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Regulation of IAA Biosynthesis in *Azospirillum brasilense* Under Environmental Stress Conditions

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Abstract

Indole-3-acetic acid (IAA) is one of the most important molecules produced by *Azospirillum* sp., given that it affects plant growth and development. *Azospirillum brasilense* strains Sp245 and Az39 (pFAJ64) were pre-incubated in MMAB medium plus 100 mg/mL L-tryptophan and treated with or exposed to the following (a) abiotic and (b) biotic stress effectors: (a) 100 mM NaCl or Na_2SO_4, 4.0% (w/v) PEG_{6000}, 0.5 mM H_2O_2, 0.1 mM abscisic acid, 0.1 mM 1-aminocyclopropane 1-carboxylic acid, 45 °C or daylight, and (b) 4.0% (v/v) filtered supernatant of *Pseudomonas savastanoi* (*Ps*) or *Fusarium oxysporum* (*Fo*), 0.1 mM salicylic acid (SA), 0.1 mM methyl jasmonic acid (MeJA), and 0.01% (w/v) chitosan (CH). After 30 and 120 min of incubation, biomass production, cell viability, IAA concentration (μg/mL), and *ipdC* gene expression were measured. Our results show that IAA production increases with daylight or in the presence of PEG_{6000}, ABA, SA, CH, and *Fo*. On the contrary, exposure to 45 °C or treatment with H_2O_2, NaCl, Na_2SO_4, ACC, MeJA, and *Ps* decrease IAA biosynthesis. In this report, growth and IAA biosynthesis in *A. brasilense* under biotic and abiotic stress conditions are discussed from the point of view of their role in bacterial lifestyle and their potential application as bioproducts.

Introduction

The genus *Azospirillum* consists of diazotrophic gram-negative bacteria that colonize the rhizosphere and intercellular root spaces of several plant species. There are numerous reports on the improvement of plant growth and crop yield upon inoculation of plant roots with *Azospirillum* sp. [5], and it is therefore classified as a plant-growth promoting rhizobacterium (PGPR) [18]. The stimulatory effects of *Azospirillum* on root development are well documented, and morphological changes have been repeatedly attributed to its production of plant-growth promoting substances. It has been shown to produce the five major plant hormones: auxins [12], cytokinins [14], gibberellins [7], abscisic acid [17], and ethylene [33]. However, observed plant response to inoculation has been mainly attributed to the activity of indole-3-acetic acid (IAA) in roots [3, 23]. One of the most important naturally occurring auxins in nature is IAA, which is implicated in many aspects of plant growth and development. In addition to being produced by plants, IAA is widespread among plant-associated bacteria [25]. Biochemical and genetic analyses of IAA biosynthesis in *Azospirillum brasilense* revealed multiple pathways that use the amino acid tryptophan (Trp) as a precursor [28], with the indole-3-pyruvate pathway being the most prevalent. The key enzyme of this pathway is indole-3-pyruvate decarboxylase (encoded by the *ipdC* gene), since an *ipdC* knock-out mutant is strongly reduced during IAA biosynthesis under all conditions tested [36]. Dobbelaere et al. [13] demonstrated that plant inoculation with the wild-type strain *A. brasilense* Sp245 enhanced root hair formation, while an *A. brasilense* Sp245 *ipdC* knock-out mutant failed to do so, which confirms the important role of bacterial IAA in plant interaction. Results obtained in *Azospirillum* field inoculation trials are numerous but somewhat inconsistent [4], suggesting that *Azospirillum* sp. or bacterial IAA biosynthesis

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may be drastically affected by environmental conditions. A significant number of reports regarding factors that influence the growth of *A. brasilense* have been published (see [5] for further details), but few of them focus on bacterial capacity to produce IAA [8, 24, 30, 31, 36]. An evaluation of biotic and abiotic stress conditions, as well as plant stress signaling molecules that frequently occur in the rhizosphere under plant stress conditions, could provide a better understanding of environmental factors regulating IAA biosynthesis and bacterial growth promotion. Our hypothesis is that biotic and abiotic environmental stress effectors, as well as plant signaling molecules related with plant responses to stress, are able to modify growth in bacteria and/or their capacity to produce IAA. The main aim of this work was to evaluate a set of these effectors and molecules which may come into play in the regulation of growth, *ipdC* expression, and IAA biosynthesis in *A. brasilense*, and to discuss their tentative role in bacterial lifestyle in the rhizosphere and their potential application in the formulation of bioproducts.

