**Research Article**

# Characterization of indole-3-acetic acid content in inoculant fractions and its effect on plant growth[1]

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## ABSTRACT

Indole-3-acetic acid (IAA) is a phytohormone produced by many rhizobacterial species to mediate plant colonization while promoting plant growth. The colorimetric assay is widely used to analyze the bacterial IAA biosynthesis because it is a straightforward, cost-effective and fast technique. However, the characterization of parts or fractions of inoculant formulations is rarely performed. This study aimed to determine the fraction of the inoculant that is effective in promoting the plant growth, whether the bacteria or media growth can induce the phytohormones to expand the radicular zone of the plant, and whether the seed bacterization or foliar application can alter the plant growth. The inoculum samples (homemade and commercial) were obtained in a Luria-Bertani growth medium with and without tryptophan-L and the inoculants were divided into two fractions: pellet and supernatant. For the IAA quantification, the pellet fraction was subjected to physical (sonication) and chemical (lysozyme) treatments alone and combined. The IAA levels were evaluated using the colorimetric assay, and the effect on plant growth was determined by the inoculation of maize seedlings. The homemade and commercial formulations showed distinct patterns, in terms of IAA synthesis. The supernatant fraction provided higher amounts of IAA and was effective in improving the root area. Lysozyme was superior to sonication in mediating the release of IAA from bacterial cells. No significant differences were observed between seed or foliar inoculation.

KEYWORDS: *Azospirillum brasilense*, seed and foliar inoculation, phytohormone.

## RESUMO

Caracterização do teor de ácido indole-3-acético em frações de inoculante e seu efeito no crescimento vegetal

O ácido indol-3-acético (AIA) é um fitohormônio produzido por muitas espécies de rizobactérias para mediar a colonização de plantas enquanto promove o seu crescimento. O ensaio colorimétrico é amplamente utilizado para analisar a biossíntese de AIA bacteriano por ser uma técnica simples, econômica e rápida. No entanto, a caracterização de partes ou frações de formulações de inoculantes raramente é realizada. Objetivou-se determinar a fração do inoculante que é eficaz em promover o crescimento da planta, se o crescimento de bactérias ou meios pode induzir os fitohormônioss a expandirem a zona radicular da planta e se a bacterização de sementes ou a aplicação foliar pode alterar o crescimento das plantas. As amostras de inóculo (caseiro e comercial) foram obtidas em meio de crescimento Luria-Bertani com e sem triptofano-L e os inoculantes divididos em duas frações: pellet e sobrenadante. Para a quantificação do AIA, a fração pellet foi submetida a tratamento físico (sonicação) e químico (lisozima) isoladamente e combinados. Os níveis de AIA foram avaliados por ensaio colorimétrico e o efeito sobre o crescimento das plantas determinado pela inoculação de plântulas de milho. As formulações caseira e comercial apresentaram padrões distintos, em termos de síntese de AIA. A fração sobrenadante forneceu maiores quantidades de AIA e foi eficaz em melhorar a área radicular. A lisozima foi superior à sonicação na otimização da liberação de AIA de células bacterianas. Não foram observadas diferenças significativas entre inoculação por sementes ou foliar.

KEYWORDS: *Azospirillum brasilense*, inoculação foliar e seminífera, fitohormônio.

## INTRODUCTION

Globally, food production is reliant on the extensive use of chemicals, such as fertilizers, which are non-renewable, expensive, petroleum-based, polluting products that require high energy inputs. Owing to the detrimental effects of these products, farmers have been advocating for economic alternatives associated with low levels of pollution (Ajmal et al. 2018). Among these alternatives, inoculant biofertilizers are eco-sustainable and inexpensive biologically active products. However, they require specific formulation before their use (Bardi & Malusà 2012, Owen et al. 2015, Vassilev et al. 2020).

[1] Received: June 07, 2022. Accepted: Sep. 16, 2022. Published: Oct. 18, 2022. DOI: 10.1590/1983-40632022v5273044.

