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Association of indolebutyric acid with rhizobacteria on the viability of herbaceous minicuttings of blueberry 'Brite Blue'

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ABSTRACT: Plant regulators used alone or associated with rhizobacteria are alternatives that maximize the rhizogenic potential of cuttings that are difficult to root, such as the blueberry. Thus, the objective of this study was to investigate if concentrations of IBA in talc, associated or not with the rhizobacteria *Azospirillum brasilense* and *Bacillus subtilis*, interfere in the viability of blueberry mini cuttings, cultivar 'Brite Blue'. The experimental design used was entirely randomized, with four repetitions. The treatments studied, designed in a bifatorial scheme (4 × 3), were concentrations of IBA (0, 500, 1,000, and 2,000 mg L^{-1}), applied as talc, associated or not with *A. brasilense* and *B. subtilis*. A significant interaction was observed between the factors IBA and inoculation with rhizobacteria regarding the presence of callus. In relation to the isolated effect of inoculation, *B. subtilis* proved to be superior to the treatments with application of IBA alone, also presenting the highest percentage of sprouted minestems. There was an isolated effect of IBA doses on the number of roots per minicutting, survival, and rooted minicutting. It is concluded that the treatments with IBA and rhizobacteria, especially *B. subtilis*, favor the viability of the 'Brite Blue' minicuttings.

Key words: *Azospirillum brasilense*; *Bacillus subtilis*; plant growth regulator; propagation; *Vaccinium* sp.

Associação de ácido indolbutírico com rizobactérias na viabilidade de miniestacas herbáceas de mirtilo 'Brite Blue'

RESUMO: Os reguladores vegetais utilizados isoladamente ou associados à rizobactérias são alternativas que maximizam o potencial rizogênico de estacas de difícil enraizamento, a exemplo do mirtilo. Dessa forma, o objetivo do trabalho foi investigar se concentrações de AIB em talco, associados ou não às rizobactérias *Azospirillum brasilense* e *Bacillus subtilis*, interferem na viabilidade de miniestacas de mirtilo, cultivar 'Brite Blue'. O delineamento experimental utilizado foi inteiramente casualizado, com quatro repetições. Os tratamentos estudados, delineados em esquema bifatorial (4 × 3), foram concentrações de AIB (0, 500, 1.000 e 2.000 mg L^{-1}), aplicados na forma de talco, associadas ou não à *A. brasilense* e à *B. subtilis*. Foi observada interação significativa entre os fatores AIB e inoculação com rizobactérias quanto à presença de calo. Em relação ao efeito isolado da inoculação, *B. subtilis* mostrou-se ser superior aos tratamentos com aplicação de AIB isolado, apresentando também a maior porcentagem de miniestacas brotadas. Houve efeito isolado de doses de AIB quanto ao número de raízes por miniestaca, sobrevivência e miniestacas enraizadas. Conclui-se que os tratamentos com AIB e rizobactérias, em especial o *B. subtilis*, favorecem a viabilidade das miniestacas 'Brite Blue'.

Palavras-chave: *Azospirillum brasilense*; *Bacillus subtilis*; regulador vegetal; propagação; *Vaccinium* sp.

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Introduction

The blueberry (*Vaccinium* sp.) is native to some regions of Europe and North America. Its fruit has been widely consumed and its production has shown a significant increase worldwide (Wang et al., 2015). Blueberry cultivation is a labor-intensive activity, but it offers high economic return in a short period of time, besides standing out due to its functional properties, its flavor, color, and the wide possibility of industrialization, making it a great alternative for small producers (Affonso et al., 2015).

In subtropical and tropical regions like Brazil, some factors have been limiting the expansion of blueberry cultivation, mainly related to technical knowledge about the crop and the difficulty of asexual propagation of most cultivars, tied mainly to the lack of efficient propagation methods both in quality and quantity, given the low rejuvenation of adult materials, as well as environment management techniques, nutritional management and survival of post-rooted cuttings (Shahab et al., 2018; Schuch & Tomaz, 2019). Among the propagation methods, staking stands out as the most used, because the technique allows the reduction of the juvenile phase of the plant and the anticipation of fruit production (Vignolo et al., 2012).

