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Aspergillus flavipes as a novel biostimulant for rooting-enhancement of *Eucalyptus*

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ABSTRACT

In recent years, forest breeding programs have increased *Eucalyptus* production for commercial purposes; however, high-performing *Eucalyptus* clones have had problems with propagation, especially when rooting. Nevertheless, studies have shown that inoculation of microorganisms producing indole-3-acetic acid (IAA) is an especially productive procedure to help these clones breed. In this study, we evaluated the production of IAA and analogues in 16 microbial strains. For the first time, a high IAA production was described in the strain *Aspergillus flavipes* (ATCC® 16814™), and *A. flavipes* was shown by LC-MS/MS to produce IAA through a tryptophan-dependent biosynthetic pathway. *A. flavipes* reached the highest IAA production when cultivated under solid-state fermentation in an optimized medium composed of soybean bran, water and tryptophan. We mixed the fermentation products in solid form (SF) and liquid form (LF) with the substrate Carolina I® and then planted the cuttings of the hybrid *Eucalyptus grandis* x *E. urophylla* (clone IPB2). In fact, treatments with 40–120 mg kg^{-1} of SF increased the adventitious rooting rate, root length and both root fresh and dry mass, while 120 mg kg^{-1} of LF increased root length and dry mass. Additionally, there was no toxicity on fibroblasts (NIH/3t3), and, therefore, the plant biostimulant was confirmed as a novel, non-toxic, and eco-friendly solution.

1. Introduction

Clonal plantation of *Eucalyptus* based on vegetative propagation is successful mainly due to well-developed forest breeding programs. However, vegetative propagation efficiency is prejudiced in some species due to specific endogenous and exogenous factors of the mother plant, alterations of the root system architecture and topophysis effects that influence the rooting potential (Peralta et al., 2012).

The clone IPB2 of the hybrid *Eucalyptus grandis* x *Eucalyptus urophylla* from ArborGen is classified as a clone SuperTree®. Such clones are selected for a higher volume of wood, disease resistance, trunk straightness, and wood quality characteristics (ArborGen, 2018). Despite the advantages in the field, the clone has difficulties in rooting, demanding research to promote the adventitious roots induction and development.

The inoculation of some classes of microorganisms can assist in overcoming this difficulty, stimulating adventitious root growth, increasing the absorption of nutrients and water, the host-plant biomass and the tolerance to stresses such as drought and diseases (Sukumar et al., 2013). When a substance can improve the plant nutritional efficiency and abiotic stress and/or influence the quality of the crop, regardless of its nutritional status, it is denominated as a plant biostimulant (du Jardin, 2015). The commercial products are currently divided into five classes: microbial inoculants, humic acids, fulvic acids, amino acids and seaweed extracts (Calvo et al., 2014). The commercial biostimulants can contain one or more microorganisms and/or substances (du Jardin, 2015).

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The global market of biological products for agriculture, which include biopesticides, biofertilizers, and biostimulants, may reach US$ 10.05 billion until 2020, with an estimated annual increase of 14.5%. The potential market growth is linked to the reduction of chemical risks, better waste management and incentives from governmental agencies (Markets & Markets, 2016).

Several studies have proven the effectiveness of microbial biostimulants with the ability to provide additional phytohormones and/or their precursors (Wong et al., 2016). Phytohormones, such as auxins, modulate the associations between plants and microorganisms and coordinate cellular and metabolic responses associated with microbial growth in different plant tissues (Boivin et al., 2016). The auxin indole-3-acetic acid (IAA) is the main regulator of all aspects of plant development, acting on both basic cellular processes and macroscopic phenomena (Sauer et al., 2013), playing a central role in the formation of adventitious roots (Pacurar et al., 2014). Beneficial effects on plants, as increases in shoot and root growth, lateral root and root hair numbers resulting from the application of microorganisms of the genus *Aspergillus*, *Trichodema*, and *Bacillus*, producers of IAA, have been described in bean (*Phaseolus vulgaris* L.) (Hoyos-Carvajal et al., 2009), mung bean (*Vigna ratiata*) (Hussein et al., 2016) and *Arabidopsis thaliana* (Salas-Marina et al., 2011).

Solid-state fermentation (SSF) is a microbial cultivation technique that has been standing out in the development of industrial bioprocesses, in the last two decades. SSF is advantageous due to the lower energy requirement, higher product yield, lower wastewater production and lower risk of bacterial contamination. In addition, it is considered an eco-friendly technique, mainly due to the use of solid agro-industrial wastes as substrate (Thomas et al., 2013).

Considering the adventitious rooting difficulties of *Eucalyptus* clones and the IAA effects on rooting, this study aimed to produce a new plant biostimulant by SSF, evaluate the biostimulant effect on the development of *Eucalyptus grandis* x *Eucalyptus urophylla* (clone IPB2) seedlings and test the product's safety for fibroblasts.

2. Materials and methods

2.1. Screening of microorganisms strains for IAA production

2.1.1. Microbial strains and inoculum preparation

The strains *Aspergillus ustus* (Bainer.) Thom & Church (IOC 4410), *Aspergillus niger* van Tieghem (INCQS 40015), *Aspergillus flavipes* (ATCC® 16814™), *Bacillus subtilis* (CCGB 0030), *Bacillus megaterium* (CCGB 0146), *Bacillus amyloliquefaciens* (CCGB 0145), *Trichoderma atroviride* (IOC 4503), *Trichoderma koningii* (INCQS 40331), *Trichoderma harzianum* Rifai (IOC 3844), were donated by Oswaldo Cruz Foundation (FIOCRUZ). The strain *Aspergillus niger* 01 (CBMAI 2084) was isolated by the Bioprocess Laboratory of São Paulo State University, Botucatu, Brazil. The strains *Bacillus subtilis* (B, C, D, E, F, E27) were donated by Campinas State University (UNICAMP), Brazil.