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In Brazil, 63 % of farmers apply seed treatment on their farms using a concrete mixer or other machines suitable for this purpose: 20 % use furrow inoculation, 15 % inoculate seeds in the sowing box, and 2 % use industrial seed treatment (ANPII 2020). Although the simultaneous treatment of seeds with fungicides and insecticides is potentially harmful and may impair the survival and metabolism of the inoculated cells, it is considered the most direct method, requiring smaller amounts of inoculant (Cassán et al. 2015, O'Callaghan 2016). To prevent the direct contact of bacteria with chemical treatments, foliar, in-furrow or foliar spray inoculation has been recommended for many crops, and the efficacy of these methods depends on the dosage, mode of action and timely administration (Vieira Neto et al. 2008, Singh et al. 2016, Preininger et al. 2018). However, the determination of the most effective mode of application has been hindered by inconsistent yield results (Müller et al. 2021).

*Azospirillum* sp. is a free-living, motile, variable, aerobic, gram-negative, plant growth-promoting proteobacteria that colonizes the rhizosphere of several plant species. It is used widely in inoculant formulations, primarily for cereals, and mediates plant growth via a beneficial association (Santos et al. 2019, Souza et al. 2017).

Indole-3-acetic-acid (IAA) is the main compound of plant growth-promoting bacteria that stimulates and facilitates plant growth by increasing the root surface area (Goswami et al. 2016). IAA secreted by rhizosphere bacteria along with the endogenous plant supply can impact the root growth either positively or negatively (Duca et al. 2014).

The biosynthesis of IAA occurs by the transformation of chorismite into tryptophan (TRP) (Spaepen et al. 2007), and the addition of TRP to growth media can favor the IAA synthesis. Five different pathways have been described for IAA production in bacteria: indole-3-acetamide (IAM), indole-3-pyruvic acid (IPyA), indole-3-acetonitrile (IAN), tryptamine (TAM) and tryptophan side-chain oxidase (TSO) pathways (Kochar et al. 2013, Li et al. 2018). In *Azospirillum* species, IAA can be synthesized via IPyA, IAM and IAN pathways along with a TRP-independent pathway involving TRP as a precursor during all stages of culture growth (Prinsen 1993, Pedraza et al. 2020). The IPyA pathway is the most significant synthesis pathway for this species (Costacurta & Vanderleyden 1995, Kunkel & Harper 2018).

Typically, bacteria-synthesized IAA is evaluated in the supernatant fraction of the bacterial growth media using the Salkowski's reagent via a colorimetric assay (Glickmann & Dessaux 1995). This methodology has been widely adopted because it is straightforward, fast and cost-effective, and allows for the analysis of a large number of bacterial species in one day. However, the characterization of different parts of the inoculum growth media to determine the fraction that is most relevant to inoculant formulations is rarely performed.

In this study, we address questions related to inoculant composition, such as the effectiveness of bacteria or growth media fraction of the inoculant in promoting the growth and production of higher levels of phytohormones, and also investigate whether seed bacterization or foliar delivery mediates differences in plant growth under *in vitro* conditions.

## MATERIAL AND METHODS

The experiments were performed at the Universidade Federal do Paraná (UFPR), in Palotina, Paraná state, Brazil, from April 2020 to November 2020. Corn (*Zea mays*) DKB225 and *Azospirillum brasilense* AbV-5 and AbV-6 were used in the experiments.

The "homemade" laboratory-prepared inoculant was obtained via the growth of *Azospirillum brasilense* (AbV-5 and AbV-6) provided by the UFPR (Curitiba, Paraná state) under *in vitro* conditions. The bacteria were maintained in a Petri dish with a specific solid NFB malate growth medium, incubated at 28 ºC for stocking, and replicated at two-week intervals. The pre-inoculum was prepared with *Azospirillum brasilense* (AbV-5 and AbV-6) in 1:1 proportion, in a 50 mL falcon tube containing 15 mL of Luria-Bertani (LB) growth medium and a colony of the bacteria that was derived from the solid medium plates. The falcon was maintained in a shaker incubator at 28 ºC and 120 rpm for 24 h. For inoculum preparation, 1 mL of the pre-inoculum was added to 50 mL of LB in a 250-mL Erlenmeyer flask.

