Leaf growth and biomass accumulation in radish (*Raphanus sativus* L.) inoculated with rhizosphere microorganisms

Crecimento foliar y acumulación de biomasa en rábano (*Raphanus sativus* L.) inoculado con microorganismos rizosféricos

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Abstract

Radish seeds (*Raphanus sativus* L. 'Var Champion') were inoculated with commercial strains of rhizosphere fungi and bacteria. It was designed a totally randomized experiment with four treatments: *Chromobacterium violaceum* + *Acinetobacter calcoaceticus* (T1), *Azospirillum brasilense* (T2), *Glomus intraradices* (T3), uninoculated control (T4) and 20 replicates with the aim of evaluating its effect on leaf growth, length and dry mass of the root, dry mass of leaves and tubers, as well as fresh mass of the tuber. The net assimilation rate (NAR), absolute growth rate (AGR) and relative growth rate (RGR) were calculated. At 33 after sowing (das) the plants inoculated with *Chromobacterium violaceum* + *Acinetobacter calcoaceticus* (T1) showed increases of 52.67% of foliar area in relation to the control; at 42 das the increase was 72.30%. At 33 das there were increases of 49.66 and 45.52% of dry leaf mass in the treatments with *Chromobacterium violaceum* + *Acinetobacter calcoaceticus* (T1) and *Azospirillum brasilense* (T2) with regard to the control. The fresh mass of the tubers was 65.03 and 63.11% higher than the control at 33 das in the same treatments and 80.70 and 74.56% at 42 das respectively. It is concluded that coinoculation with *Chromobacterium violaceum* + *Acinetobacter calcoaceticus* and with *Azospirillum brasilense* increases the growth of radish tubers as a result of a greater surface and foliar biomass and the increase of the Net Assimilation Rate.

Keywords
Biostimulants • Growth Rates • Net Assimilation Rate • *Raphanus sativus* L.

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Resumen

Se inocularon semillas de rábano (*Raphanus sativus* L. `Var Champion´) con cepas comerciales de hongos y bacterias de la rizosfera. Se diseñó un experimento totalmente aleatorizado con cuatro tratamientos: *Chromobacterium violaceum + Acinetobacter calcoaceticus* (T_1), *Azospirillum brasilense* (T_2), *Glomus intraradices* (T_3), un tratamiento control sin inocular (T_4) y 20 repeticiones con el objetivo de evaluar su efecto en el crecimiento foliar, la longitud y masa seca de la raíz, la masa seca de hojas y de tubérculos, así como masa fresca del tubérculo. Se calcularon la tasa de asimilación neta (TAN), tasa absoluta de crecimiento (TAC) y tasa relativa de crecimiento (TRC). A los 33 después de la siembra (dds) las plantas inoculadas con *Chromobacterium violaceum + Acinetobacter calcoaceticus* (T_1) mostraron incrementos del 52,67% del área foliar con relación al control; a los 42 dds el incremento fue de 72,30%. A los 33 dds se registraron aumentos del 49,66 y 45,52% de la masa seca foliar en los tratamientos con *Chromobacterium violaceum + Acinetobacter calcoaceticus* (T_1) y *Azospirillum brasilense* (T_2) con respecto al control. La masa fresca de los tubérculos fue 65,03 y 63,11 % superior al control a los 33 dds en los mismos tratamientos y de 80,70 y 74,56% a los 42 dds respectivamente. Se concluye que la coinoculación con *Chromobacterium violaceum + Acinetobacter calcoaceticus* y con *Azospirillum brasilense* incrementa el crecimiento de los tubérculos de rábano como consecuencia de una mayor superficie y biomasa foliar y el incremento de la Tasa de Asimilación Neta.

Palabras clave

Bioestimulantes • Tasas de Crecimiento • Tasa de Asimilación Neta • *Raphanus sativus* L.

Introduction

Radishes (*Raphanus sativus* L.) - originally from South Asia - have a thick, dense root of varied sizes and shapes, and are red, pink, purple, or white depending on the variety. They have basal leaves with petioles, and lobed leaves with one to three pairs of lateral segments with jagged edges (10). They grow well in humid climates and optimum temperature is 18° to 22 °C. Radishes have a short growth cycle - 20 to 70 days according to the variety, and adapt to any soil type, although deep neutral clay soils are ideal (10). Radishes have one of the highest vitamins and mineral contents of all vegetables. Their rapid growth makes them useful for researching plants` physiological responses to inoculation of growth promoting microorganisms (24).