The products from the fungal growth in potato dextrose agar (PDA) slants for 96 h at 30 °C and the bacterial growth in tryptic soy agar (TSA) slants for 24 h at 33 °C were suspended in 6 mL of sterilized water. The suspension was adjusted to 10^7 spores mL^{-1}.

2.1.2. Quantification of IAA and analogues

The production of IAA and analogues by the microorganisms was determined by colorimetric measurement at 535 nm using Salkowski's reagent (2% of $FeCl_3$ in 35% of perchloric acid), according to an adaptation of the methods described by Hoyos-Carvajal et al. (2009) and Sarwar et al. (1992). Petri plates with PDA media, for fungi, and TSA media, for bacteria, with tryptophan (1%, m/v) were inoculated with 100 μL of the microbial suspensions.

After 96 h of incubation at 30 °C, for fungi, and 24 h of incubation for bacteria at 33 °C, 20 mL of Salkowski's reagent were added to the plates at room temperature. The reaction occurred for 30 min in the dark.

The plates content were transferred to Falcon tubes and centrifuged for 15 min at 6,000 rpm. Blanks were prepared by the addition of Salkowki's reagent in Petri plates with the culture media plus tryptophan (1%, m/v). The absorbance readings were compared to a standard curve of indole-3-acetic acid (Sigma Aldrich®) and the results were expressed as μg of indole-3-acetic acid and analogues mL^{-1}.

2.2. Selection of the highest IAA producer for developing the biostimulant

2.2.1. Solid-state fermentation (SSF)

The microorganism with the highest production of auxins in the pre-test was evaluated for IAA and analogues production using different agro-industrial residues (cassava bagasse, wheat bran, soybean bran, distillers dried grains with solubles (DDGS) of maize and DDGS of sorghum) as substrates for SSF. The amino acid tryptophan (1%, m/m) was added to the substrates as an IAA biosynthesis inductor. The SSF media was composed of 50% of substrate and 50% of distilled water (m/m). Erlenmeyer flasks (250 mL) with the substrate (10 g) and water (10 mL) were sterilized for 20 min at 121 °C.

The microbial suspensions (2 mL) were inoculated in the substrate and incubated at 30 °C for 120 h. Then, 50 mL of distilled water was added and the content was filtered in two layers of cotton gauze.

The Salkowski's reagent (4 mL) was added to the filtration products (1 mL), the absorbance reading was performed in spectrophotometer at 535 nm and the results were expressed as μg of indole-3-acetic acid and analogues mL^{-1}.

2.2.2. Factorial planning

The SSF substrate with best production of auxinic compounds was selected to be optimized by factorial planning. The pH was measured both in the substrate and in the fermentation product. The particle size (0.5, 1.0 and > 1.0 mm), the amount of water added to the substrate (5, 10, 15 mL) and the tryptophan percentage (0.5, 1.0 and 1.5%, m/m) were selected as the independent variables, using the complete factorial planning of the 2^3 type with three replications at the central point. Erlenmeyer flasks (250 mL) with the substrate (10 g) and water (10 mL) were sterilized for 20 min at 121 °C. The microbial suspensions (2 mL) were inoculated in the substrate and incubated at 30 °C for 120 h.

2.2.3. Quantification of IAA by liquid chromatography and mass spectrometry (LC-MS/MS)

The selected SSF conditions that gave the best preliminary results were prepared with and without tryptophan in order to confirm the IAA production by a metabolic pathway with tryptophan as a precursor. The extracts were centrifuged at 6,000 rpm for 10 min and filtered through a 0.22 μm membrane. IAA was quantified according to an adaptation of the method described by Prado et al., 2019.

The samples (20 μL, pH 6.3) were injected in a LC-MS/MS system consisting of a High Efficiency Liquid Chromatograph, model Proeminence UFLC (Shimadzu®, Kyoto, Japan), equipped with two pumps LC-20AD, a self-injector SIL-20AC, a degasser DGU-20A5, a controller system CBM-20A and an oven CTO-20AC., coupled to a mass spectrometer 3200 Q TRAP (Applied Biosystems®, Foster City, USA).

The LC separation was carried out by a Synergi 2.5 μm Fusion RP 100 Å chromatographic column (50 × 4.6 mm, Phenomenex) at 40 °C. The samples were eluted with 1% of acetic acid diluted in milli-q water (v/v) (phase A) and 1% of acetic acid diluted in methanol (v/v) (phase B) at the flow rate of 0.65 mL min^{-1}. The gradient program was as followed: 0–2 min, 45% phase B; 2–8 min, 95% phase B; and 8–10 min, 45% phase B. The total run time was 10 min, and the retention time of the compound in the chromatographic column was 2.51 min. The electrospray ionization (ESI) was operated in the negative ion mode. Mass parameters were Q1 mass (174.19), Q3 mass (130.0), declustering potential (DP, −65 V), entrance potential (EP, −10 V), collision energy (CE, −14 V), collision cell exit potential (CXP, −9 V). Analyst software (version 1.4.2) was used to control sample acquisition and data analysis.

2.3. Biostimulant effect in the hybrid Eucalyptus grandis x E. urophylla (clone IPB2)

2.3.1. Application forms

The aim of this experiment was to test if the SSF product is more effective when applied in *Eucalyptus* in solid form (SF), with smaller contact surface and longer contact time, or in liquid form (LF), with wider contact surface and a shorter contact time.

The crude/solid form (SF) was processed as described below to obtain the liquid form (LF). The LF was prepared by adding distilled water to the fermentative product in the proportion of 5:1 (5 mL of water for each g of fermentation product), followed by filtration in two layers of cotton gauze, the yield of the filtration process was 80%. Both preparations were mixed to the substrate Carolina I® (Sphagnum peat, rice husk, and vermiculite), both were adjusted to the doses 0, 40, 80, 120 and 160 mg kg^{-1} of IAA. Both forms of the fermentation products were placed in plastic tubes of 55 m^3 and moistened with nebulizers.