Materials and Methods

Strains and Plasmids

The strains and plasmids used in this study are listed in Table 1. *A. brasilense* was routinely maintained on L-malate minimal medium (MMAB) [37]. For the genetically modified strains (pFAJ64), the antibiotic tetracycline (Tc) was added to a final concentration of 10 µg/mL.

Conjugation of *A. brasilense* Az39 with pFAJ64

To study the expression of the *ipdC* gene, we used *A. brasilense* Sp245 (pFAJ64) [36] and moved the plasmid pFAJ64, which contains an *ipdC–gusA* fusion, from *E. coli* to the strain *A. brasilense* Az39 by tri-parental conjugation [37].

Bacterial and Fungal Growth and Culture Conditions

The bacteria were grown at 30 °C with 200 rpm orbital shaking in Luria broth (LB) supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ (L* medium), until they had reached exponential growth phase corresponding to OD_{595} 1.2. Cells were harvested by centrifugation (4000×*g*, 5 min), washed with sterile phosphate buffer solution (containing 1.24 g K_2HPO_4, 0.39 g KH_2PO_4, and 8.80 g NaCl per liter) and re-suspended to obtain a concentration of 10^9 colony forming units (CFU/mL). Aliquots were taken and transferred to a 1000 mL capacity flask containing 250 mL minimal MMAB medium and MMAB supplemented with 100 mg/mL L-tryptophan (L-Trp) [34]. In keeping with the treatment protocol, bacteria were grown at 30 °C with 200 rpm shaking until early exponential growth phase (OD_{595} 0.6) or stationary growth phase (OD_{595} 1.4–1.5). In the case of the early exponential growth phase, 5 mL aliquots were transferred into 10 mL sterile glass tubes, supplemented with 100 mg/mL L-Trp and pre-conditioned at 30 °C with 230 rpm shaking for 60 min. Then, cultures were incubated for 2 h at 30 °C with 200 rpm shaking in the presence of the following (a) abiotic and (b) biotic stress effectors (i.e., stress molecules, stress conditions, or plant signaling molecules), summarized in Table S1: (a) 100, 200, 400 mM NaCl or Na_2SO_4; 2.0, 4.0, and 8.0% (w/v) PEG_{6000}; 0.5 mM H_2O_2; 0.05, 0.1, and 0.2 mM abscisic acid (ABA); 0.1 mM 1-aminocyclopropane 1-carboxylic acid (ACC); high temperature (45 °C) or daylight exposure (75 W, at 22.5 cm) and (b) 2.0, 4.0 and 8.0% (v/v) filtered supernatant of *Pseudomonas savastanoi* (*Ps*) or *Fusarium oxysporum* (*Fo*); 0.1 mM salicylic acid (SA); 0.05, 0.1 and 0.2 mM methyl jasmonic acid (MeJA); and 0.01%

Table 1 Bacterial and fungal strains and plasmid used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Bacterial strains		
Azospirillum brasilense Sp245	Wild-type strain, isolated from surface-sterilized wheat roots, Brazil	[2]
Azospirillum brasilense Sp245 (pFAJ64)	Tc^r, pLAFR3 containing *ipdC-gusA* translational fusion	[36]
Azospirillum brasilense Az39	Wild-type strain isolated of wheat roots and used for inoculants formulation, Argentina	WDCM31[a]
Azospirillum brasilense Az39 (pFAJ64)	Tc^r, pLAFR3 containing *ipdC–gusA* translational fusion	This study
Pseudomonas savastanoi pv. PT300	Wild-type strain and IAA-producer pathogen for plants	CMPG[b]
Plasmid		
pFAJ64	Tc^r, *ipdC–gusA* translational fusion	[36]
Fungal strain		
F. oxysporum f. sp. matthiolae	Wild-type strain phytopathogen	CMPG[b]

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(w/v) chitosan (CH). In the case of the stationary growth phase, cultures were maintained at 30 °C with 200 rpm shaking until a final OD_{595} between 1.4 and 1.6 was obtained. In all cases, biomass production (OD_{595}), viability (CFU/mL), IAA concentration (µg/mL), and *ipdC* gene expression in Miller Units (MU) were evaluated and compared between treatments. The fungal strain was grown in potato dextrose broth (PDB) agar. The supernatant was obtained from cultures of *F. oxysporum* grown in PDB medium flasks on a rotary shaker at 120 rpm and 25 °C. Then, cultures were centrifuged at 12,000×*g* for 20 min and the supernatant was collected. It should be noted that all experiments were performed to generate untimely and fast stress (i.e., non-adaptive stress conditions) at the exponential and early stationary growth phases. This strategy excluded the lag phase, because exposure to stress at the beginning of the bacterial culture would have selected only the resistant bacteria from the population and deviated behavior after exposure to stress. It should also be clarified that photo-oxidative stress was evaluated only in *A. brasilense* Az39.