The blueberry is a species that presents difficulty in rooting, since for the success of its propagation by cuttings it is necessary to form adventitious roots. However, due to the special root architecture of blueberry, which consists mainly of fine roots, it generally requires certain environmental conditions, especially soil moisture, permeability, and pH, which generally lead to a lower percentage of adventitious rooting (Braha & Rama, 2016; An et al., 2019). One of the alternatives to enhance seedling formation is the use of plant regulators, such as indolebutyric acid (IBA) (Vignolo et al., 2012). Although the natural auxin widely found in plants is indoleacetic acid (IAA), the exogenous application of synthetic auxins is very common in vegetative propagation by cuttings, as they act similarly to the natural regulator (Lazaj et al., 2015).

Auxins are the group of plant regulators with the greatest effect on root formation in cuttings. They are active in the formation of adventitious roots, in the activation of cambium cells and in the promotion of plant growth, besides influencing the inhibition of lateral buds and leaf and fruit abscission. The use of plant regulators is intended to increase the percentage of cuttings that form roots, accelerate their initiation, increase the number and quality of roots formed, and uniformity in rooting (Fachinello et al., 2013).

Another alternative that can favor the rooting of cuttings is the use of plant growth promoting rhizobacteria (PGPR). The benefits provided by PGPR are linked to the production of endogenous plant regulators, such as auxins, gibberellins, and cytokinins, capable of stimulating plant growth, especially of the roots, making the acquisition of nutrients and water from the soil more efficient (Mariosa et al., 2017).

Studies have shown promising results from the association of phytohormones with *Azospirillum brasilense*. This interface promotes root increment due to increased production of plant regulators, providing plant growth and development (Fukami et al., 2018). Thus, the use of PGPR may be a promising biological alternative to increase the rooting efficiency of cuttings and thus reduce seedling production costs (Mariosa et al., 2017). However, there are few studies that relate the use of PGPR and plant regulators in blueberry. For example, Koyama et al. (2019b) evaluated the feasibility of producing ‘Powderblue’ blueberry seedlings from cuttings with different doses of IBA in association with *A. brasilense* and found that the application of IBA associated with the microorganism increased the length and number of roots of the cuttings. Due to this scarcity of information in the literature, it is necessary to fill the existing gap on the interaction of microorganisms with phytohormones in order to enhance the production of blueberry seedlings.

Therefore, the objective of this study was to investigate if IBA concentrations in talc, associated or not with the rhizobacteria *A. brasilense* and *Bacillus subtilis*, interfere in the viability of blueberry minicuttings, cultivar ‘Brite Blue’.

Materials and Methods

The experiment was conducted from April to October 2019, during early fall to early spring, in the Fruit Sector of the Center of Agricultural Sciences, Universidade Estadual de Londrina (UEL), Paraná, Brazil (latitude 23° 23’ S, longitude 51° 11’ W, and 566 m of altitude). Herbaceous minicuttings of 5 cm were taken from the median part of the branches of nine-year-old mother plants of blueberry cultivar ‘Brite Blue’, grown in pots with manual irrigation. The mother plants were kept at the same institution in a protected environment, from the blueberry cultivars collection belonging to Embrapa Clima Temperado, Pelotas, Rio Grande do Sul, Brazil.

The treatments studied, designed in a bifatorial scheme (4 × 3), were concentrations of IBA (0, 500, 1,000, and 2,000 mg L^{-1}), applied as talc, associated or not with *A. brasilense* (Azo) and *B. subtilis* (Bac). The experimental design used was entirely randomized, with four repetitions. Each sample plot was composed of 10 minicuttings.

Before minicuttings were collected, the hydroalcoholic solution of IBA was prepared by weighing 0.05, 0.10, and 0.20 g of concentrated IBA (99.9% purity; Sigma-Aldrich®, St. Louis, USA) on a semi-analytical balance, and dissolved in 50 mL of 100% ethanol. After the complete dissolution of IBA, the volume was adjusted to 100 mL with distilled water, obtaining the concentrations of 500, 1,000, and 2,000 mg L^{-1} of IBA, respectively. Then, inert industrial talc (Quimidrol®, Joinville, Brazil) was added to the solution to form a paste and then transferred to a drying oven at 40 °C, where it remained until complete evaporation of the solvent.