The "commercial" Nitro1000™ inoculant formulation used in this experiment comprised *Azospirillum brasilense* AbV5/AbV6 strains, vitamins, minerals and water, as well as a carbon source, thickener, preservative and polyvinylpyrrolidone (PVP) stabilizer, in an aqueous solution containing $2 \times 10^8$ colony forming units (CFU) $mL^{-1}$, as indicated

in the packaging label. For assays, pre-inoculum was prepared by adding 1 mL of the industrial inoculant to 50 mL of LB medium (10 g of NaCl, 10 g of peptone and 5 g of yeast extract).

The homemade and commercial inoculant samples were derived from the pre-inoculum medium. The optical density (OD) was calculated and the CFU adjusted to $10^6$ $mL^{-1}$ for both formulations. TRP was added to the LB medium at concentrations of 1.25 and 2.5 mM, and the bacteria were grown for 24 h with agitation (120 rpm).

An aliquot of 2 mL was obtained. After centrifugation, two fractions were considered: 1) pellet (the decanted part of the sample after centrifugation); 2) supernatant (the liquid fraction obtained after centrifugation), which was used as control. For the IAA quantification, the pellet fraction was resuspended in 1 mL of saline solution (0.9 %) and subjected to physical and chemical treatments as it follows: 1) pellet alone (no treatment); 2) pellet sonicated for 1 min; 3) pellet treated with lysozyme (50 mg $mL^{-1}$); 4) pellet treated with lysozyme (50 mg $mL^{-1}$) + sonication (1 min); 5) supernatant alone. Sonication was performed using an ultrasonic bath with a heating system (LGI™) at a frequency of 80 kHz.

The IAA content was evaluated via the method reported by Sarwar & Kremer (1995). The OD (600 nm) of both the homemade and commercial formulations were measured, and 2 mL of the inoculant media were centrifuged at 10,000 *g* (4 ºC), for 10 min, to separate the supernatant and pellet fractions. To 1.5 mL solution of each of these fractions, 1 mL of the Salkowski reagent was added, and the samples were maintained for 25 min in the dark for color development. The intensity of the color increased with increasing levels of IAA (Kuss et al. 2007). IAA was quantified via measurement of absorbance at 535 nm and plotting a standard curve (0-0.3 mg $mL^{-1}$) (Asghar et al. 2002).

Another aliquot of 1 mL was centrifuged, and the pellet was frozen at -20 ºC for subsequent analysis of total proteins (Lowry et al. 1951). To compare the results, the standard curve was prepared using bovine serum albumin (BSA) with concentrations ranging 0-1.0 µg 100 $\mu L^{-1}$. The assays were conducted in quadruplicate.

The serial dilutions technique evaluated the bacterial populations from the homemade and commercial inoculants after a growth period of 24 h. Nalidixic acid (5 µg $mL^{-1}$) was added to the LB solid medium, and the plates were maintained at 30 ± 2 ºC, for 24 h. The evaluation was performed in triplicate. The CFU were counted using a stereoscopic magnifying glass (Quimis™, São Paulo, Brazil).

To verify the effect of auxin present in different fractions, maize seedlings were inoculated with the supernatant and pellet of both the homemade and commercial formulations. Under both conditions, the inoculum was prepared with $10^6$ CFU $mL^{-1}$ after 24 h of growth in the media, to ensure that each seedling was treated with the same bacterial/auxin concentration.

Seed asepsis and germination were conducted as it follows: 5 min in 70 % alcohol; 20 min in a mixture of 2 % sodium hypochlorite, two drops of Tween™ 80 and 1 mL of Lysoform™; 10 min in the commercial fungicide, namely, 2 mL $kg^{-1}$ of seed Standak® Top BASF, following the modified protocol of Vendruscolo et al. (2008). The seeds were then stored in plates containing a 1 % agar-water medium in the dark until germination (three days).