2.3.2. Planting of cuttings

The experiment was conducted in the greenhouse of the company Avam Flora, located in Águas de Santa Bárbara county, São Paulo State, Brazil (22°52′50″ S and 49° 14′20″ W). The clone IPB2 of the hybrid *Eucalyptus grandis* x *E. urophylla* from ArborGen was selected for the experiment due to its large capacity of cellulose production and the low performance in greenhouse due to problems in adventitious rooting.

Apical cuttings, from 3 to 6 cm length and from 1.5 to 2.5 mm diameter were selected and planted in the prepared substrates. The experiments were implemented in a completely randomized design with five treatments (four doses of IAA plus the control) and three biological replicates (n = 88). The cuttings were taken to the greenhouse, where they remained for 30 days at 25-30 °C and relative humidity above 75% and then, were transferred to the shade house (approximately 50% of full sun) for another 10 days, totalizing 40 days of the experiment. The water management was performed according to the need of irrigation, by observing the water saturation in the substrates.

2.3.3. Assessment of rooting and development

The survival percentage was evaluated in the transition from the greenhouse to the shade house. At the end of the experiment, 15 central seedlings were removed from each experimental unit to perform the destructive evaluations. The plants were removed from the plastic tubes and washed in running water to remove the substrate residues. Then, the seedlings were sectioned at the interface between root and aerial part and determined, immediately after cutting, the rooting percentage, the mean lengths of the shoots and roots, and the mean of fresh root mass. The roots were drained and then placed in a lyophilizer for 24 h at −60 °C and weighted in analytical balance for the dry mass determination.

2.4. Product safety

2.4.1. Cell viability

The biostimulant cytotoxicity in fibroblasts (NIH/t3t) was evaluated as described by Mosmann (1983). The cells were seeded 48 h before treatments in a 96-well plate at 5 × 10^4 cells mL^{-1}. These cells were selected because the mixing operation of the product to the substrate requires contact of the operator's hands with the product.

The microbial extracts were centrifuged at 6,000 rpm for 10 min and filtered through a 0.22 μm membrane. The cells were exposed to the biostimulant and to synthetic IAA (Sigma-Aldrich®, St Louis, USA) at 0, 40, 80, 120 and 160 mg kg^{-1}, the same concentrations of the greenhouse experiments. After the exposure time of 24 h, the cell viability was subjected to MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (1 mg mL^{-1}) treatment for 3 h. Thereafter, the MTT solution was removed and 0.1 mL of DMSO (dimethyl sulphoxide) was added into each well for the solubilization of the dye. Then the absorbance readings were performed at 570 nm using a microplate reader (SYNERGY-HTX multi-mode reader, Biotek, USA).

2.5. Statistical analysis

The results were expressed as the mean values of three biological replicates (mean ± standard deviation). Data were subjected to analysis of variance (ANOVA) and, when the F test was significant, means were compared by the Scott-Knott test ($p \leq 0.05$). The Statistica software (version 10, Statsoft®) was used to perform the analysis.

3. Results

3.1. Production of IAA and analogues

All tested strains produced IAA and analogues in culture media supplemented with tryptophan (1%, m/v). A high auxin derivative production was reported for the first time by the strain of *Aspergillus flavipes* (ca 71 μg mL^{-1}), while the productivity of the other strains varied from ca 3 (*T. harzianum*) to 23 μg mL^{-1} (*B. subtilis* E27) (Fig. 1). Thus, the fungus *A. flavipes* was selected for the continuation of the study and cultivated under SSF.

The *A. flavipes* strain was cultivated in cassava bagasse, wheat bran, soybean bran, maize DDGS and sorghum DDGS. There was no microbial growth in cassava bagasse and maize DDGS. The higher indolic compound production occurred using soybean bran as substrate (183 μg mL^{-1}), being increased about 2-fold in comparison to wheat bran (87 μg mL^{-1}) and 5-fold in comparison to sorghum DDGS (36 μg mL^{-1}) ($p \leq 0.05$) (Fig. 2). Thus, soybean bran was selected for the optimization of IAA and analogues production using factorial planning.

3.2. IAA and analogues production using factorial planning

In the factorial planning ($R^2 = 0.86$), the variables particle size (mm), addition of water (mL) and tryptophan (%) and the interaction among all the variables, were not statistically significant for the production of IAA and analogues using soybean bran as substrate. However, there was a great variation in the productivity of IAA and analogues due to the variation of the culture medium conditions. The lowest particle size (0.5 mm), highest water addition (15 mL) and lowest tryptophan addition (0.5%) induced the lowest IAA and analogues productivity (237 μg mL^{-1}), while, under the same

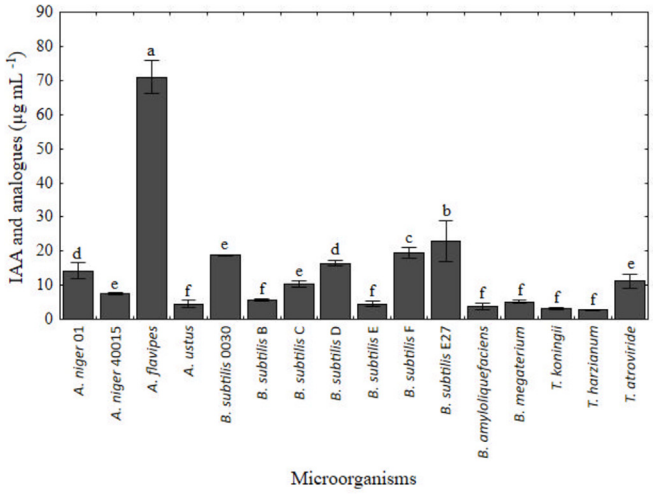

Fig. 1. Quantification of IAA (indole-3-acetic acid) and analogues (μg mL^{-1}), produced by the strains *A. niger* (01 and INCQS 40015), *A. ustus* (IOC 4410), *A. flavipes* (ATCC 16814), *B. subtilis* (CCGB 0030, B, C, D, E, F and E27), *B. megaterium* (CCGB 0146), *B. amyloliquefaciens* (CCGB 0145), *T. harzianum* (IOC 3844) and *T. atroviride* (IOC 450). Mean followed by the same letter do not differ statistically according to the Scott Knott test ($p \leq 0.05$).