The suspensions of the rhizobacteria *A. brasilense* and *B. subtilis* were produced in the Biochemistry Laboratory at UEL. The bacterial isolates were grown in DYGS medium (glucose 2.0%, peptone 1.5%, yeast extract 2.0%, KH_2PO_4 0.5%, $MgSO_4$

$7H_2O$ 0.5%, glutamic acid 1.5%, pH 6.8), according to Kuss et al. (2007), with 120 g shaking at 28 °C, until a population density of 109 CFU mL^{-1} was reached. The microorganism *B. subtilis* (strain ZK) was isolated from the rhizosphere of sunflower plants and characterized as a growth-promoting bacterium by Goes et al. (2012); it is a bacterium with auxin and siderophore production capabilities. The strain is stored under cryopreservation in the Laboratory of Molecular Biochemistry at UEL. *A. brasilense* Ab-V5 is a microorganism isolated from corn plants and registered for commercial inoculant use with Ministério da Agricultura, Pecuária e Abastecimento (MAPA), with wide application in inoculation studies with different plant species (Hungria et al., 2010).

To prepare the minicuttings, a bevel cut was made at both ends, just below the node, with the goal of not accumulating water on the upper part and to increase the surface area of the lower part. The leaves of the basal part were discarded, keeping a pair of leaves in the upper part per minicuttings. With the help of a stylet, a superficial lesion was made at the base, in order to remove a part of the bark about 2 mm wide and 0.5 cm long, in order to expose the cambium tissue. The minicuttings were prepared in the morning, from 9:00 to 10:00 am, and immediately placed in trays with water to avoid dehydration.

After preparation, the bases of the minicuttings were immersed for 10 seconds in suspensions of *A. brasilense* and *B. subtilis* and then placed in contact with concentrations of IBA with talc, except for the treatments containing only IBA and the treatments containing only rhizobacteria.

The minicuttings were placed in perforated plastic boxes (44 × 30 × 7 cm) containing medium-grained vermiculite substrate for rooting and subjected to an intermittent misting system controlled by a timer and a solenoid valve. The valve was programmed to nebulize for 10 seconds every 6 minutes. The sprayer used (Model DAN-7755 Modular Greenhouse Sprinkler, Tel Aviv, Israel) had a flow rate of 35 L h^{-1}. The misting chamber was kept in a protected environment with a semicircular arched roof with transparent polyethylene film 150 microns thick and 30% coverage, installed in a north-south direction.

After 150 days the survival of the minicuttings (% of live minicuttings), rooted minicuttings (% of minicuttings with at least one root), sprouted minicuttings (% of minicuttings with shoots), number of roots per minicuttings, root length (cm), and callused minicuttings (% of callused minicuttings) were evaluated.

The data obtained were submitted to variance analysis ($p < 0.05$) considering the inoculation as a qualitative factor and the dose as a quantitative factor. The assumptions of normality of errors and homogeneity of variances were tested by Shapiro-Wilk & Bartlett ($p > 0.05$). When a significant effect for inoculation was found, the averages were compared using Tukey test ($p < 0.05$). When there was an effect of the dose or interaction between the factors, polynomial regression analysis was performed ($p < 0.05$). The analyses were processed using the R software (R Core Team, 2020).

Results and Discussion

There was a significant effect of the interaction between the factors IBA and inoculation with rhizobacteria only for the presence of callus. There was also an isolated effect of inoculation on the number of budded minicuttings and the number of roots per minicuttings. In relation to the IBA doses, a significant effect was observed for number of roots, survival and rooted minicuttings (Table 1).

A significant interaction was observed between the IBA factors and rhizobacteria inoculation for the variable callus presence. However, in the unfolding of the interaction, only with *B. subtilis* there was an effect of the doses of IBA, being possible to adjust a quadratic polynomial regression, with a reduction in the number of callus as the doses of IBA were increased, until the estimated dose of 1,025.243 mg L^{-1}, which resulted in 56.71% of callus (Figure 1).

The intense callus formation, especially in the cuttings treated with the highest dose of IBA (2,000 mg L^{-1}) and inoculated with *B. subtilis* may be as a result of the hormonal