One day after the germination, the samples were transferred to test tubes containing 10 mL of the Murashige & Skoog medium and a mixture of sterilized sand and vermiculite (2:1), which served to mimic soil. Two inoculation conditions were evaluated: 1) seed bacterization, where the inoculant was provided directly to the seeds at 2 h before transferring the germinated seeds to the test tubes; 2) foliar, where the fractions were pipetted into the foliar area (corn VE-phenological stage) at 72 h after transferring the seedlings to the test tubes.

For both inoculation treatments, 15 randomly organized test tubes were inoculated. The control group was subjected to the same procedure, but without inoculation. The growing seedlings were maintained for 12 days in a growth room at 25 ± 2 ºC, with a 16 h light/8 h dark photoperiod. The experimental design was entirely randomized, with seven treatments (two formulations, two fractions, two inoculation methods and control without inoculation).

Five 15-day-old plantlets were randomly chosen from each treatment group, and a graduated ruler was used to determine the total length (cm), shoot length (cm) and root length (cm). The plant shoot and root fresh and dry weights (g) were determined using a precision scale. The total area of the root surface was obtained using the ImageJ

software (Abràmoff et al. 2004), and the dry weight was assessed after drying at 65 ºC, in an incubator, for 72 h.

The data were evaluated using analysis of variance (Anova), and the means were grouped via the Tukey test at 5 % of probability using the Genes statistic software (Cruz 2013).

## RESULTS AND DISCUSSION

The IAA concentrations observed in different fractions after treatment are shown in Figure 1. A higher IAA concentration was observed in the supernatant fraction of the homemade formulation without the addition of TRP, reaching 8.6 µg $mg^{-1}$ of protein (Figure 1A). Under the same conditions, the IAA contents in the untreated pellet fraction or after sonication and lysozyme treatment were 0.10 and 0.3 µg $mg^{-1}$ of protein, respectively. The IAA levels in the supernatant were 86-fold higher than those in the untreated pellet, and 28-fold higher in the pellet after lysozyme and sonication treatment with no addition of TRP. These results indicate that higher levels of IAA were present outside the cells during bacterial growth.

When 1.25 mM of TRP was added to the homemade formulation, the IAA content was similar in fractions treated with lysozyme and lysozyme + sonication and in the supernatant (Figure 1A). The levels differed from those in the untreated pellet and sonicated pellet fractions. At a higher concentration of TRP (2.5 mM), the IAA content was lower in the pellet fraction after all treatments (sonication, lysozyme, sonication + lysozyme), and only the supernatant showed a significant level (1.3 µg $mg^{-1}$ of protein).

In the commercial formulation, the highest levels of IAA in the pellet fraction were observed in the absence of TRP (1.02 µg $mg^{-1}$ of protein) (Figure 1B). Pellets treated with lysozyme and sonication (1.1 µg $mg^{-1}$) showed a higher IAA content in the presence of 1.25 mM of TRP, when compared to untreated pellets. At 2.5 mM of TRP, the IAA concentration was higher, although not significantly, in the pellet fraction subjected to all treatments, when compared to that in the supernatant (0.6 µg $mg^{-1}$).