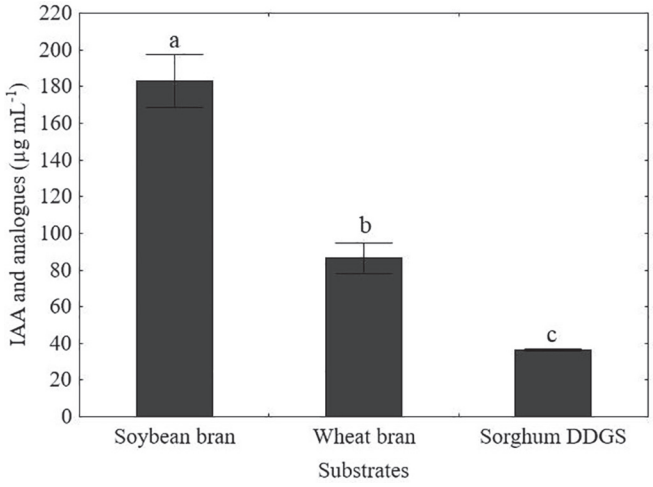

Fig. 2. Quantification of IAA (indole-3-acetic acid) and analogues (μg mL^{-1}) in extracts from the solid-state fermentation of *A. flavipes* (ATCC® 16814™) in soybean bran, wheat bran, and sorghum DDGS.
Means statistically compared by Scott Knott test ($p \leq 0.05$).

conditions of particle size and water and the highest tryptophan concentration (1.5%) was reported the highest production of IAA and analogues (655 μg mL^{-1}) (Table 1).

In addition, there was an increase by about ca 3.6-fold in the productivity of auxinic compounds in comparison to the initial conditions of soybean bran (183 μg mL^{-1}) (Fig. 2). Thus, although not statistically significant by factorial planning, the cultivation conditions that resulted in higher IAA production were selected for the study continuation.

3.3. Quantification of indole-3-acetic acid by LC-MS/MS

Three biological replicates were prepared (using the conditions of the factorial planning that most increased the production of auxin analogues) for the quantification of indole-3-acetic acid by LC-MS/MS. The IAA productivity by *A. flavipes* (516 μg mL^{-1}) (Fig. 3B), confirmed the results of the colorimetric analysis (654 μg mL^{-1}), about the high microbial production of IAA.

It was also verified that the production of IAA by *A. flavipes* occurs using a tryptophan-dependent pathway. In the absence of tryptophan, the production of IAA by *A. flavipes* reached 0.3 μg mL^{-1}, while, adding 1.5% of tryptophan, the production was increased to 515.5 μg mL^{-1} (Fig. 3A).

3.4. Rooting and development in response to the potential biostimulant

The fermentation process decreased soybean bran pH from 6.5 to 6.3. The treatments with both SF and LF resulted in changes in adventitious rooting and plant development (Figs. 4 and 5).

There was no significant difference between the treatments and the control for both survival (Fig. 4A) and average shoot length (Fig. 4C). There was an increase in the percentage of rooting by the addition of 40 (89%), 80 (91%) and 120 mg kg^{-1} (82%) of SF in comparison to the control (62%), while treatment with 160 mg kg^{-1} of IAA was statistically equal to the control (Fig. 4B). The same pattern was followed in the variables average root length, roots fresh and dry mass (Fig. 4D, E, and 4F). The treatments with 40 (11.0 cm), 80 (10.9 cm), and 120 mg kg^{-1} (10.0 cm) were higher than the control (6.7 cm) and the application of 160 mg kg^{-1} (7.2 cm) of SF, for average root length (Fig. 4D). There was an increase in root mass, fresh and dry, in treatments with 40 (19.1 and 1.7 mg, respectively), 80 (20.0 and 1.8 mg) and 120 (22.1 and 1.7 mg) mg kg^{-1} of IAA, in comparison to the control (14.0 and 1.3 mg) (Fig. 4E–F), while 160 mg kg^{-1} was similar to the control (12.3 and 1.1 mg).

For the LF treatments, there was no significant difference for the percentage of survival (Fig. 5A), rooting percentage (Fig. 5B) and fresh root mass (Fig. 5E). The treatments with 80, 120 and 160 mg kg^{-1} of IAA (whose averages were, respectively, 9.5, 9.7 and 9.7 cm) did not alter the average length of the aerial part in relation to the control (10.0 cm), while the treatment with 40 mg kg^{-1} of IAA induced length reduction (8.5 cm) (Fig. 5C). The average root length and the roots dry mass were only increased by the application of 120 mg kg^{-1} of IAA (11.3 cm and 1.8 mg, respectively), in comparison to the control (6.7 cm and 1.3 mg, respectively), while the other treatments did not cause changes in those variables (Fig. 5D–F).

