional organisms have the capacity to synthesize gibberellin and cytokinins, which stimulate cell elongation and the plant's juvenility, which in turn increases plant size and longevity (Glick, 2012). Furthermore, the bioregulatory activities of *A. brasilense* and *Chlorella* sp. perform synergistic mechanisms, promoting a positive effect, assisting in the assimilation and greater absorption of secondary metabolites that assist in plant development (Guimarães et al. 2018). According to Silva et al. (2015), the stomatal conductance behavior determines the transpiration demand to which leaves are potentially submitted, which in turn controls loss of water to the environment in the transpiration process and increases the water use efficiency, a fact observed in seedlings cultivated with 12 mL of *A. brasilense*.

Multifunctional organisms, represented in the present study by *A. brasilenses*, are capable of enhancing *gs*, allowing greater CO_2 absorption and increasing photosynthetic efficiency (Castiglione et al. 2021). In this condition, there is also increase in the leaf water potential due to the regulation of aquaporin genes (Barzana, Aroca and Ruiz-lozano, 2015), promoted by *A. brasilense* (Castiglione et al. 2021), increasing the ability to modulate water relations, making it a favorable mechanism for the species, especially when exposed to drought or other stress conditions (Eke et al. 2019). Thus, with this synergistic action, bio-inputs help and improve photosynthetic aspects, increasing photosynthesis, water use efficiency and consequently carboxylation efficiency. The greater root length is due to the fact that microalgae contain amino acids, lipids, proteins, vitamins and antioxidants, as well as hormones (Ru et al. 2020), which stimulate root growth, and this greater root vigor gives the plant greater rusticity. The symbiotic relationship between *A. brasiliense* and the plant induces the biosynthesis of auxin precursor agents, favoring an increase in root extension (Coniglio et al. 2019).

Mutale-Joan et al. in 2020 investigated the effects of using biostimulants derived from microalgae extracts on plant growth, nutrient absorption capacity and metabolic profile of tomato plants (*Solanum lycopersicum*). The results obtained revealed that the application of these extracts exerted a significant positive influence on several parameters. There was a significant increase in root length, shoot development, as well as nutrient absorption efficiency. Furthermore, a marked improvement in the quality and productivity of the tomato crop was observed.

Root development is one of the important factors for seedling survival, especially considering that when transplanted to field conditions, roots are responsible for connecting the plant to the soil and perform the function of support, absorption and reserve of water and nutrients (Billings et al. 2018). According to Andivia et al. (2021), for the selection of seedlings in reforestation programs or recovery of degraded areas, it is necessary to choose vigorous root systems to mitigate the effects of stress due to abiotic factors, enhance the absorption of water and nutrients and improve soil aggregation. Therefore, seedlings with root systems with high morphological and physiological standards have greater capacity to produce new roots quickly after planting, ensuring growth potential and production of new roots (Grossnickle, 2018).

The highest DQI values with bio-inputs are due to the best biomass production and leaf area, under the same cultivation conditions. When carrying out microalgae inoculation and application, these bio-inputs favor improvements in seedling nutrition. Plant nutrition, mainly nitrogen and phosphorus, play a fundamental role in the production and synthesis of ATP and constitution of reserve carbon compounds (Zonta et al. 2020). According to Soares et al. (2021), higher DQI values characterize ideal allometric indicators, configuring seedlings that possibly have greater survival capacity under field conditions, which reflect less need for replanting, that is, favoring the planting of the species in recovery areas. However, studies aimed at the field phase should be carried out in order to add information to the *H. courbaril ex situ* cultivation protocol.

Given the above, *A. brasilienses* inoculation and the use of *Chlorella* sp. in the production of forest seedlings represents an innovative and promising approach, demonstrating that the integration of these bio-inputs in the production process of forest nurseries, offers several benefits, highlighting improving seedling quality, environmental sustainability and reducing negative impacts from the excessive use of fertilizers. Thus, the integration of bio-inputs also has a positive impact on the restoration of forest ecosystems and the preservation of biodiversity.

Conclusion

Inoculation with 6 and 9 mL of *Azospirillum brasiliense*, regardless of *Chlorella* sp. microalgae application favored the growth of *H. courbaril* seedlings.