Comparing the physical and biochemical treatments applied to the pellet fraction, a combination of lysozyme and sonication treatments was efficient for overflowing the IAA content from the pellet in

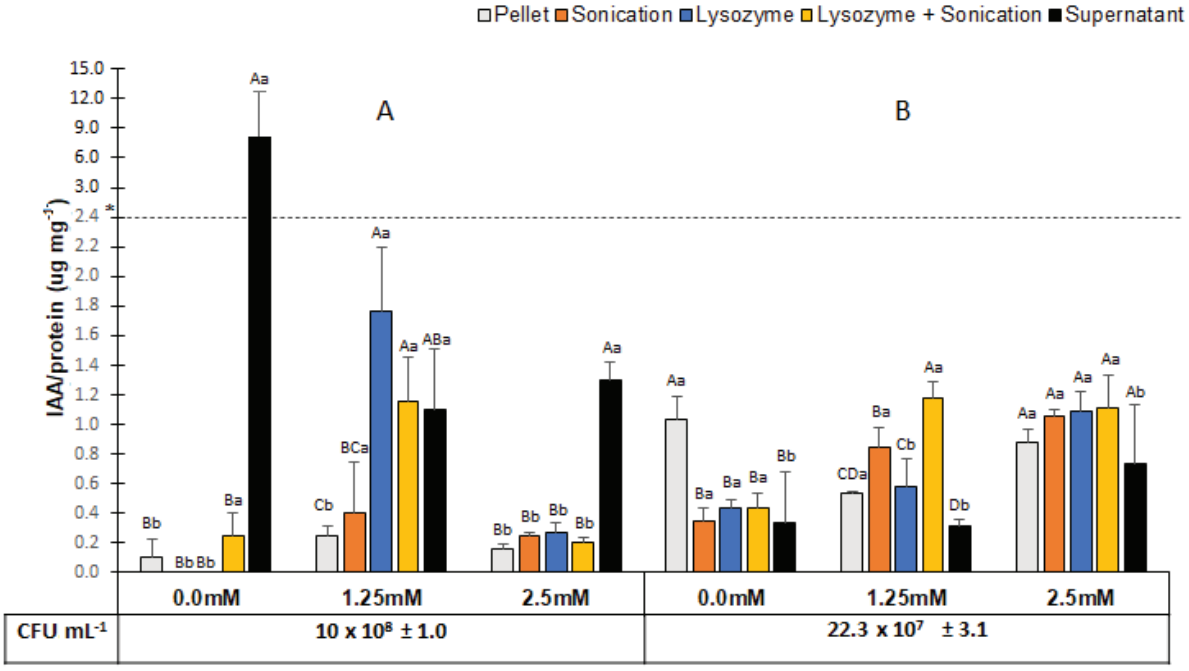


Figure 1. Quantitative results for IAA production and bacterial growth in different inoculant fractions. The homemade formulation is depicted as A, and the commercial formulation as B. Media followed by the same capital letter (treatments in the same formulation) and lowercase letter (homemade and commercial formulations) in the column did not differ significantly by the Tukey test ($p > 0.05$). The dotted line highlighted by "*" marks a breakpoint in scale to improve the data visualization.

the commercial formulation, particularly at 1.25 mM of TRP (Figure 1B). Only sonication was sufficient to disrupt cells in the homemade formulation (1.6 μg $mg^{-1}$) at the same TRP concentration (Figure 1A).

The homemade and commercial formulations showed distinct behaviors, in terms of bacterial growth and IAA content. At 2.5 mM of TRP in the homemade formulation, the pellet fraction exhibited an increased IAA content, when compared to the supernatant.

The formulations were initially adjusted to $10^6$ CFU $mL^{-1}$ and, after 24 h of growth, they reached $10 \times 10^8$ CFU $mL^{-1}$ (homemade) and $23 \times 10^7$ CFU $mL^{-1}$ (commercial). The results indicated an increased bacterial growth, leading to 10 × more bacteria in the media. Differences were observed in the IAA levels in the inoculum fractions (untreated and treated pellets and supernatant). The IAA content varied from 0 to 8.60 μg $mg^{-1}$ of protein in the homemade formulation and from 0 to 1.1 μg $mg^{-1}$ of protein in the commercial formulation. In the literature, the IAA production among *Azospirillum* species varies 107-1,038 μmol $L^{-1}$ (Souza et al. 2017), 32-40 ng $mL^{-1}$ (Fallik et al. 1989) and 24 μg $mL^{-1}$ (Aguilar-Piedras et al. 2008). These variations may be partially attributed to the quantification methodology, bacterial growth conditions, and strain or species used in the assay. The pellet fraction that was considered a bacterial mass showed a continuous biosynthesis of IAA phytohormone (Costacurta & Vanderleyden 1995, Shahab et al. 2009, Duca et al. 2014).