The need to supply food to the planet`s billions of people has led to use of highly efficient genetic material in agriculture, pest and disease resistant varieties with short production cycles, and agrochemicals that provide nutrients to plants and protect them against biotic and abiotic factors (7). However, many of these agricultural strategies have negative environmental impacts. For example, increasing use of chemical fertilizers is contaminating the environment (13). The environmental effects of modern intensive agriculture are still not fully understood (7). In order to avoid negative impacts, many researchers, extension agents, and farmers have sought to develop holistic agroecological management strategies that reduce the need for chemical inputs (6, 19, 20, 22). One such strategy is use of rhizospheric microorganisms, which enhances plant growth, development, and yield (3).

Aguirre *et al*. (2015) found that the bacteria *Azospirillum brasilense* and the fungus *Rhizophagus intraradices* enhanced plant growth upon using either one alone to inoculate seeds of *Leucaena leucocephala* (Lam.) de Wit. However, using the fungus and bacteria together induced greater growth in plants than either alone, and more than the control. The difference between the effect of co-inoculation and inoculation of a single microorganism on dry biomass accumulation was greatest 15 days after planting.

The objective of this study was to identify the physiological effects of inoculating radishes with different rhizospheric microorganisms on growth and biomass accumulation, in order to advance our knowledge of effective methods of biological fertilization which may help reduce use of chemical fertilizers and increase nutritional properties of agricultural crops.

Materials and Methods

This study was carried out in the experimental area and the Sciences Laboratory of the Villa Corzo campus of the University of Sciences and Arts of Chiapas in the Mexican state of Chiapas, located at 16° 09´43.7´´ N latitude and 93° 16´37.8´´ W longitude, at an altitude of 583 m.a.s.l. The principal climate in the study area is A (w2) warm sub humid with abundant summer rain. Total annual precipitation ranges from 1200 mm to 3000 mm, which falls 100 to 200 days per year (14).

An inert solution was prepared consisting of 60 g of carboxyl adherent powder dissolved in 1.5 L of sterile distilled water and left to sit two hours. Before covering Champion variety radish seeds with a 95% germination rate and 99% purity level with this solution, they were disinfected four minutes with 2% sodium hypochlorite and washed with sterile distilled water. The seeds were then covered with the different treatments of fungus (*G. intraradices*) or rhizospheric bacteria (*C. violaceum+A. calcoaceticus,* or *A. brasilense*) and left to dry in Petri dishes at room temperature (30 ± 2°C).

The radish seeds were planted in August 20, 2015 in polyethylene bags with a volume of 950 cm^3 (17 x 7 x 8 cm) filled with sifted sterilized sandy loam soil from a plot cultivated with maize. Two seeds were planted per bag, one of which was later randomly weeded out. Bags were watered daily until germination, and then every three days. Plants were manually weeded and no chemical fertilizers or pesticides were applied.

We used a completely random experimental design with 20 repetitions and four treatments: T_1: BiogosfoBuap®: *Chromobacterium violaceum+Acinetobacter calcoaceticus* (1 x 10^8 CFU of the total consortium of microorganisms), T_2: AzoFer: *Azospirillum brasilense* (5 x 10^8 CFU), T_3: MicorrizaFer: *Glomus intraradices* (3x10^4 CFU), and T_4: control without inoculation. The experimental unit consisted of 20 bags per treatment with one plant per bag.

Biomass accumulation

Thirty three and 42 days after sowing (das), five plants were randomly selected per treatment. Leaves, tuber, and taproot of each plant were separated and washed with sterile distilled water, left to dry at room temperature, and placed in a forced air stove at 80°C to dehydrate 72 h. At this point they had a constant weight, determined with a digital Sartorius® scale with a precision level of 0.01 g. The tuber and taproot were each weighed before and after placing them in the stove to determine fresh and dry weight.

Leaves growth

For each plant, total number of leaves was counted, length and width of each leaf was measured (cm), and total leaf area was calculated (cm^2). A millimeter ruler was used for linear measurements, and leaf area determined using a digital Placom KP-80N Planimeter.

Growth indexes

Using the values for total accumulated weight and leaf area per plant 33 and 42 das, relative growth rate (RGR, $g\,g^{-1}d^{-1}$), absolute growth rate (AGR, $g{\cdot}day^{-1}$), and net assimilation rate (NAR, $g{\cdot}cm^{-2}day^{-1}$) were calculated, according to Hunt (1978).