ipdC Gene Expression

We measured *ipdC* gene expression in *A. brasilense* Sp245 (pFAJ64) and Az39 (pFAJ64) grown in MMAB medium and exposed to abiotic stress conditions or modified by the presence of stress effectors and plant signaling molecules. A quantitative analysis of β-glucuronidase activity was assayed in microtiter plates using the *gusA* extraction buffer and *p*-nitrophenyl-β-D-glucuronide as a substrate [15]. The measured β-glucuronidase activity [20] was used to monitor *ipdC* gene expression and represents the mean of three replicates.

IAA Biosynthesis

The bacterial cultures were centrifuged at 10,000 rpm for 10 min to collect the supernatants. Initially, 1 mL of supernatant was used to quantify IAA with the use of the colorimetric Salkowski reaction, as described by [27]. Further confirmation was conducted by a reverse-phase high-pressure liquid chromatography (HPLC) following Jensen et al. [16]. Briefly, an Agilent 1200 Series HPLC system with a Quaternary Pump and an Agilent Eclipse XDB-C18 column (4.6 mm diameter, 150.0 mm length and 5.0 µm particle size) was used at a flow rate of 1 mL/min. Elution was performed with a mixture of H_2O and MeOH (60:40) containing 0.5% acetic acid and monitored at 280 nm.

Statistical Analysis

Experimental data were analyzed with ANOVA, followed by Tukey *post hoc* test at $P < 0.05$. Values shown represent mean ± standard error of mean (SEM). Analyses and graphs were performed with GraphPad Prism 5.0 software. Experiments were carried out in triplicate.

Results

Bacterial Growth, IAA Biosynthesis, and *ipdC* Gene Expression

The addition of L-Trp to the culture medium did not have any effects ($P < 0.05$) on the growth of *A. brasilense* Sp245 and Az39 in terms of biomass (OD_{595}) or cell viability (CFU/mL) (Table 2). However, biomass values were higher for

Table 2 Bacterial growth and IAA biosynthesis of *A. brasilense* cultured in MMAB medium

	A. brasilense Sp245		*A. brasilense* Sp245 (pFAJ64)		*A. brasilense* Az39		*A. brasilense* Az39 (pFAJ64)	
Maximum value	MMAB	MMAB + L-Trp	MMAB	MMAB + L-Trp	MMAB	MMAB + L-Trp	MMAB	MMAB + L-Trp
Optical density (OD_{595})	1.513 ± 0.012^{b}	1.542 ± 0.021^{b}	1.443 ± 0.014^{a}	1.473 ± 0.022^{a}	1.613 ± 0.018^{c}	1.635 ± 0.021^{c}	1.521 ± 0.012^{b}	1.483 ± 0.035^{ab}
Cell number log_{10} (CFU/mL)	9.08 ± 0.11^{a}	9.13 ± 0.09^{a}	9.07 ± 0.14^{a}	9.11 ± 0.10^{a}	9.12 ± 0.06^{a}	9.10 ± 0.12^{a}	9.09 ± 0.09^{a}	9.10 ± 0.017^{a}
IAA concentration (µg/mL)	*	5.17 ± 0.39^{a}	*	6.03 ± 0.31^{b}	*	6.27 ± 0.26^{bc}	*	6.41 ± 0.28^{c}
ipdC expression (Miller units)	*	*	*	238.00 ± 1.31^{a}	*	*	*	196.00 ± 4.31^{b}

Average of OD_{595} at the start of the experiment (stationary growth phase): 1.201 ± 0.118

*IAA concentration below detection limit

Different letter differs significantly by Tukey post hoc test at $P < 0.05$

Az39 than for Sp245, independently of the addition of L-Trp to the culture medium. A significant increase in *ipdC* gene expression was observed in Sp245 compared to Az39, but interestingly, Az39 was able to produce the same amount of IAA (6.03 ± 0.31 vs. 6.41 ± 0.28) even though the *ipdC* gene was less expressed than in Sp245 (238.00 ± 1.31 vs. 196.00 ± 4.31).