Chlorella sp. microalgae application at intervals of 14 and 21 days favored the responses of seedlings inoculated with *Azospirillum brasilenses*.

The association between *Azospirillum brasiliense* and *Chlorella* sp. promoted gains in seedling production and quality.

Declarations

Authors' contributions

R.M.B., S.C.S, E.S.M, C.C.S, S.P.Q.S contributed to the study’s conception and design. Experiments were conducted by R.M.B, B.L.P, R.L.C and E.P.T.Material preparation and analyses were performed by R.M.B., S.C.S, E.S.M, C.C.S, E.P.T. and S.P.Q.S The first draft of the manuscript was written by R.M.B., S.C.S, E.S.M, C.C.S. B.L.P., R.L.C., E.P.T. and S.P.Q.S writing—original draft preparation were performed by R.M.B., S.C.S, E.S.M, C.C.S. B.L.P., R.L.C., E.P.T. and S.P.Q.S All authors commented on previous versions, read and approved the manuscript.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of interest

The authors declare no conflict of interest

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Figures

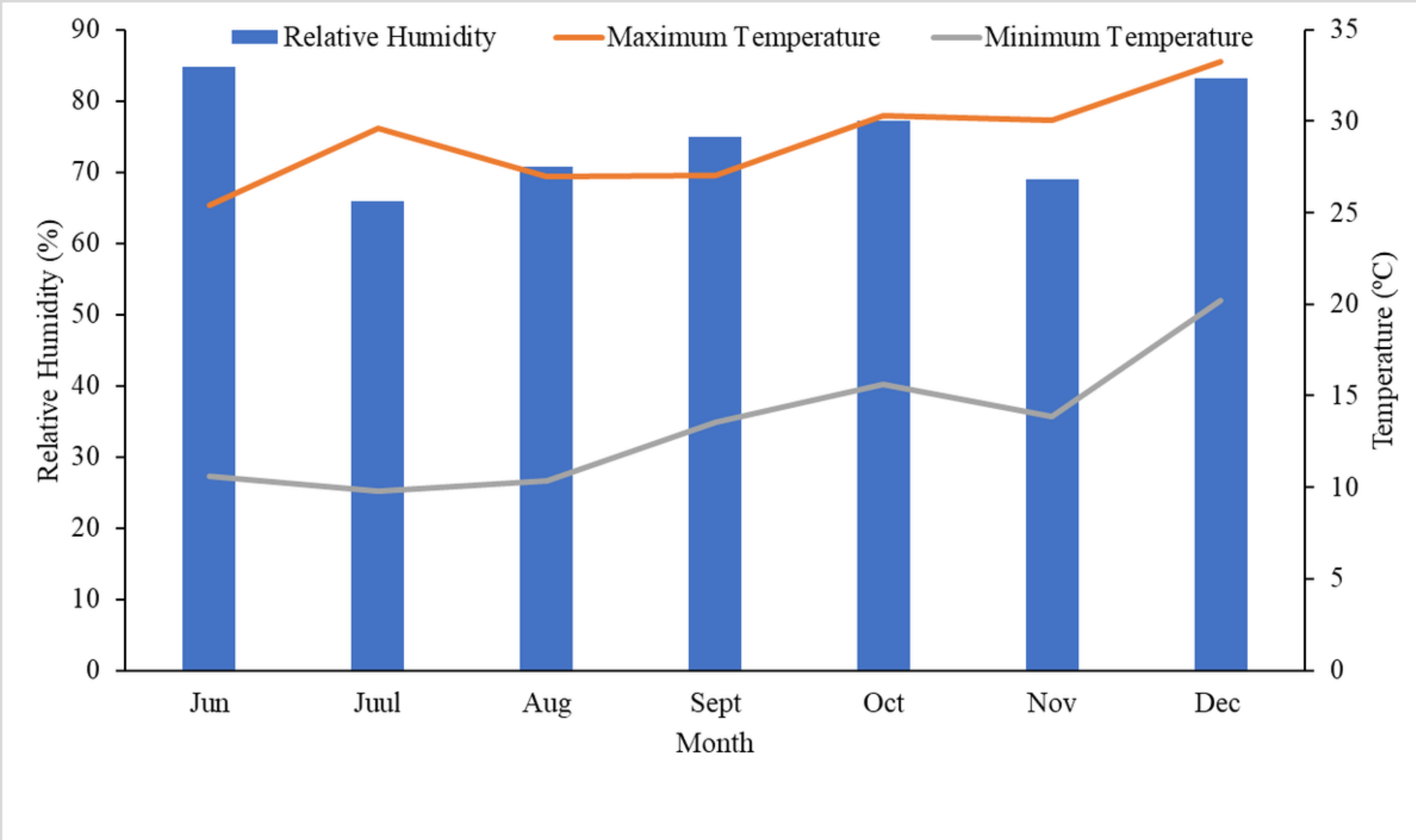

Figure 1

Relative humidity, Maximum temperature, and Minimum temperature for the months of June to December 2022