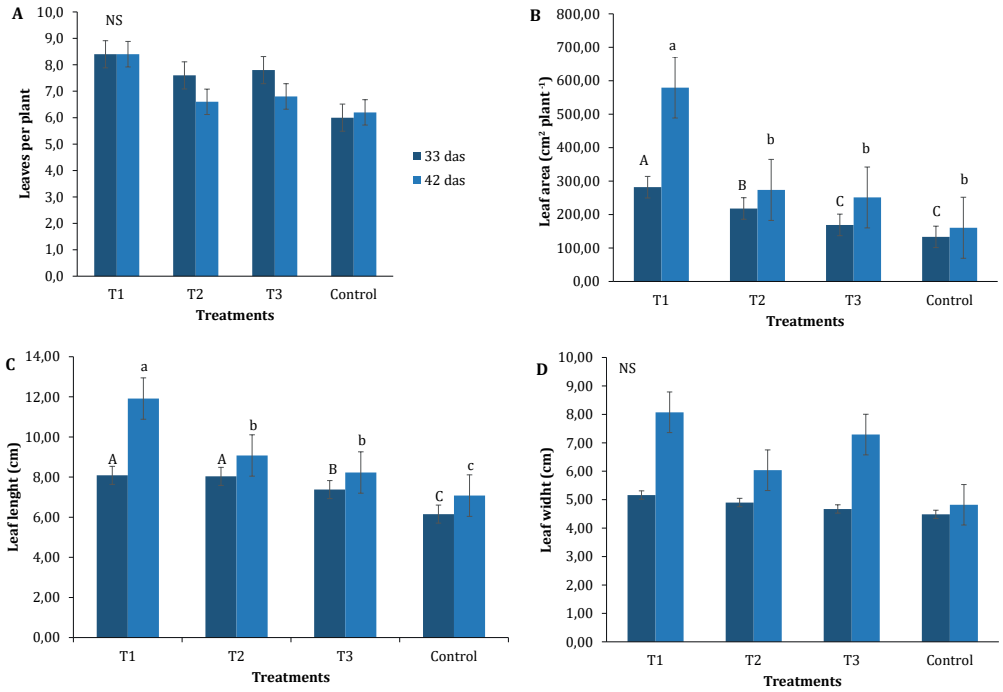
Statistical analysis

A one way ANOVA was carried out and mean values of the treatments were compared using Tukey´s test ($p \leq 0.05$). A simple linear regression analysis was carried out between dry weight of tubers as a dependent variable, and leaf area and dry weight of the aboveground part of the plant as independent variables. For these analyses, the STATISTICA® software was used.

Results and Discussion

Although a slightly greater number of leaves was observed in plants inoculated with *C. violaceum+A. calcoaceticus* (T_1) and *A. brasilense* (T_2) than in those inoculated with *G. intraradices* (T_3) and the control 33 and 42 das (figure 1A, page 81), differences were not

statistically significant among treatments 33 das ($F_{3;16}$=1.699; p=0.21) or 42 das ($F_{3;16}$=0.42; p=0.74). Leaf area was significantly greatest 33 das in plants inoculated with *C. violaceum+A. calcoaceticus* (T_1) ($F_{3;16}$=14.50; p≤ 0.01), followed by *A. brasilense* (T2) and *G. intraradices* (T_3), with no differences between the latter two treatments; T_1 surpassed the control by 52.67, and T_2 and T_3 by 38.82% (figure 1 B).



Vertical lines represent standard error of the mean. N = 5. T_1: *Chromobacterium violaceum* + *Acinetobacter calcoaceticus*, T_2: *Azospirillum brasilense*, T_3: *Glomus intraradices*.

Las líneas verticales representan el error estándar de la media. N = 5. T_1: *Chromobacterium violaceum* + *Acinetobacter calcoaceticus*, T_2: *Azospirillum brasilense*, T_3: *Glomus intraradices*.

Figure 1. Number of leaves (A), leaf area (B), leaf length (C), and leaf width (D) 33 and 42 days after sowing (das) in radish plants inoculated with different rhizospheric microorganisms.

Figura 1. Número de hojas (A), Área foliar (B), Longitud de hoja (C), y Ancho de hoja (D) en plantas de rábano a los 33 y 42 días después de sembradas (dds) con microorganismos rizosféricos.

At 42 das, leaf area of T1 and T2 was also statistically greater ($F_{3;16}$=16.23; p≤0.01) than that of the control - 72.30 and 41.36%, respectively, while leaf area of T_3 was 36.10% greater than the control. These results suggest that the microorganisms had more influence on growth in leaf area than on number of leaves (figure 1B), which was corroborated upon statistically comparing the effect of the treatments on linear dimensions (length and width) of leaves (figure 1C). Leaf length 33 das ($F_{3;16}$=4.797; p≤0.01) as well as 42 das ($F_{3;16}$=13.136; p≤0.01) was greatest in T_1. Nevertheless, leaf width 33 das ($F_{3;16}$=1.697; p=0.20) and 42 das ($F_{3;16}$=1.70; p=0.20) did not statistically differ among treatments.