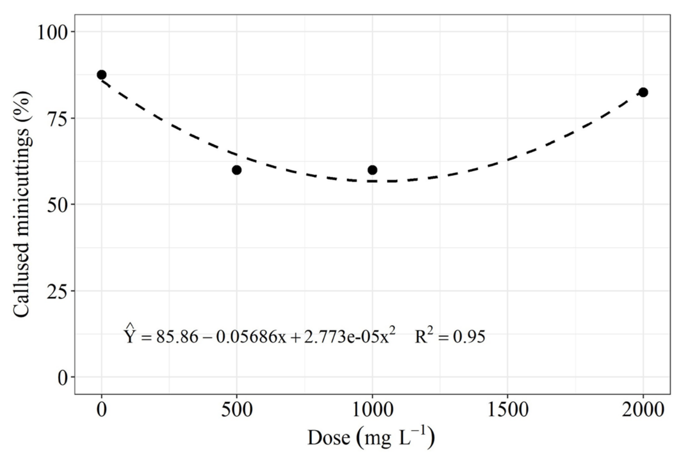


Figure 1. Presence of callus on blueberry minicuttings 'Brite Blue' inoculated with *B. subtilis* as a function of different doses of IBA.

Table 1. Summary of analysis of variance regarding viability of blueberry 'Brite Blue' minicuttings produced with IBA and PGPR.

Sources of variation	SM (%)[1]	RM (%)	SPM (%)	NRM	RL (cm)	MC (%)
Inoculation (A)	3.024 ns	9.875 ns	52.125 **	16.542 *	7.068 ns	27.125 **
Dose (B)	12.171 **	20.063 *	15.417 ns	15.083 *	21.794 ns	17.063 *
A × B	5.762 ns	15.625 ns	9.708 ns	6.792 ns	30.905 ns	21.875**
Residue	19.000	68.750	96.000	61.500	370.870	51.750

[1] SM: survival of the minicuttings; RM: rooted minicuttings; SPM: sprouted minicuttings; NRM: number of roots per minicuttings; RL: root length; MC: minicuttings with callus. ns, * and ** = not significant and significant at 1 and 5% probability by the F test, respectively.

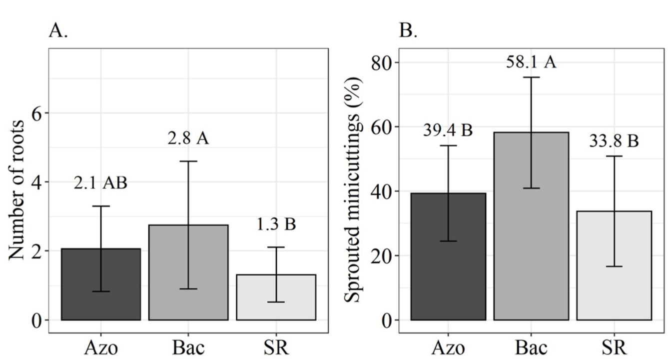
disproportion caused due to the exogenous increase of auxin provided by the plant regulator and the rhizobacterium (Fachinello et al., 2013). Thus, this increase in auxin content, in addition to the endogenous levels present in the plant, may have increased the hormone levels above the ideal concentration, leading to greater callus formation without, however, increasing the number of roots. This result was not observed for *A. brasiliense*, one of the possible causes is that this bacterium may have acted by regulating the auxin balance (Ghosh et al., 2019), keeping hormone levels stable.

Minicuttings treated with *B. subtilis* had a greater number of roots than those produced without the microorganisms (Figure 2A). The root stimulation provided by *B. subtilis* may be due to the production of IAA, inhibition of ethylene synthesis, decreased IAA oxidase activity, and nutrient mineralization by the bacteria. The production of IAA by the bacteria works in conjunction with endogenous auxin in the plant to stimulate root growth and development, cell division, and nutrient uptake (Hussein et al., 2016).

The highest percentage of sprouted minicuttings was obtained with *B. subtilis* inoculation, with an average of 58.1% (Figure 2B). This result may be associated with the increased number of roots in minicuttings treated with the rhizobacterium, resulting in better water acquisition and possible increase in carbohydrate production and assimilation, reflecting in vigorous vegetative growth (Mariosa et al., 2017).

B. subtilis exerts a direct effect on plant growth through the production of IAA. However, it can also contribute through biological nitrogen fixation. Studies have revealed that although bacteria do not provide all the nitrogen needed by plants, these microorganisms can make a significant contribution (Goswami et al., 2016). Thus, the increase in sprouts in cuttings inoculated with *B. subtilis* may result from the greater accumulation of nitrogen fixed by the bacteria.

In general, the minicuttings inoculated with *B. subtilis* showed better viability than those inoculated with *A. brasilense*. This can be explained by the fact that *Bacillus* sp. species have the ability to colonize plant roots and can remain viable under a variable range of environmental conditions, mainly due to the presence of endospores. These bacteria are able to adapt to plant physiological conditions and remain viable during distinct seasons (Goswami et al., 2016).