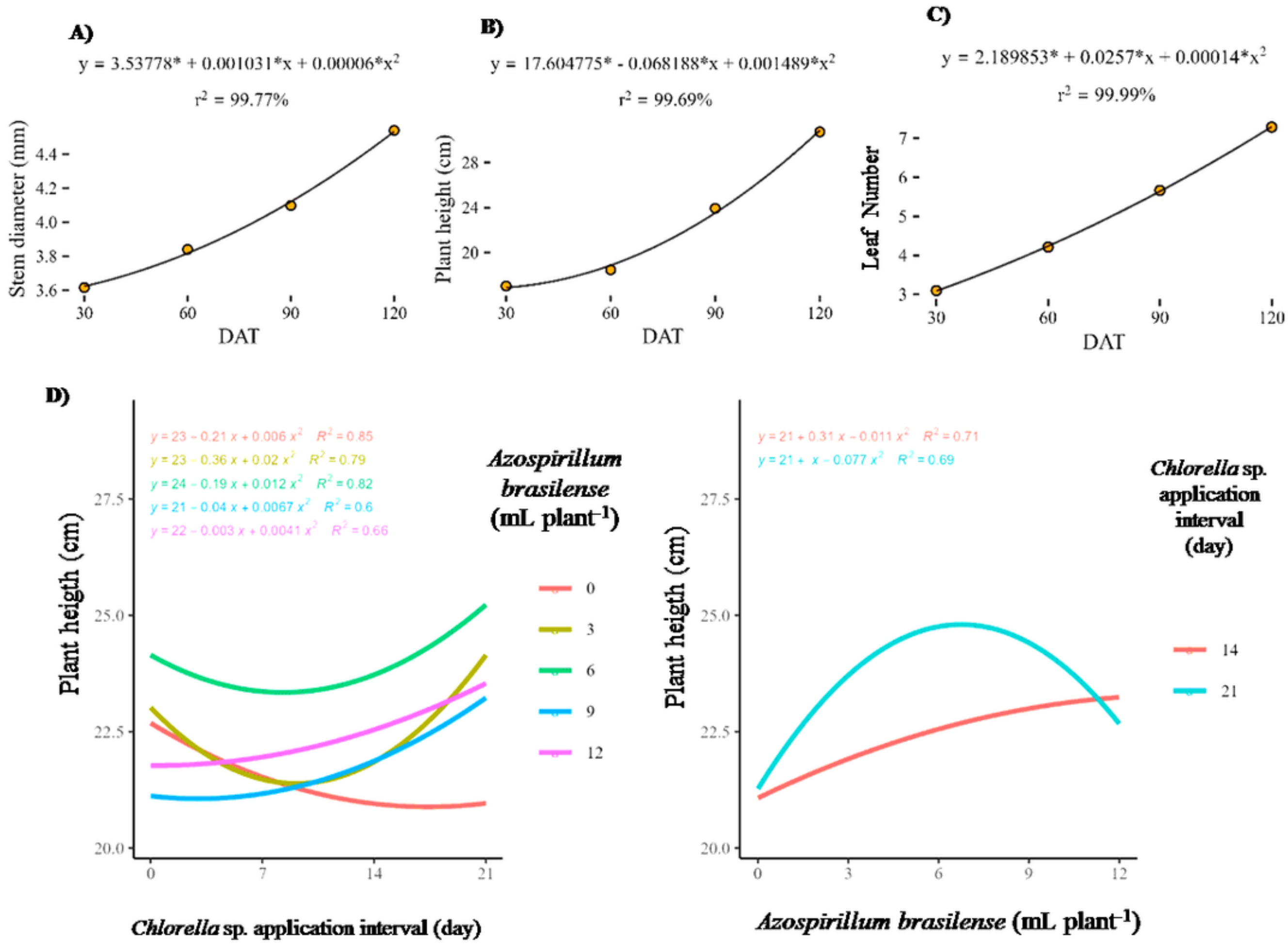

Figure 2

Diameter (A), Plant Height (B), Number of Leaves (C) assessed at different evaluation times. Height (D) of *Hymenaea courbaril* L. seedlings inoculated with increasing doses of *Azospirillum brasilense* at different intervals of *Chlorella* sp. application.