These results would suggest that the greater growth observed in plants inoculated with *C. violaceum+A. calcoaceticus* and *A. brasilense* it could be due to greater hormone production, in concordance with results published by Nuncio *et al.* (2015), who found an association between production of indoleacetic acid (IAA) and nitrogenase activity of rhizobacteria on seed germination.

Dry leaf weight 33 das was significantly greater (p<0.01) in T_1 (figure 2A, page 82) than in the other treatments and 42 das significantly (p<0.01) higher leaf dry weight was observed for T1 T2 treatments compared to the rest of the treatments. Thirty three das, dry leaf weight was 49.66 %, 45.52%, and 19.43% greater in T_1, T_2, and T_3, respectively, than the control, while 42 das, these differences were 68.14%, 41.36 %, and 36.10% (T_4) (figure 2A, page 82). Similar results were published by Aguirre, *et al.* (2011) and Ibarra *et al.* (2014) for *Coffea arabica* L., and by Chattopadhyay *et al.* (2006) for *Coffea canephora* (Pierre) ex Froehner; these authors reported increases in leaf biomass in plants inoculated with *Azospirillum*.

Vertical lines represent standard error of the mean. N=5. T_1: *Chromobacterium violaceum + Acinetobacter calcoaceticus*, T_2: *Azospirillum brasilense*, T_3: *Glomus intraradices*, das: days after sowing.

Las líneas verticales representan el error estándar de la media. N=5. T_1: *Chromobacterium violaceum + Acinetobacter calcoaceticus*, T_2: *Azospirillum brasilense*, T_3: *Glomus intraradices*, dds: días después de sembrado.

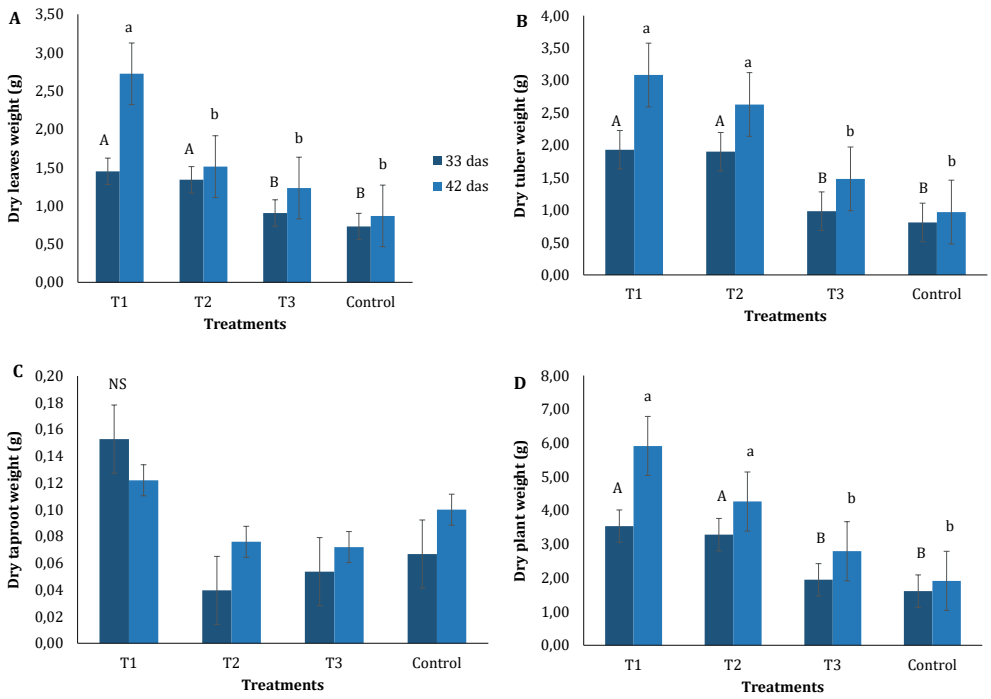


Figure 2. Accumulation of dry weight in leaves (A), tubers (B), taproots (C), and entire plant (D) of radishes inoculated with different rhizospheric microorganisms.

Figura 2. Acumulación de peso seco en Hojas (A), Tubérculos (B), Raíces primarias (C), y toda la planta (D) de plantas de rábanos inoculados con diferentes microorganismos risozféricos.

The greater influence of *C. violaceum+A. calcoaceticus* than of other microorganisms on plant growth is likely due to enhanced effects of co-inoculation as compared to inoculation with a single organism. Co-inoculation has been demonstrated to result in synergies between soil fungi and bacteria, for example between C*. violaceum* and *A. calcoaceticus* (12, 18, 25). For soybean, a better response has been observed with the symbiosis between *Bradyrhizobium sp*. and arbuscular mycorrhizal fungi (AMF) than by either one alone (4, 5). The physiological effect of co-inoculating with rhizospheric bacteria and fungi is definitely a complex process (23) that depends on many factors, including the plant species and physical and chemical soil properties. However, the present study evaluates only co-inoculation with two rhizobacteria.