A wider range of IAA concentrations was observed with the homemade formulation, which showed a possibly superior bacterial viability for bacterial growth and production of IAA (Figure 1). The highest levels of IAA in the supernatant derived from homemade formulations indicated an increased bacterial growth because of the freshness and viability of the inoculum obtained from a fresh bacterial colony in an LB medium without protective molecules.

In contrast, the commercial formulation showed a smaller range of variation in the IAA levels after the majority of the treatments. This formulation comprised a liquid and viscous growth medium containing carboxymethyl cellulose (CMC) (Sanz et al. 2005, Fernandes Júnior et al. 2009). O'Callaghan (2016) commented that the protective properties of biopolymers can aid in maintaining optimal water activity levels, thereby improving the survival of bacteria. However, components found in this media can interfere with IAA biosynthesis, despite the storage of the commercial formulation package for only one of the six months before its expiry date, when used in the assays.

Lysozyme was more efficient than sonication for disrupting the outer bacterial membrane and mediating a release of IAA from cells, as observed after the TRP addition (homemade formulation) (Figure 1). Sonication did not appear to interfere with the release of IAA. The effects observed after the lysozyme plus sonication treatment may be primarily attributed to the presence of lysozyme.

Comparing the supernatant fractions from both formulations, a contrasting behavior was observed, whereby the homemade formulation showed a reduction in IAA content levels with an increase of TRP, and the commercial formulation showed an increase in IAA levels of 2.4 folds with 2.5 mM of TRP, if compared to that without TRP. These results differ from those reported by Ona et al. (2005), who observed that the IAA synthesis was enhanced by several folds after 18 h with TRP supplementation (200 μg $mL^{-1}$) in *Azospirillum brasilense* SP245. Prinsen (1993) observed the highest optical density (OD) (0.6-0.8) after 60 h in association with the highest IAA content (> 480 nmol 10 $mL^{-1}$ $OD^{-1}$) in the *A. brasilense* mutated strain SpM7918.

The plant biomass was positively affected by both inoculation methods, with a higher biomass observed in both formulation groups, when compared to that in control plants (Figures 2A and 2B). The largest increments were observed with seed inoculation in the pellet fraction of the homemade formulation, although the biomass was similar to that observed after foliar application. The same tendency was observed with root biomass.

Control plants had less root and shoot dry biomass, in comparison to plants inoculated with the pellet from the homemade formulation (Figure 2A). The supernatant also increased the fresh biomass in both inoculation sites (seed and leaves), but no significant difference was observed in the fresh root or dry shoot and root biomass. The treatment with pellet showed a higher shoot and root fresh biomass and shoot dry biomass, if compared to the supernatant treatment. Seeds and leaves inoculated with the pellet fraction from the homemade formulation showed