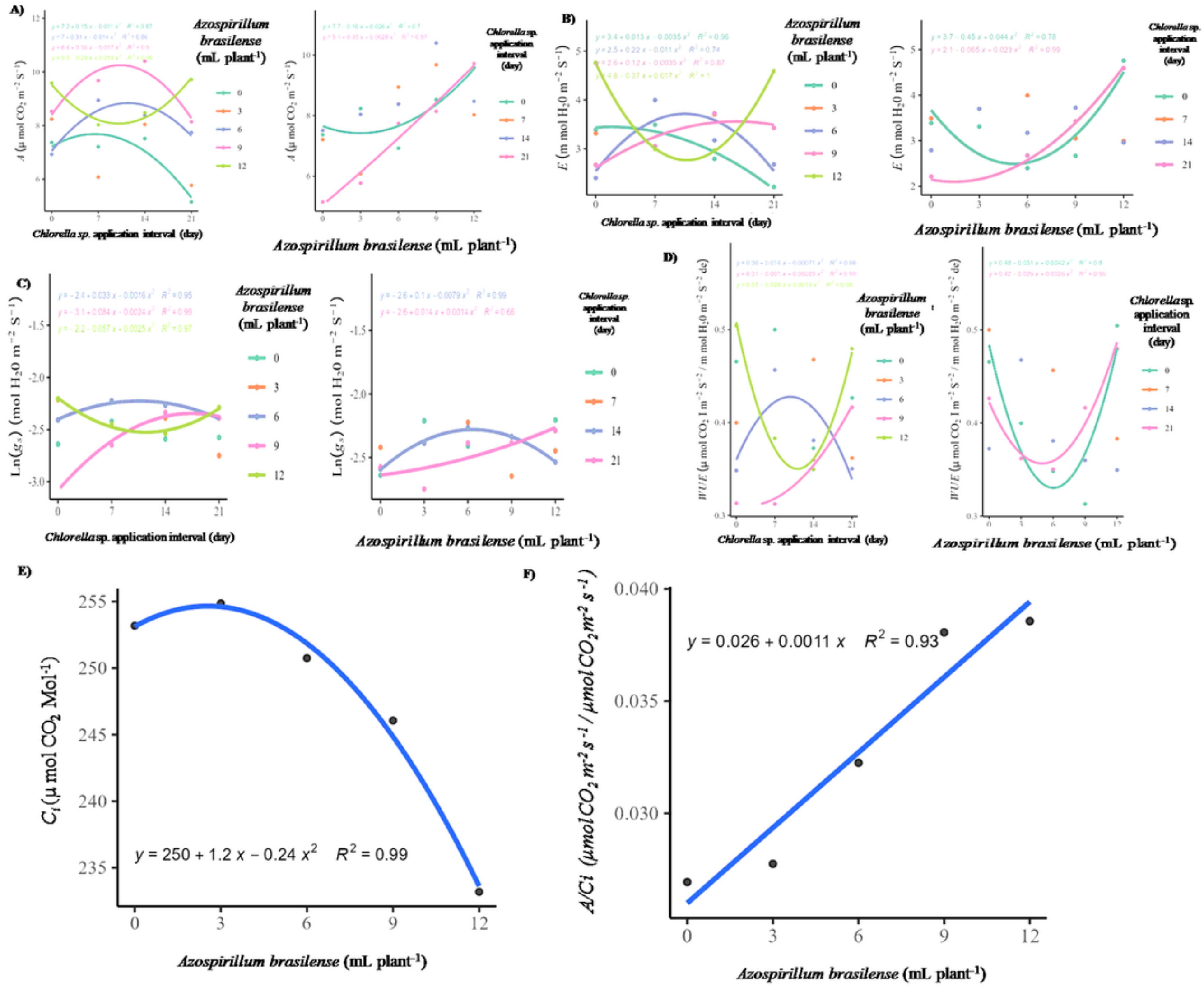

Figure 3

A (A), *E* Transpiration (B), *gs* (C), *WUE* (D), of *Hymenaea courbaril* L. seedlings inoculated with increasing doses of *Azospirillum brasilense* and different application intervals of *Chlorella* sp. *Ci*(E) and *A/Ci* (F), of