Dry weight of tubers (figure 2B) was also statistically greater in T_1 and T_2 than in the control: 33 das ($F_{3;16}$ =9.77; p≤0.01) they were 58.07% and 57.41% greater, respectively, and 42 das ($F_{3;16}$=13.42; p≤0.01) they were 68.57% and 63.12% greater. While differences in dry weight between T_1 and T_2 were not statistically significant, dry weight of tubers was statistically greater in T_1 and T_2 than in T_3 and the control.

Dry weight of the taproot (figure 2C) was not statistically different among treatments 33 das or 42 das, although a tendency toward greater root biomass accumulation was observed in T_1 33 days. These results indicate that inoculating plants with *C. violaceum + A. calcoaceticus* tends to favor accumulation of biomass in the tuber at the cost of growth of the taproot.

Total plant dry weight, in both 33 das ($F_{3;16}$ =13.04; p≤0.01) and 42 das ($F_{3;16}$ =20.57; p≤0.01) was significantly higher in T1 and T2 treatments than in T3 and control treatments (Figure 2D). However, taproots length (figure 3A, page 83) of plants inoculated with microorganisms are not longer than those of the control 33 days ($F_{3;16}$=0.77; p=0.52) or 42 days ($F_{3;16}$=0.73; p=0.54). This could show that inoculation with the microorganisms used in this study induces transfer of photosynthates from the above ground part of the plant to the tuber to produce biomass. Aguirre *et al*. (2005) found similar results in beans inoculated with *Glomus macrocarpum.* The effect of microorganisms on plant root growth has been documented by other studies, presumably due to increased production of phytohormones (15). Nuncio *et al*. (2015) found that root and stem growth were favored by *Azospirillum sp.*, although no differences in plant growth were found upon varying the concentration of this inoculate.

Tuber fresh weight 33 days ($F_{3,16}$=15.690, p≤0.01) as well as 42 das ($F_{3,16}$=24.05, p≤0.01) was significantly greater in T_1 and T_2 than in T_3 or the control; 33 das, T_1 and T_2 were 65.03% and 63.11% heavier, respectively, than the control, while 42 das, these differences were 80.70% and 74.96% (figure 3B).

Vertical lines represent the standard error of the mean. N=5. T_1: *Chromobacterium violaceum + Acinetobacter calcoaceticus*, T_2: *Azospirillum brasilense*, T_3: *Glomus intraradices*, das: days after sowing.

Las líneas verticales representan el error estándar de la media. N=5. T_1: *Chromobacterium violaceum + Acinetobacter calcoaceticus*, T_2: *Azospirillum brasilense*, T_3: *Glomus intraradices*, dds: días después de sembrado.