3.5. Cell viability

The fibroblasts (NIH/3t3) viability differed in response to both *A. flavipes* and synthetic IAA (Fig. 6A–B). The highest dose of synthetic auxin (Fig. 6A) reduced the viability of NIH/3t3 in relation to controls. The doses 120 and 160 mg kg^{-1} reduced NIH/3t3 viability in comparison to 40 mg kg^{-1}, while the application of 120 and 160 mg kg^{-1} of *A. flavipes* IAA (Fig. 6B) increased NIH/3t3 viability in relation to controls and other doses. Thus, for fibroblasts, the IAA produced by *A. flavipes*, at the doses tested, can be considered non-toxic and advantageous in relation to the synthetic IAA, which was shown as cytotoxic.

4. Discussion

4.1. Production of IAA by Aspergillus flavipes

Auxins can be analyzed by various methods such as qualitative (Hoyos-Carvajal et al., 2009), spectrophotometric (Sarwar et al., 1992), gas spectrometry (GC) (Barkawi et al., 2010), and high performance liquid chromatography (HPLC) (Gupta et al., 2011; Lu et al., 2010) both coupled to mass spectrometry. However, the

Table 1
Factorial planning for IAA and analogues (μg mL^{-1}) optimization under solid-state fermentation, using *A. flavipes* and soybean bran and modifying the substrate conditions: particle size (mm), addition of water (mL) and tryptophan (%).

Assay	Particle size (mm)	Water (mL)	Tryptophan (%)	IAA and analogues (μg mL^{-1})
1	0.5	5	0.5	317.77
2	>1.0	5	0.5	285.83
3	0.5	15	0.5	237.30
4	>1.0	15	0.5	307.84
5	0.5	5	1.5	365.52
6	>1.0	5	1.5	264.74
7	0.5	15	1.5	654.56
8	>1.0	15	1.5	384.56
9	1.0	10	1.0	450.84
10	1.0	10	1.0	383.86
11	1.0	10	1.0	359.09

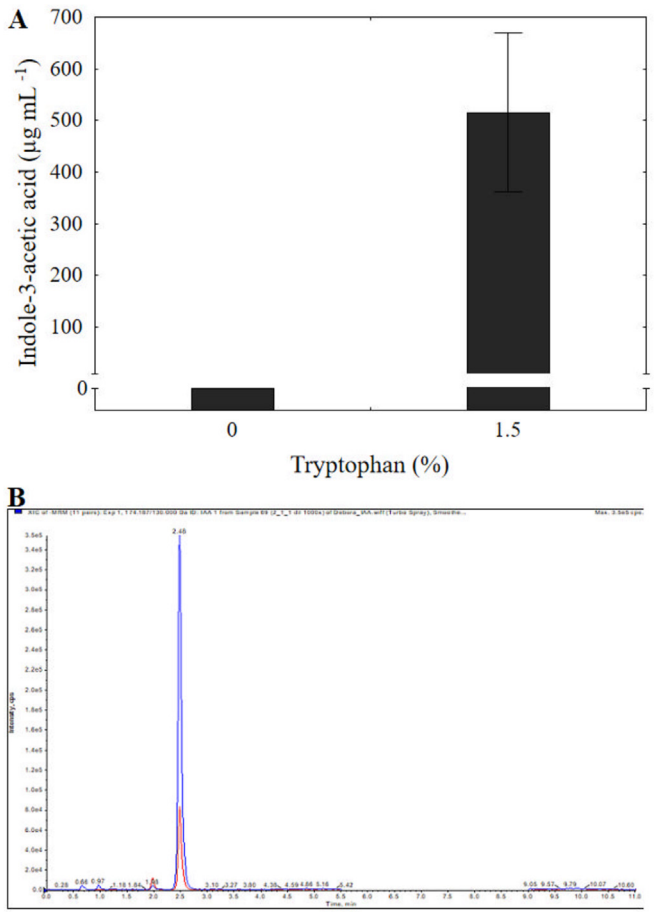

Fig. 3. Production of indole-3-acetic acid (IAA) by *A. flavipes* cultivated in soybean bran (0.5 mm) supplemented with 15 mL of water, in the presence and absence of tryptophan (1.5%) (A), and the chromatogram of the extract with tryptophan (MM = 175,187) and the secondary ions (MM = 130.0 e 127.9) (B).

better the efficiency of the method, the higher are the costs to perform them. In our study, due to a high number of strains, we firstly performed the quantification of IAA and analogues by a spectrophotometric method in order to select the strain with the highest IAA production for tests under solid-state fermentation.

The selected strain, *A. flavipes*, was reported to produce IAA under solid-state fermentation for the first time. Previously, the production of IAA was reported by several microbial species, among them, *Bacillus subtilis* (106.36 μg mL^{-1}), *Aspergillus ustus* (7.94 μg mL^{-1}) (Salas-Marina et al., 2011), *Aspergillus nidulans* (5 pmol mL^{-1}) (Eckert et al., 1999), *Bacillus megaterium* (2.2 μg 100 mL^{-1}) and *Bacillus cereus* (0.8 μg 100 mL^{-1}) (Karadeniz et al., 2006). Thus, in addition to unprecedented, the productivity of *A. flavipes* under optimized conditions, in soybean bran (515 μg mL^{-1}), is higher than the productivity of other microorganisms.

The auxin production of *A. flavipes* was exponentially increased (from 0.3 to 515.5 μg mL^{-1}) by tryptophan addition. IAA can be synthesized using L-tryptophan as a precursor and other tryptophan-independent pathways (Zhao, 2010). Although the biosynthetic pathways of IAA production by microorganisms have not yet been fully elucidated, it is described that tryptophan is used as the main precursor for both bacteria and fungi, through intermediates such as indole-3-pyruvic acid (IPA), indole-3-acetamide (IAN), and tryptamine (TAM) (Spaepen and Vanderleyden, 2010). Thus, our study indicates that the production of IAA by *A. flavipes* is most likely performed by a tryptophan-dependent biosynthetic pathway.