Abiotic Stress

In relation with bacterial growth, both strains showed similar behavior under similar abiotic stress conditions (Table 3). We observed that NaCl and Na_2SO_4 (saline stress) significantly increased bacterial growth (OD_{595}) and cell viability (CFU/mL) in Sp245, while Az39 maintained a tendency

Table 3 Bacterial growth, IAA biosynthesis, and *ipdC* gene expression of *A. brasilense* Sp245 (pFAJ64) and Az39 (pFAJ64) growing in MMAB medium exposed to abiotic and biotic stress conditions or modified by presence of stress effectors and plant signaling molecules

	A. brasilense Sp245 (pFAJ64)				*A. brasilense* Az39 (pFAJ64)			
	Optical density	Cells viability	IAA biosynthesis	*ipdC* gene expression	Optical density	Cell viability	IAA biosynthesis	*ipdC* gene expression
Control abiotic	1.272 ± 0.014[d]	8.23 ± 0.16[b]	4.714 ± 0.281[d]	167.61 ± 9.05[d]	1.321 ± 0.017[d]	8.88 ± 0.23[c]	4.869 ± 0.280[c]	157.87 ± 5.05[d]
NaCl 100 mM	1.373 ± 0.007[e] +7.4%	8.91 ± 0.12[c] +6.9%	3.763 ± 0.213[c] − 20.1%	139.65 ± 1.84[c] − 16.6%	1.354 ± 0.026[d] +2.5%	8.91 ± 0.18[c] +0.3%	3.369 ± 0.281[b] − 30.8%	129.47 ± 4.12[c] − 18.0%
Na_2SO_4 100 mM	1.416 ± 0.016[f] +10.5%	9.02 ± 0.10[c] +8.2%	3.662 ± 0.135[c] − 22.3%	139.81 ± 3.85[c] − 17.0%	1.330 ± 0.019[d] +0.7%	8.94 ± 0.12[c] +0.6%	3.310 ± 0.286[b] − 32.0%	124.83 ± 3.47[c] − 21.0%
PEG_{6000} 4.0%	1.192 ± 0.014[c] − 6.3%	7.52 ± 0.21[a] − 10.3%	5.484 ± 0.254[f] +16.2%	196.72 ± 6.19[f] +17.0%	1.198 ± 0.028[d] − 9,3%	7.87 ± 0.08[b] − 11.4%	5.294 ± 0.287[d] +8.7%	178.51 ± 3.16[e] +13.0%
Temperature 45 °C	1.053 ± 0.071[b] − 17.2%	< 7.00	0.491 ± 0.610[a] − 89.5%	90.73 ± 1.85[b] − 45.8%	1.154 ± 0.025[b] − 12,6%	7.05 ± 0.03[a] − 20.6%	3.173 ± 0.283[b] − 34.8%	114.38 ± 6.17[b] − 27.5%
H_2O_2 0.5 mM	0.949 ± 0.014[b] − 23.5%	< 7.00	0.425 ± 0.883[a] − 90.9%	8.88 ± 0.53[a] − 94.7%	1.038 ± 0.012[a] − 21.4%	< 7.00	2.478 ± 0.285[a] − 49.1%	11.32 ± 4.01[a] − 92.9%
ABA 0.1 mM	1.205 ± 0.014[c] − 5.2%	7.58 ± 0.09[c] − 7.9%	5.286 ± 0.252[c] +12.1%(*)	184.88 ± 6.41[e] +10.7%(*)	1.247 ± 0.004[c] − 5,6%	8.35 ± 0.12[d] − 5.9%	5.465 ± 0.284[d] +11.2%(*)	185.05 ± 10.5[e] +17.2%(*)
ACC 0.1 mM	1.431 ± 0.028[f] +11.6%	8.19 ± 0.08[b] +0.5%	2.971 ± 0.152[b] − 36.9%	135.61 ± 6.00[c] − 19.1%	1.406 ± 0.014[d] +6.4%	9.11 ± 0.15[d] +2.6%	3.251 ± 0.281[b] − 33.2%	117.37 ± 4.23[b] − 25.6%
Control Biotic	1.314 ± 0.009[c]	8.72 ± 0.11[c]	5.646 ± 0.071[c]	226.26 ± 6.41[a]	1.349 ± 0.012[b]	8.36 ± 0.13[bc]	5.980 ± 0.071[b]	175.95 ± 4.12[a]
MeJA 0.1 mM**	1.135 ± 0.024[a] − 13.6%	8.07 ± 0.21[a] − 7.4%	3.681 ± 0.189[a] − 34.0%	261.64 ± 11.3[b] +15.3%	1.210 ± 0.017[a] − 10.3%	7.89 ± 0.17[a] − 4.54%	5.602 ± 0.071[a] − 16.5%	199.16 ± 5.33[b] +13.2%
SA 0.1 mM**	1.268 ± 0.011[b] − 3.5%	8.53 ± 0.19[c] − 2.1%	6.263 ± 0.097[d] +10.9%	235.63 ± 4.05[a] +4.1%	1.347 ± 0.022[b] − 0.15%	8.21 ± 0.09[b] − 1.79%	6.086 ± 0.071[b] +5.3%	178.56 ± 1.10[a] +1.5%
Chitosan 0.01%	1.243 ± 0.032[b] − 5.4%	8.24 ± 0.15[ab] − 5.5%	6.336 ± 0.112[d] +12.2%	229.13 ± 3.32[a] +1.2%	1.309 ± 0.031[b] − 2.7%	8.08 ± 0.07[a] − 3.5%	6.208 ± 0.071[c] +11.5%	183.41 ± 2.19[a] +4.2%
P. savastanoi supernatant 4.0%	1.396 ± 0.012[d] +6.2%	9.21 ± 0.13[b] +5.6%	3.663 ± 0.145[a] − 35.1%	265.85 ± 2.11[b] +17.5%	1.4219 ± 0.007[c] +5.3%	8.94 ± 0.13[d] +6.9%	5.621 ± 0.071[a] − 18.1%	201.81 ± 4.65[b] +14.7%
F. oxysporum supernatant 4.0%	1.349 ± 0.030[c] +2.9%	8.75 ± 0.19[c] +0.3%	5.953 ± 0.065[b] +5.4%	236.68 ± 4.23[a] +4.6%	1.403 ± 0.015[c] +4.0%	8.44 ± 0.10[c] +0.9%	6.210 ± 0.071[c] +11.6%	181.36 ± 6.01[a] +3.0%