Hymenaea courbaril L. seedlings inoculated with increasing doses of *Azospirillum brasilense.*

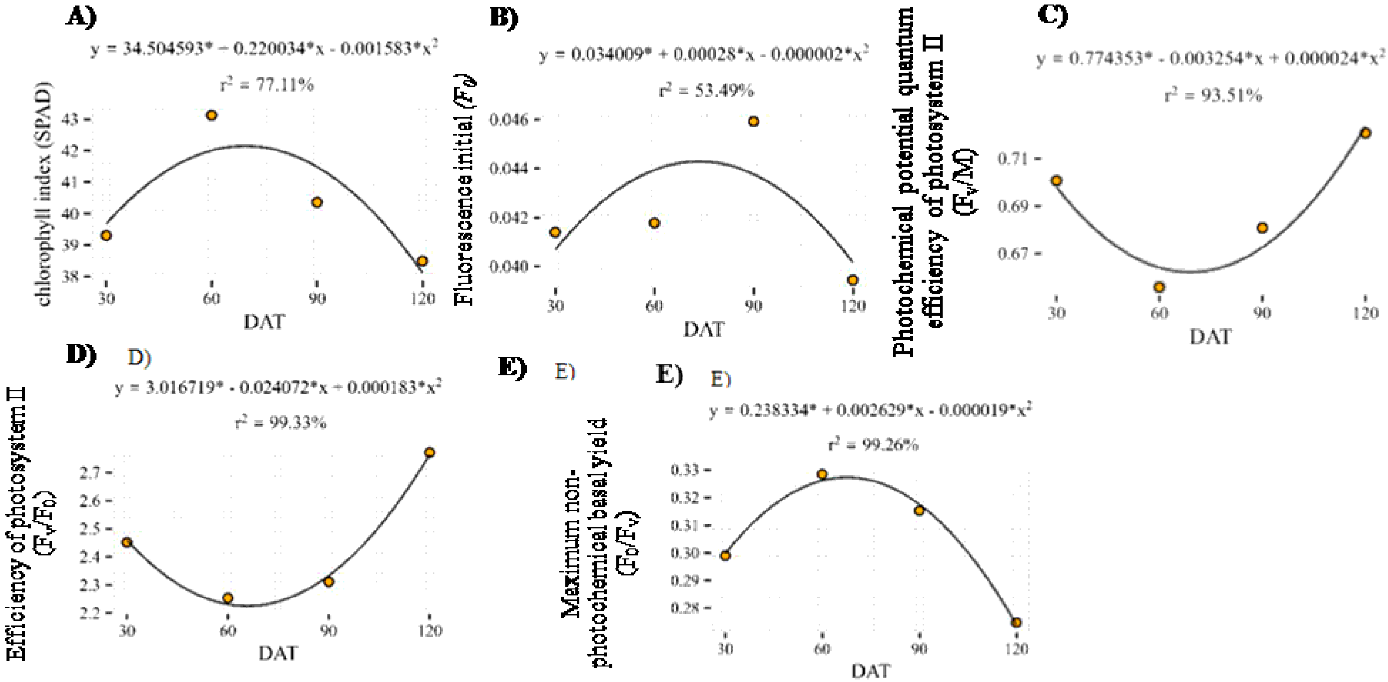

Figure 4

Chlorophyll index (A), F0 (B), Fv/F0 (C), FV/Fm (D) of *Hymenaea courbaril* L. seedlings assessed at different evaluation times.