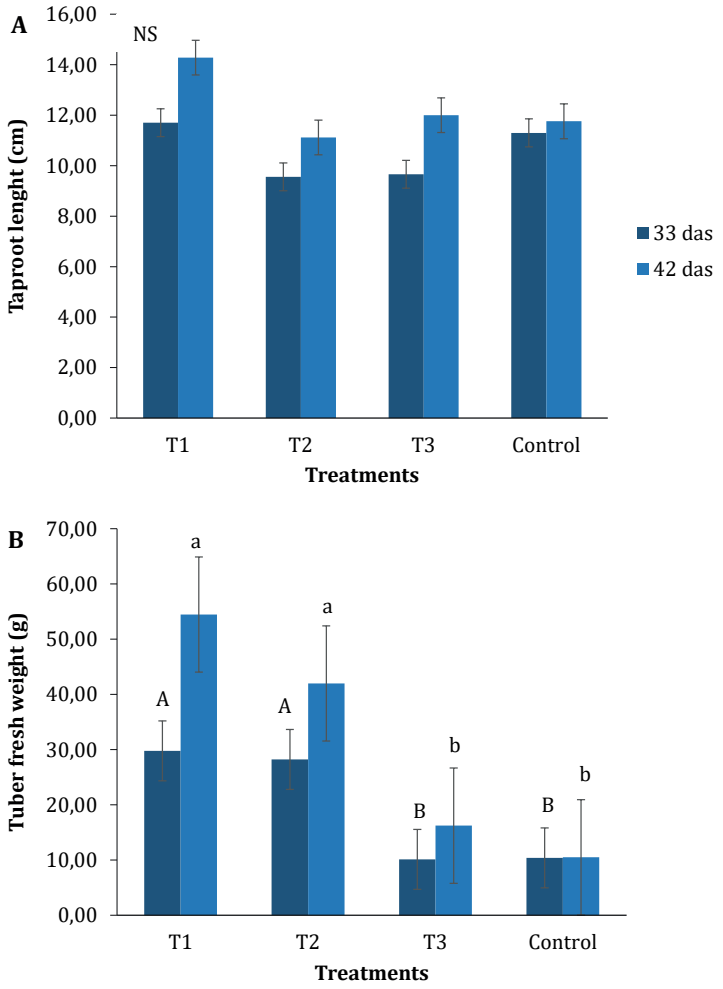


Figure 3. Length of taproot (A) and fresh weight of tubers (B) of radish plants inoculated with different rhizospheric microorganisms.

Figura 3. Longitud de la raíz principal (A) y peso fresco de los tubérculos (B) de plantas de rábano inoculadas con diferentes microorganismos rizosféricos.

Adjustments to linear equations used to identify possible correlations between dry tuber weight and leaf area per plant 33 and 42 das (figure 4, page 84), as well as between dry tuber weight and leaf weight per plant 33 and 42 das (figure 5, page 84), showed that plants with greater leaf area and greater dry leaf weight produced tubers with more biomass.

Linear regression analysis between dry tuber weight and above dry weight showed determination coefficients ranging from 21% to 64%. While the regression was not significant for T_3 (figure 4, page 84), tuber biomass clearly increased as a function of leaf area for T_1 and T_2. This demonstrates the importance of achieving an optimum leaf area in crops in order to guarantee assimilation and transfer of assimilates to produce large tubers.

Adjustments to the regression equations between dry weight of tubers and dry weight of leaves resulted in determination coefficients ranging from 39% to 67% (figure 5, page 84), validating the finding that tuber weight increases as a function of leaf area, as well as the hypothesis that size of the source influences growth of tuber, which is the main nutrients source for plants.

A = T_1: *Chromobacterium violaceum + Acinetobacter calcoaceticus*, B = T_2: *Azospirillum brasilense*, C = T_3: *Glomus intraradices*, D = T_4: control.

A = T_1: *Chromobacterium violaceum + Acinetobacter calcoaceticus*, B = T_2: *Azospirillum brasilense*, C = T_3: *Glomus intraradices*, D = T_4: control.

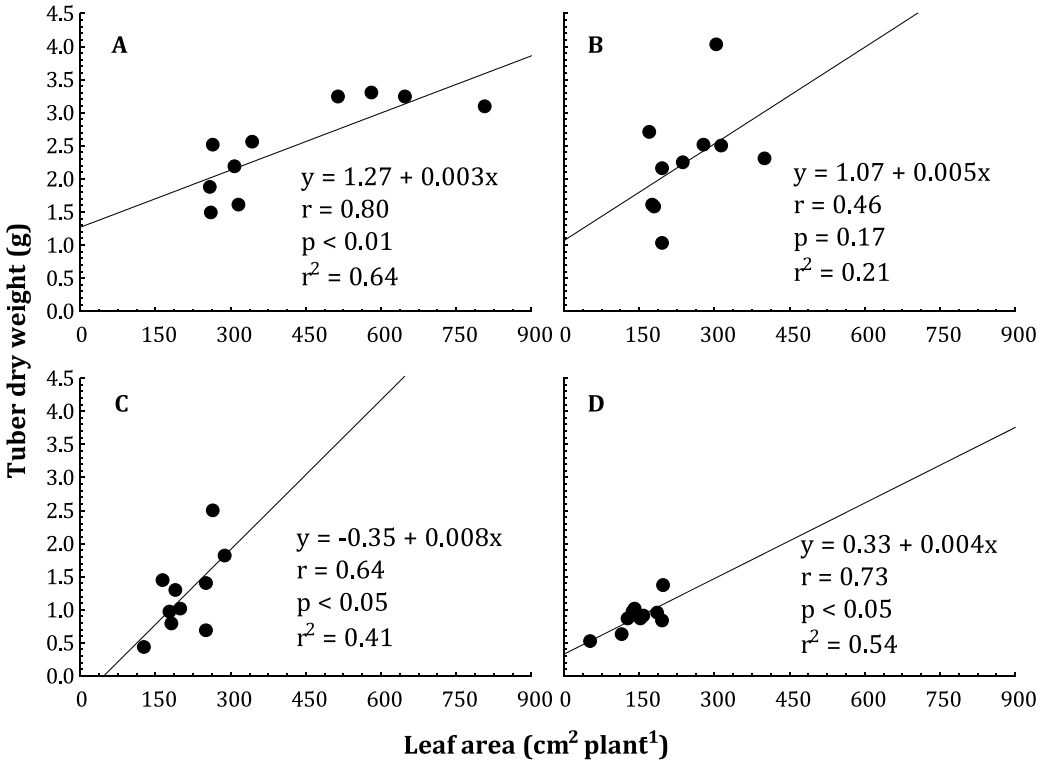


Figure 4. Linear regression fittings between dry weight of tubers and leaf area for different treatments 42 days after planting.