Averages followed by the same letter do not differ by Tukey test (p ≥ 0.05).

Figure 2. Number of roots (A) and percentage of sprouted minicuttings (B) of blueberry 'Brite Blue' inoculated with *A. brasilense* (Azo), *B. subtilis* (Bac), and without the presence of rhizobacteria (WR).

Regression analysis showed that for blueberry 'Brite Blue', the number of roots (Figure 3), the percentage of survival (Figure 4), and the percentage of rooted minicuttings (Figure 5) were influenced by the application of IBA,

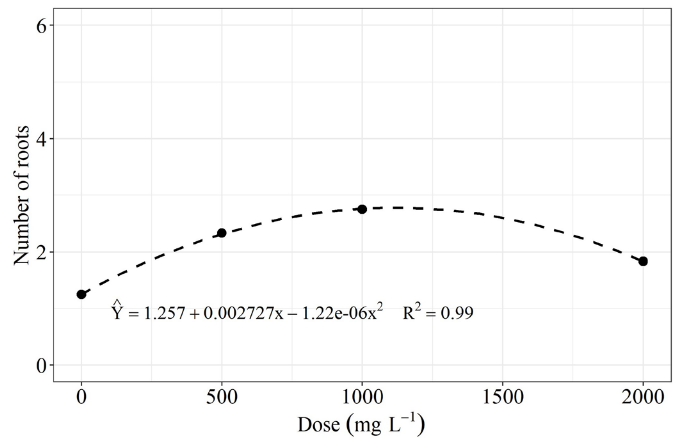


Figure 3. Number of roots of blueberry 'Brite Blue' minicuttings as a function of different doses of IBA.

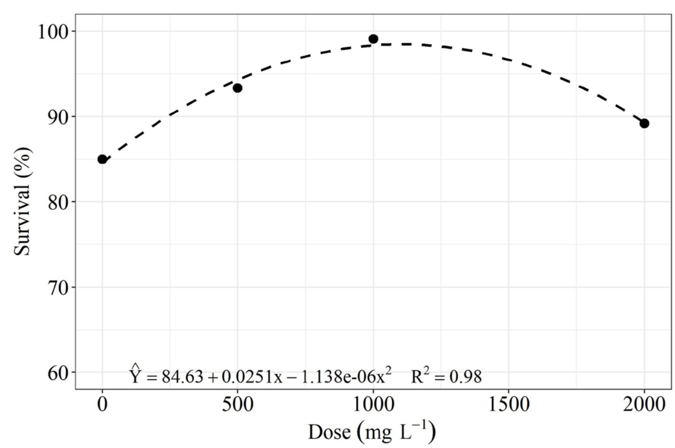


Figure 4. Survival of blueberry 'Brite Blue' minicuttings as a function of different doses of IBA.

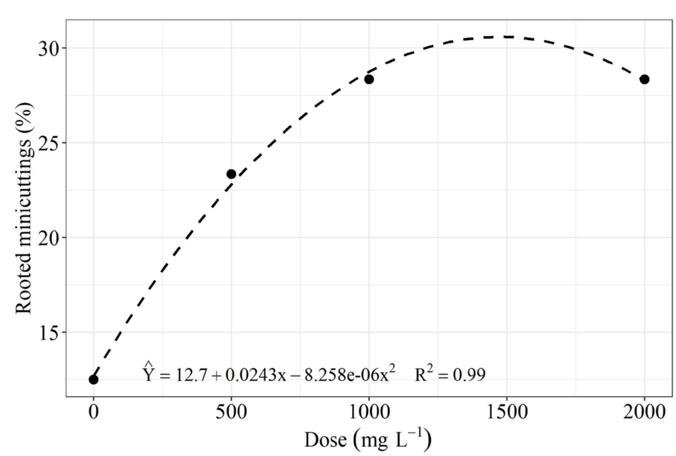


Figure 5. Rooting of blueberry 'Brite Blue' minicuttings as a function of different doses of IBA.

showing quadratic behavior. Increasing the concentration of exogenous auxin, applied to cuttings, causes root stimulating effect up to a maximum value, from which any auxin addition has an inhibitory effect (Fachinello et al., 2013).