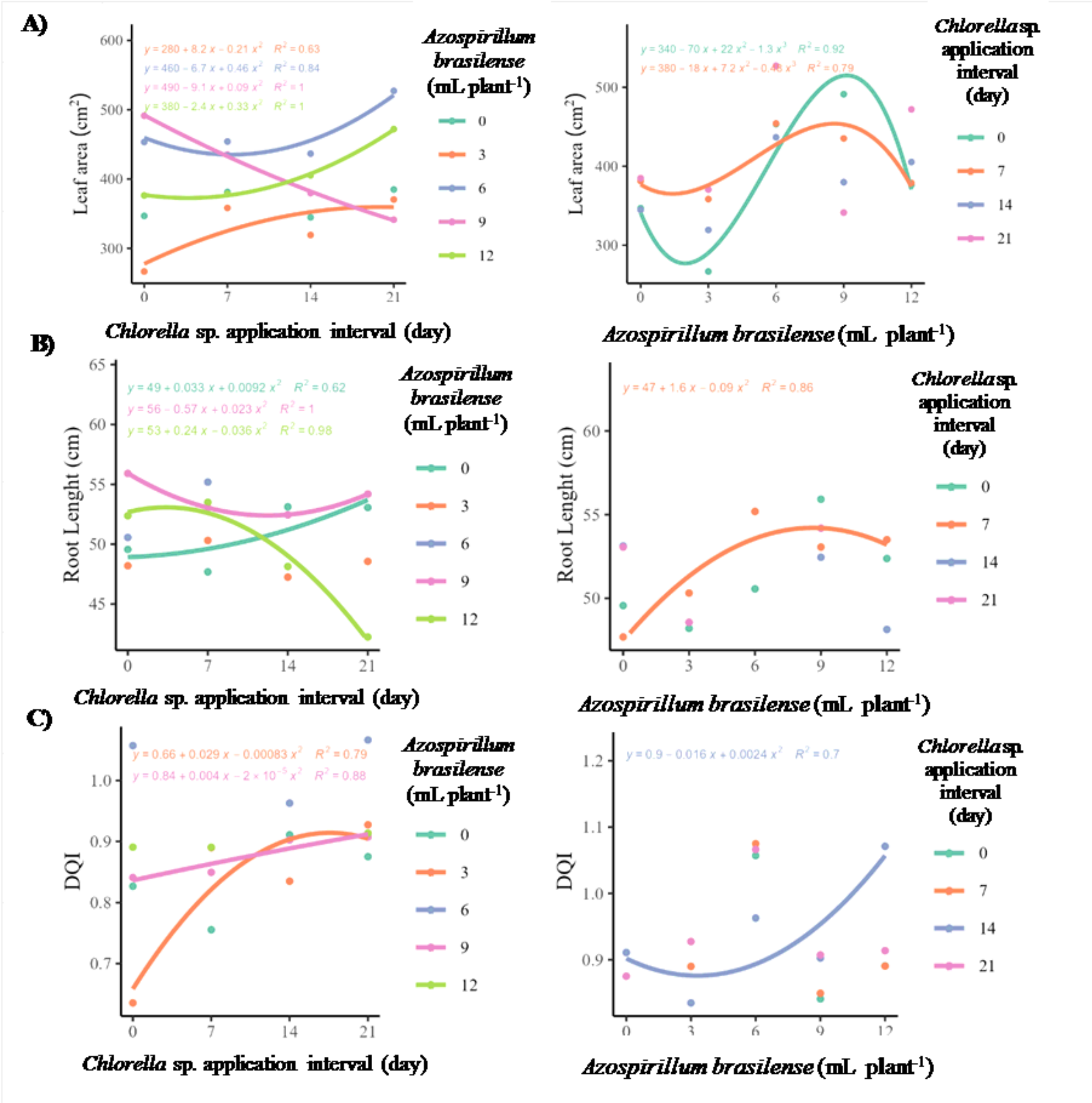

Figure 5

Leaf area (A), Root length (B), and DQI(C), of *Hymenaea courbaril L.* seedlings inoculated with increasing doses of *Azospirillum brasilense* and different application intervals of *Chlorella* sp.