Figura 4. Regresión lineal entre el peso seco de los tubérculos y el área foliar de diferentes tratamientos a los 42 días después de la siembra.

A = T_1: *Chromobacterium violaceum + Acinetobacter calcoaceticus*, B = T_2: *Azospirillum brasilense*, C = T_3: *Glomus intraradices*, D = T_4: control.

A = T_1: *Chromobacterium violaceum + Acinetobacter calcoaceticus*, B = T_2: *Azospirillum brasilense*, C = T_3: *Glomus intraradices*, D = T_4: control.

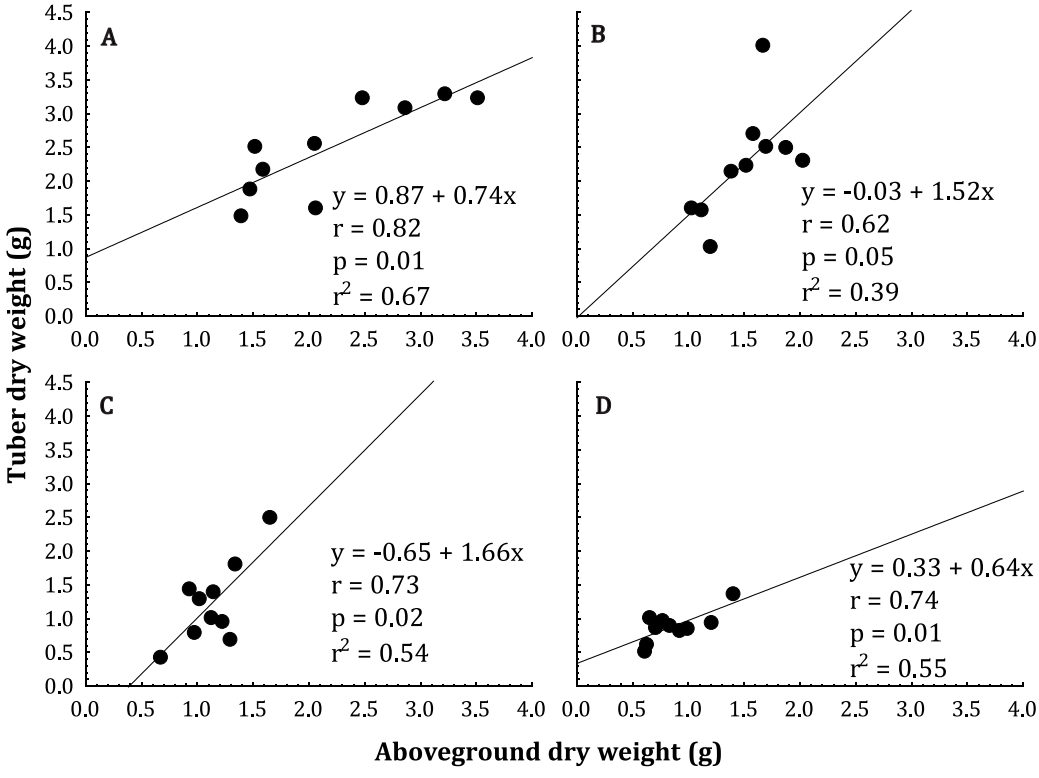


Figure 5. Linear regression fittings between dry weight of tubers and dry weightabove ground in different treatments 42 days after planting.

Figura 5. Regresión lineal entre el peso seco de los tubérculos y el peso seco sobre el suelo en diferentes tratamientos a los 42 días después de la siembra.

This hypothesis was further validated upon confirming the significant effect of inoculation with *C. violaceum+A. calcoaceticus* on net assimilation rate, relative growth rate (RGR), and absolute growth rate (AGR) (figure 6). T_1 showed significant differences in NAR from the other treatments ($p \leq 0.05$), doubling the quantity of biomass accumulated per unit of leaf area per day as compared to T_2, and quadrupling that of the control. As Díaz *et al.* (2015) affirm in a study with soy plants inoculated with different microorganisms, this could be partially due to the fact that co-inoculated plants have greater N, P, and K content in the leaves than do plants inoculated with a single organism (5, 9).