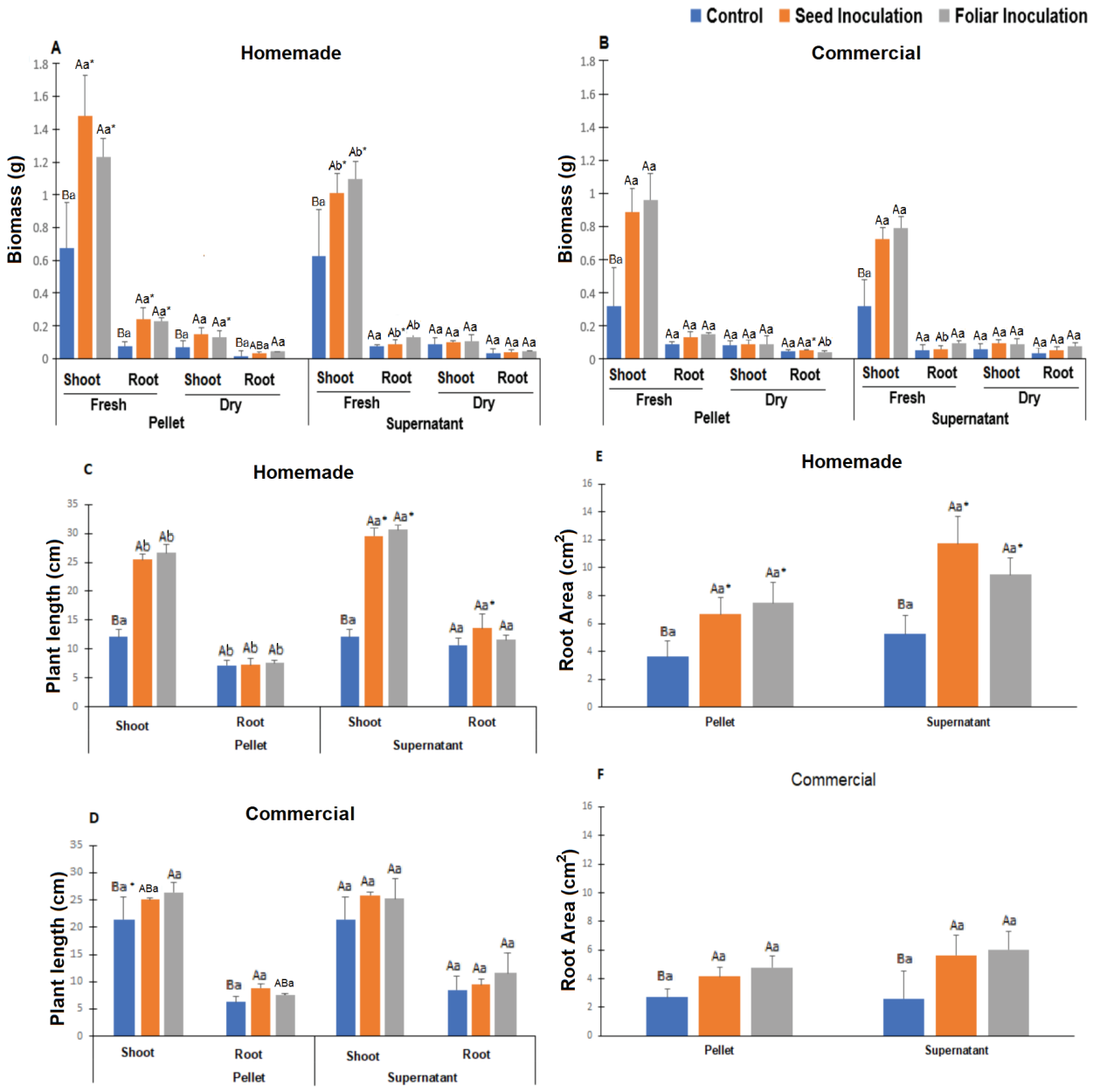


Figure 2. Morphological characteristics of corn plantlets inoculated with the different fractions. A and B refer to the biomass of corn plantlets; C and D to plant length; E and F to root area. The corn plantlets were inoculated with different fractions (pellet and supernatant) of homemade and commercial formulations via seed bacterization or foliar inoculation. Media followed by the same capital letter (treatments in the same formulation) and lowercase letter (homemade and commercial formulations) in the column did not differ significantly by the Tukey test ($p > 0.05$). Media followed by the same * (supernatant and control) differed significantly by the Tukey test ($p > 0.05$). $n = 4 \pm SD$.

a 3- and 2.7-fold higher biomass, when compared to control plants. The same tendency was observed with the supernatant, but with smaller increments (2.4- and 2.3-fold, respectively), in comparison to the control. The treatment with supernatant and pellet fractions of the commercial formulation revealed a significant increase for shoot fresh biomass; however, no differences were observed with respect to the fresh root or dry shoot and root biomass (Figure 2B). Plant biomass was similar among all fractions.