4.2. Biostimulant effect in the hybrid Eucalyptus grandis x E. urophylla (clone IPB2)

Both application forms (SF and LF) of the biostimulant (*A. flavipes* cultivated in soybean bran) modified the growth and development of the hybrid *Eucalyptus grandis* x *E. urophylla* (clone IPB2). Thus the process and the product were patented and deposited in the National Industrial Property Institute (INPI) under the register BR 10 2018 007927 1. In the literature, there are many reports about the effect of diverse categories of biostimulants, with direct or indirect auxin effects, on plant growth and development (Calvo et al., 2014).

Protein hydrolysates (PHs) are biostimulants composed of peptides and amino acids. PHs have been associated to direct effects in plants as increases in growth, yield, quality, and tolerance to environmental and chemical soil stresses; and indirect effects as the stimulus of beneficial microorganisms growth, such as N_2-fixing, P-solubilizing and IAA-producing bacteria (Colla et al., 2015).

Seaweed extracts can also have biostimulant function. Extracts from *Laminaria* and *Ascophyllum nodosum*, in general, stimulated root growth, nutrition, esterase activity, and sugar content of maize (*Zea mays* L.) plants. An elevate content of IAA (32.43 nM) was found in the most effective extract of *A. nodosum* for promoting root morphology traits (Ertani et al., 2018).

The commercial formulation Humyk-Fer (Duclos International), composed of 73% humic acid (HA), 27% fulvic acid (FA) and metal ions (Fe^{2+}, K^{+}, Na^{+}), increased the growth of *L. camara* (Costa et al., 2008). In another study, IAA was detected in various extracts of HA and all samples promoted root growth and proton pump activity in maize vesicles (Jindo et al., 2012).

As reported in our study, some species of *Aspergillus* have also

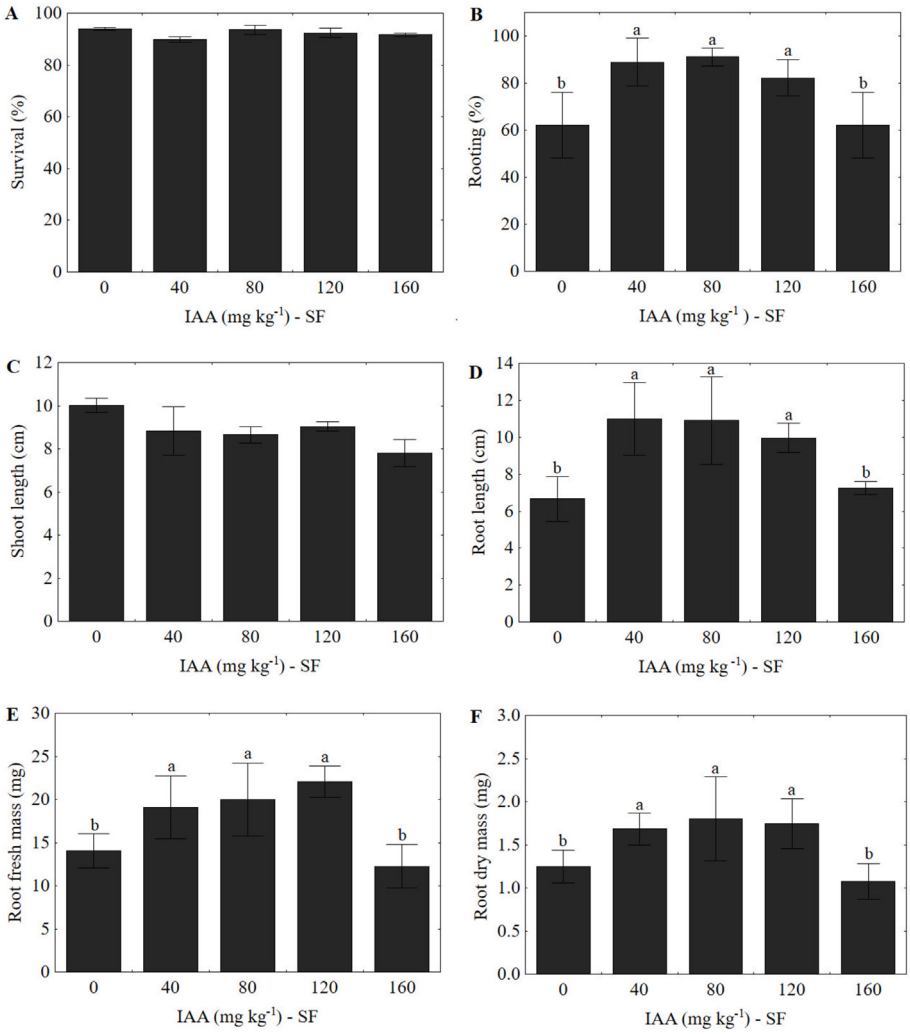

Fig. 4. Survival (%) (A), rooting (%) (B), average shoot length (C), average root length (D), average root fresh mass (mg) (E) and average root dry mass (F), in response to treatments with solid form (SF) of the biostimulant at 0, 40, 80, 120 and 160 mg kg^{-1} of IAA, 40 days after planting.
Mean followed by the same letter do not differ statistically according to the Scott Knott test ($p \leq 0.05$).

been described in the literature as inoculants with biostimulant function. The fungus *Aspergillus ustus* induced growth of shoots and roots and increased the number of lateral roots and root hairs of *Arabidopsis thaliana* and *Solanum tuberosum* (Salas-Marina et al., 2011), while the coinoculation of *Aspergillus niger* and *Trichoderma harzianum*, both producers of IAA, increased shoot length, root length and dry weight of shoot and root of chickpea (*Cicer arietinum*) (Yadav et al., 2011).

Indeed, there are growing evidences about the effectiveness of diverse classes of biostimulant on plant growth and development. In the future, biostimulant research should focus in the combination of biostimulant categories with complementary characteristics, such as microbial inoculants with seaweed extracts or humic substances (Calvo et al., 2014).