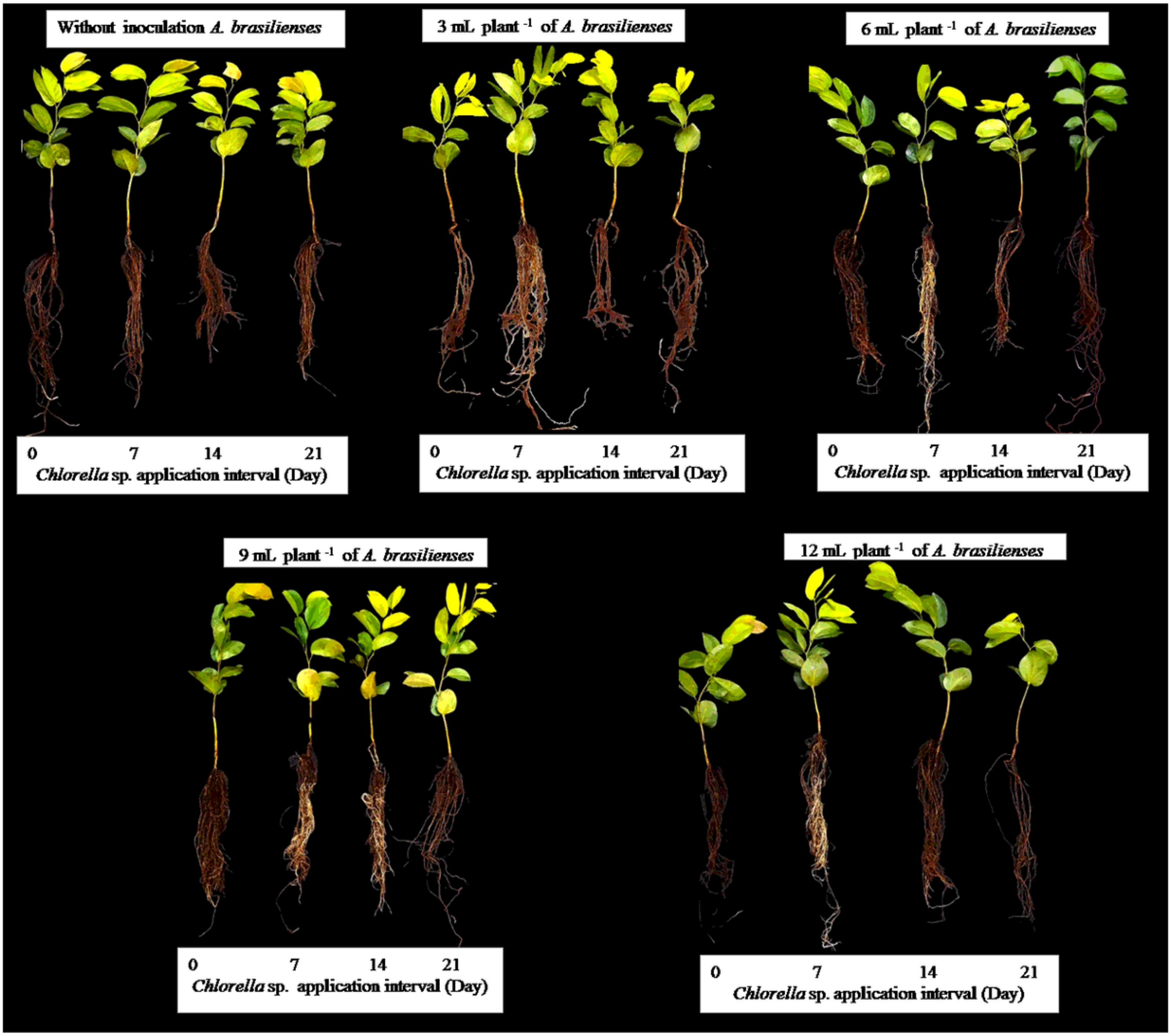

Figure 6

Visual assessment of *Hymenaea courbaril L.*seedlings inoculated with increasing doses of *Azospirillum brasilense* and different application intervals of *Chlorella* sp.