The fresh shoot biomasses obtained from seeds and leaves inoculated with pellet fraction derived from homemade formulation were 67 and

28 % higher, respectively, than that obtained from the seeds and leaves inoculated with the pellet fraction derived from the commercial formulation. When the supernatant fraction was applied to seed and plantlets leaves, the increments in plant biomass were approximately 30 % for both the inoculation methods. The homemade pellet fraction increased the fresh root biomass by 82 and 53 %, while the supernatant showed increments of 43 and 37 % via seed and foliar inoculation, respectively.

The data for plant height evaluated under all conditions are shown in Figure 2C. With regard to the homemade formulation, control plants were 2.3- and 2-fold smaller after the seed and foliar application, when compared to plants inoculated with the pellet fraction. The root height showed increments of 6 % (foliar) and 1.6 % (seed bacterization); however, the increments were not significant. The supernatant increased plant height by approximately 2.5 folds with the seed and foliar applications, in comparison to the height of the control plants. The root height was similar in all treatment groups corresponding to the homemade formulation.

The inoculated plants showed a larger root area with all application methods and inoculant fractions (Figures 2E and 2F). Both treatment fractions (supernatant and pellet) of the commercial formulation resulted in lower but significant increments in the root area after seed and foliar inoculation. The root area increased by 1.7- and 1.5-folds with foliar and seed inoculation of the pellet and 2.3- and 2.5-fold with supernatant, respectively, if compared to that of the control plants.

A positive effect of formulation fractions (pellet or supernatant) applied to the seeds and leaves was observed with respect to the morphophysiological features of plantlets cultivated *in vitro*. The control plants (without any inoculation) had lower leaf and root biomass, shoot and root length, and root area than the plants inoculated in both fractions and formulations. These data confirm that the bacterial interaction alters the plant structure because of growth inducers (Hayat et al. 2010).

Although our plant assays were conducted on early corn seedlings (12 days), the supernatant fraction resulted in higher increments in shoot length than in root length (Figures 2C and 2D). The primary effect on roots involved an increase in the lateral root expansion and, consequently, an increase in the root area (Figures 2E and 2F). Similar results were reported by Fukami et al. (2016) in corn roots. Spaepen et al. (2007) commented that bacterial IAA primarily affects the surface area by increasing lateral roots, as opposed to the biomass itself. Also, some authors have cited the effect of *Azospirillum* sp. on morphological changes in the roots of various plants, such as tomato (Pedraza et al. 2020), mung bean (Shahab et al. 2009), corn (Marques et al. 2020) and grass (Okon & Kapulnik 1986).

For the conditions of the present assay, it was not possible to find significant differences between seed and foliar inoculation. The plant biomass, plant height and root area did not favor a specific inoculum application. These data are similar to those reported in other studies conducted with *A. brasilense* (Zuffo et al. 2016, Machado et al. 2017). Our results highlighted the fact that the supernatant fraction (the liquid part of inoculant formulations) could be useful for inducing root development in cuttings or grafting in orchid seedlings. Furthermore, it may also be useful in *in vitro* culture in other plant species.

## CONCLUSIONS

1. Indole-3-acetic acid (IAA) secreted by *Azospirillum brasilense* can alter plant fitness;
2. The homemade and commercial formulations showed distinct patterns of IAA synthesis due to differences in cell growth and viability conditions;
3. The supernatant fraction showed high amounts of IAA and was effective in improving the root area, suggesting its influence on lateral root formation;
4. Lysozyme was superior to sonication in releasing IAA from the bacterial cells;
5. No significant differences were observed between seed and foliar inoculation.

## ACKNOWLEDGMENTS

To the Fundação Agrisus, for the financial support and for granting a scholarship to Pedro Henrique Pedron Mattiuzzi (Grant number PA 2893/19), and Editage, for the English language editing.

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