The number of roots, as a function of the IBA doses, showed a quadratic adjustment, in which the maximum response observed was 2.78 roots per minicuttings at the estimated dose of 1,117.62 mg L^{-1} (Figure 3). In relation to the percentage of survival of the minicuttings as a function of the IBA doses, a quadratic adjustment was observed, in which the maximum response observed was 98.47%, at the estimated dose of 1,102.81 mg L^{-1} (Figure 4).

The increase in the number of roots as well as the percentage of survival at doses close to 1,100 mg L^{-1} can be justified due to doses close to this favoring hormonal balance in blueberry cuttings, as reported by Koyama et al. (2018). The use of plant regulators is one of the most common ways to favor the plant's hormonal balance, stimulating rooting by raising the auxin content in the tissue. The moment auxin is applied, there is an increase in its concentration at the base of the cuttings and, if the other physiological requirements are met, callus formation occurs, resulting from the activation of cambium cells, and adventitious roots (Fachinello et al., 2013), which increases the survival rate, as seen in the present study.

Although the percentage of survival was high (Figure 4), there was a large number of minicuttings without roots at the time of evaluation, showing only callus, which indicates that the environment of the cuttings presented adequate conditions for prolonged maintenance of the minicuttings and that these still had sufficient reserves for their survival. For some difficult rooting species, such as blueberry, root formation can occur on callus, although callus formation is not a sure harbinger of adventitious root formation (Fachinello et al., 2013).

The percentage of rooted minicuttings as a function of the IBA dose showed a quadratic adjustment, in which the maximum response observed was 30.58%, at the estimated dose of 1,471.30 mg L^{-1} (Figure 5). The results of this study demonstrate that rooting and development of 'Brite Blue' blueberry minicuttings were influenced by the application of IBA. However, there are authors who have reported that they have not found any increase with the application of the plant regulator for the same cultivar. Koyama et al. (2019a) did not observe any influence of IBA treatment at the concentration of 1,000 mg L^{-1} on 'Brite Blue' blueberry cuttings. Similarly, Trevisan et al. (2008) found that IBA applications of up to 7,500 mg L^{-1} did not interfere with the rooting percentage of blueberry seedlings, such as 'Climax', 'Bluebelle', and 'Brite Blue'.

The different results recorded by some authors for the same cultivar may be related to the use of plant regulators (type, concentration, and application methods), collection season and vegetative stage of the parent plants, substrates (since different materials have different physicochemical properties), and period to access rooting performance (Colombo et al., 2018). Therefore, it is necessary to evaluate each one of the factors to enable the multiplication of the blueberry, a species considered difficult to propagate.

Regarding the treatments with *A. brasilense*, it is believed that the conditions under which the experiment was conducted did not favor its maximum potential, as previous research has indicated that more stressful environmental conditions can exert greater effect of rhizobacteria on plant growth (Khademian et al., 2019). Therefore, further studies under different environmental conditions are needed to elucidate the effect of rhizobacteria on growth promotion and root development of blueberry 'Brite Blue' minicuttings.

The results of this experiment presented promising results regarding the inoculation with *B. subtilis*, suggesting that this microorganism has the potential to be used commercially in the production of blueberry seedlings, since there was the formation of seedlings of better quality, overcoming one of the main limiting factors of the culture. Moreover, because most of the variables did not show significant interaction between the factors, it is possible to use *B. subtilis* with exogenous application of IBA, since the efficiency of action of the bacteria was not influenced by the plant regulator.

Conclusions

B. subtilis improves the quality and viability of the seedlings formed, especially in the number of roots and the percentage of sprouted minicuttings, demonstrating its potential use for the blueberry seedling grower.

A. brasiliense does not show positive responses on the quality of blueberry seedlings.

The IBA doses increase most of the quality variables, with an estimated optimum dose close to 1,100 mg L^{-1}.

Compliance with Ethical Standards

Author contributions: Conceptualization: RK, SRR; Data curation: MTH; Formal analysis: GDS; Investigation: MTH, LTMR, HRG, EJR, DBBP; Methodology: LTMR, HGR, EJR, DBBP; Project administration: MTH, RK; Resources: SRR; Supervision: MTH; Validation: SRR; Visualization: MTH, GDS; Writing – original draft: MTH, LTMR; Writing – review & editing: MTH, RK, SRR.

Conflict of interest: The authors declare that there is no conflict of interest.

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