4.2.1. Application forms

Regarding the application forms, the SF from 40 to 120 mg kg^{-1} of IAA, increased rooting percentage, root length and fresh and dry root masses (Fig. 4); while LF increased root length and root dry mass only in treatment with 120 mg kg^{-1} of IAA equivalents (Fig. 5).

Natural and synthetic auxins have been applied to cuttings, both in solution and in solid form, mixed with talc, aiming at inducing rooting. Woody pecan cuttings (*Carya illinoinensis*) were treated with aqueous solutions of IAA and indole-3-butyric acid (IBA) (0.03%, 0.06%, and 0.09%) and naphthalene acetic acid (NAA) (0.06%, 0.09%, and 0.12%), where the rhizogenesis occurred sooner with 0.09% of NAA (Zhao and Zhang, 2015). In *Azadirachta indica* A. Juss (Neem), aqueous solutions of IBA, IAA, and NAA (100, 250, 500, 750, 1,000 and 1,500 mg L^{-1}) were applied to cuttings and treatment with 500 mg L^{-1} of IBA induced a higher percentage of rooting (Gehlot et al., 2015). Rooting and root system quality of black pepper (*Piper nigrum* cv. Bragantina) were improved when cuttings were exposed to 4,000 mg kg^{-1} of IBA mixed with talcum powder (Freire et al., 2017). In guava cuttings (*Psidium guajava* L.) CV. Safeda different auxins (IBA, IAA and, NAA 100 mg per 100 g talcum powder) were applied. All auxins improved rooting, but cuttings treated with IAA reached the highest survival percentage and number of roots, 27.6 roots, while the treatments with IBA and NAA resulted in 23 roots (Zamir et al., 2017).

Although both aqueous and solid forms of IAA have shown promising results for cuttings rooting in several studies until now there are no comparative discussions between the methods. For the hybrid *Eucalyptus grandis* x *E. urophylla* (clone IPB2), the SF treatment stood out in relation to the LF, exerting better effects and

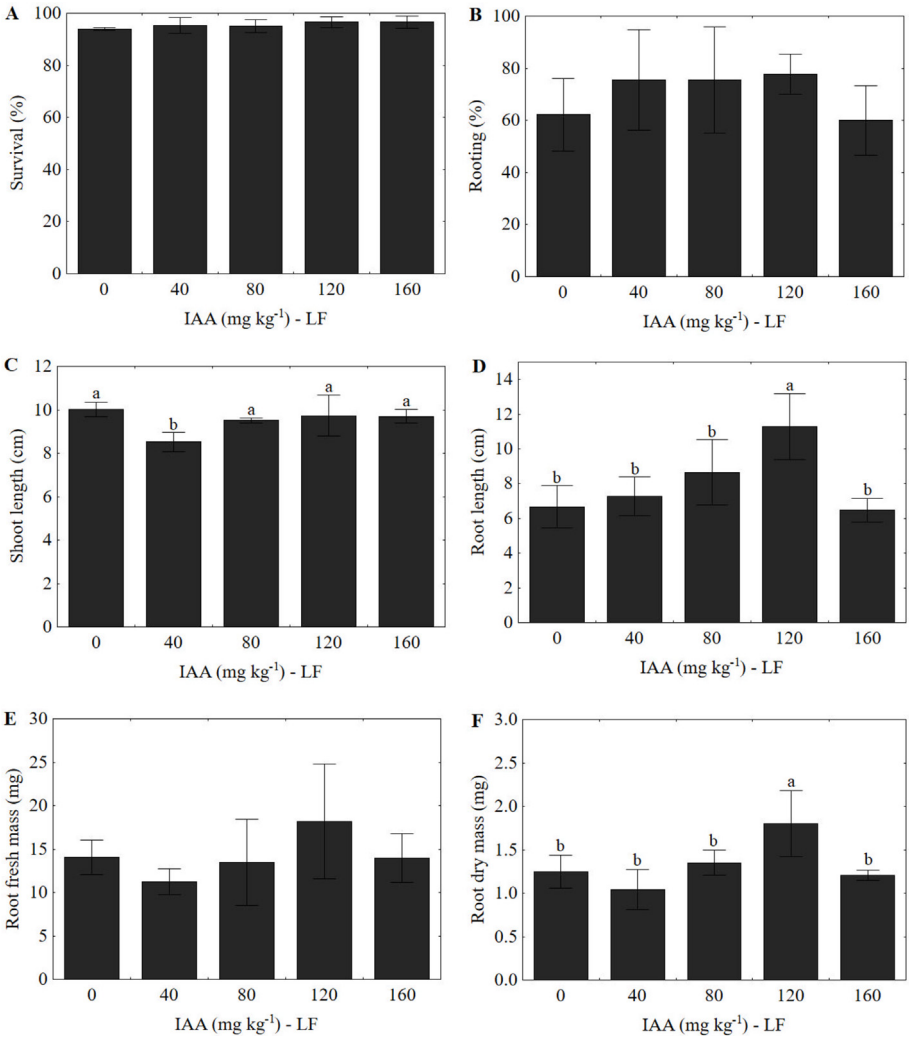

Fig. 5. Survival (%) (A), rooting (%) (B), average shoot length (C), average root length (D), average root fresh mass (mg) (E) and average root dry mass (F), in response to treatments with liquid form (LF) of the biostimulant at 0, 40, 80, 120 and 160 mg kg^{-1} of IAA, 40 days after planting.
Mean followed by the same letter do not differ statistically according to the Scott Knott test ($p \leq 0.05$).