Vertical lines represent standard error of the mean. N=5. T_1: *Chromobacterium violaceum + Acinetobacter calcoaceticus*, T_2: *Azospirillum brasilense*, T_3: *Glomus intraradices*.

Las líneas verticales representan el error estándar de la media. N=5. T_1: *Chromobacterium violaceum + Acinetobacter calcoaceticus*, T_2: *Azospirillum brasilense*, T_3: *Glomus intraradices*.

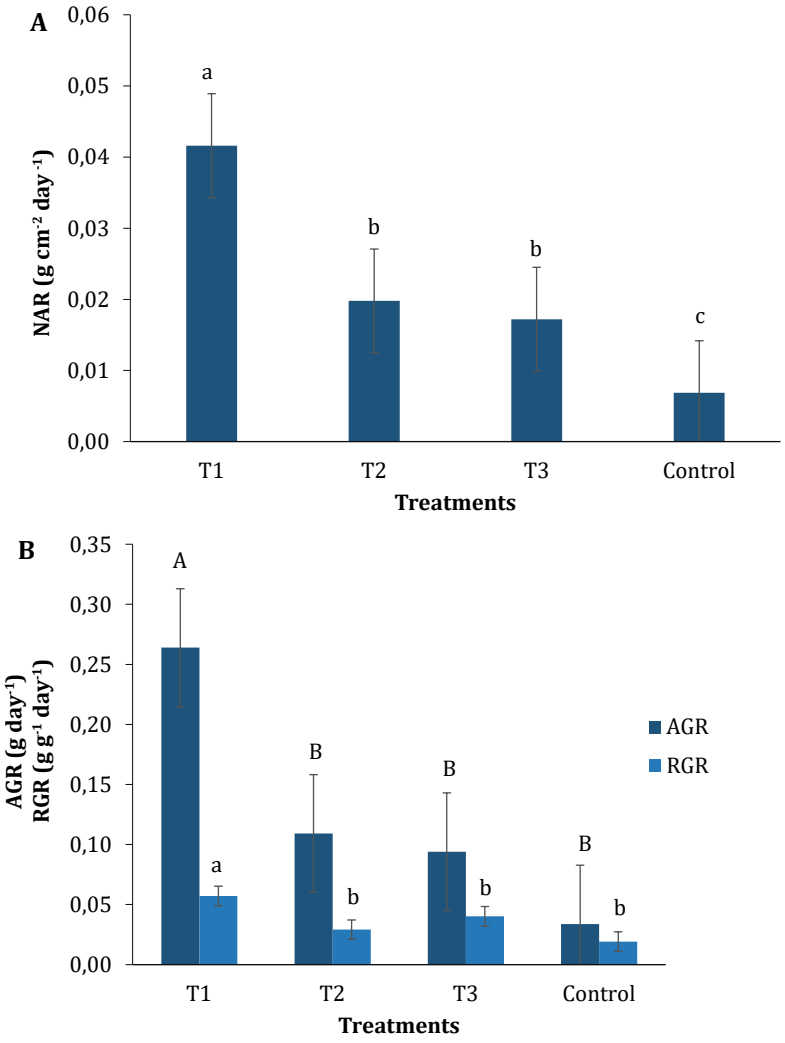


Figure 6. Net assimilation rate (g cm^{-2} day^{-1}) (A), absolute growth rate (AGR; $g^{-1}day^{-1}$) and relative growth rate (RGR; $gg^{-1}{\cdot}day^{-1}$) (B) in radish plants inoculated with different rhizospheric microorganisms.

Figura 6. Tasa de asimilación neta (g cm^{-2} $día^{-1}$) (A), tasa absoluta de crecimiento (TAC; g^{-1} $día^{-1}$) y tasa relativa de crecimiento (TRC; $g.g^{-1}$ $día^{-1}$) (B) en plantas de rábano inoculadas con diferentes microorganismos rizosféricos.

Conclusions

Co-inoculation with *C. violaceum+A. calcoaceticus,* as well as with *A. brasilense* alone, increased leaf area of radish plants due to an increase in leaf length, though not in width or number of leaves per plant.

Inoculation with these microorganisms favored accumulation of biomass in leaves and tubers, as well as total plant biomass, although not biomass or taproot length.

Fresh and dry weight of tubers were greater in treatments with *C. violaceum+A. calcoaceticus* and *A. brasilense* than in the control as a result of greater leaf area, greater dry leaf weight, and greater photosynthetic activity due to increases in net assimilation rate.

Radish plants inoculated with *C. violaceum+A. calcoaceticus* and with *A. brasilense* grew faster and had a higher relative growth rate and absolute growth rate. It was clearly demonstrated that radish growth is greater when the plants are simultaneously inoculated with more than one rhizospheric microorganism.

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