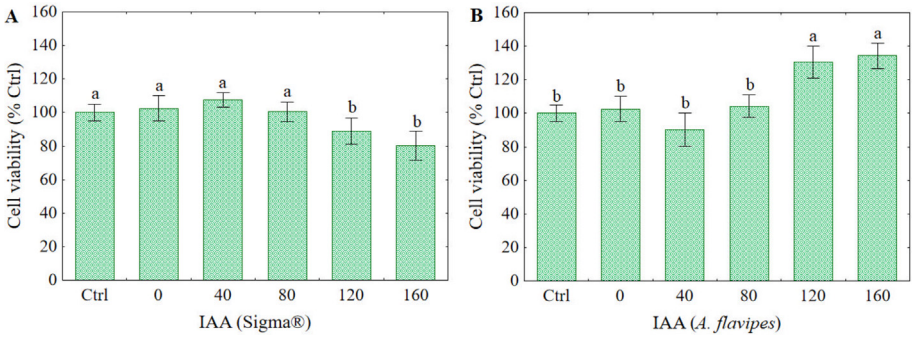

Fig. 6. Viability of fibroblasts (NIH/3t3) under increasing doses (0, 40, 80, 120 and 160 mg kg^{-1}) of synthetic indole-3-acetic acid (IAA) (Sigma®) (A) and produced by *A. flavipes* (B).
Means statistically compared by Scott Knott test ($p \leq 0.05$).

requiring one-step less of processing. In addition, a carrier substrate of the inoculant is important to provide a stable environment for the inoculum and extend the product shelf-life (Malusá et al., 2012). Between the SF doses, we consider 40 mg kg^{-1} of IAA the most advantageous for rooting *E. grandis* x *E. urophylla*, for promoting similar effects to 80 and 120 mg kg^{-1} at a lower cost of production.

4.2.2. Inhibitory tendency

There was an inhibitory tendency, on the majority of the analyzed variables, due to the application of the highest dose of both SF and LF (160 mg kg^{-1} of IAA). At low concentrations, auxins stimulate growth and development processes in plants, but with increasing concentration and activity in the tissues, the plant

growth is disturbed and the plant may be lethally affected (Grossmann, 2010). Therefore, our results suggest that the highest doses of SF and LF inhibited *E. grandis* x *E. urophylla* rooting and development.

Synthetic auxin-like compounds are among the most successful herbicides used in agriculture, as they have greater stability in plants than IAA, as well as systemic mobility and selective action (Grossmann, 2010). However, the most widely used auxinic herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D) generates waste, contaminating air, water, soil and food and the exposure to these wastes can cause adverse effects on human health (Bukowska, 2006), as genotoxicity (Madrigal-Bujaidar et al., 2001), neurotoxicity (Tayeb et al., 2010), hepatotoxicity (Bortolozzi et al., 2001) and renal toxicity (Uyanikgil et al., 2009). In contrast, microbial inoculants have been outstanding for solving environmental problems (Calvo et al., 2014).

Due to the inhibitory effects of the highest dose on rooting, it is possible that in addition to the biostimulating effect, the product described in the patent BR 10 2018 007927 1, in high concentrations, could disturb the plant development, acting as herbicide. Thus, in order to verify this effect, future studies should test higher doses of the product and investigate the possible effects on environmental toxicity and human health.

4.3. Fermentation product effects on fibroblasts

Plant hormones are present in all vegetables, and consequently, are ingested in the diet, with consequences for human physiology, but their mode of action is not fully elucidated (Chanclud and Lacombe, 2017). Some adverse effects for IAA have been reported. Higher doses of IAA, 2-naphthoxyacetic acid and 2,4-D caused significant modifications in biomembrane properties (Flasinski and Paulina, 2017). Neutrophils and lymphocytes incubated with IAA showed depolarization of mitochondrial transmembrane potential and increased activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase, showing that the IAA induced cell death process involves the production of reactive oxygen species (ROS) (Melo et al., 2004).

However, IAA has recently been used in treatments for human skin, and recognized as a new photosensitizer with minimal adverse effects (Jang et al., 2011). IAA associated with photodynamic therapy (PDT) was pointed out as a possible treatment for actinic keratoses (form of squamous cell carcinoma that occurs in chronically photoexposed skin) (Grandi et al., 2016), facial seborrheic dermatitis (Kwon et al., 2014) and mild to moderate acne (Jang et al., 2011), while free radicals induced by IAA associated with the enzyme *horseradish peroxidase* lead to the apoptosis of human melanoma cells (Kim et al., 2004).

In our study, in healthy cells, there was evidence of minimal adverse effects on the cell viability in result to IAA application. NIH/t3t viability was decreased only using the two highest doses of synthetic IAA, while the fungal IAA increased cell viability. Fibroblast assays have been shown to be efficient in the analysis of skin irritation caused by a large number of agents (Bason et al., 1991; Lee et al., 2000), therefore, the product can be considered safe for handling.

5. Conclusion

In this study, we reported the production of indole-3-acetic acid (IAA) by the fungus *A. flavipes* (ATCC® 16814™) under solid-state fermentation, for the first time. Among the tested conditions, we reached the best IAA yield using soybean bran (0.5 mm), with 15 mL of water and 1.5% of tryptophan.

We confirmed the biostimulating effect of the fermentation product in the clone IPB2 of the hybrid *Eucalyptus grandis* x *Eucalyptus urophylla*, increasing rooting. We consider the solid form (SF) advantageous in comparison to the liquid form (LF), for showing better-rooting results and one-step less of processing. The biostimulating effect of SF occurs in the range of 40–120 mg kg^{-1} of IAA, being recommended the lowest IAA dose due to the greater preparation facility, less need of labor resources and lower production costs.

In addition to the positive effects on rooting, the product is non-toxic to fibroblasts (NIH/3t3), while the synthetic IAA showed toxicity in NIH/3t3 from the dose 120 mg kg^{-1}. The biostimulant can also be considered environmentally sustainable and inexpensive, due to the use of agro-industrial wastes for its production.

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