Treatments were started at exponential growth phase ($OD_{595} \approx 1.0$) and incubated 2 h at 30 °C and 200 rpm in each condition

Units: Optical density (OD_{595}); cells viability (log_{10} CFU/mL); IAA biosynthesis (µg/mL); *ipdC* gene expression (Miller units)

*At exponential growth phase ABA reduced both IAA biosynthesis and *ipdC* gene expression with an average of 10.0% after 240 min incubation. At stationary growth phase, ABA induced IAA biosynthesis and *ipdC* expression after 30 min incubation but reduced these parameters at 120 min

**SA and JA were homogenized in 70:30 (v/v) of alcohol:water solution. A control solution was previously compared with water control to eliminate a possible deleterious effect of the methanol on bacteria

Different letter differs significantly by Tukey post hoc test at $P < 0.05$

towards increase, but there were no significant differences with the control treatment. When ACC (an ethylene precursor) was added to the culture medium of Sp245, only OD_{595} increased, while an enhancement in cell viability was registered for Az39. Daylight (photo-oxidative stress) had a similar effect on biomass to that produced by ACC (Fig. 1). It increased biomass production by *A. brasilense* Az39 close to 30, 6, and 11.0% after 24, 48, and 72 h of exposure, respectively, in comparison with the control treatment which was cultured in total absence of light. Nevertheless, the number of viable cells was similar under light or darkness culture conditions (7–8E+9 CFU/mL). IAA production by Az39 was higher under daylight than darkness treatment after 24, 48, or 72 h of exposure, but reached a maximum increase value of 50.0% at 48 h. On the contrary, PEG_{6000} (osmotic stress), high temperature, H_2O_2 (oxidative stress), and ABA (a plant signaling molecule related with plant abiotic stress) had a negative effect on bacterial growth. Temperature and H_2O_2, in particular, were lethal for both strains. With regard to IAA biosynthesis and *ipdC* gene expression, the results indicate that both parameters decreased significantly in the presence of NaCl and Na_2SO_4 in the culture medium as well as precursor ACC, high temperature, and H_2O_2 (Table 3). In the case of exposure to high temperature (45 °C) and the presence of H_2O_2, the effect was severe, with a 90.0% reduction related to bacterial inability to grow and accumulation of secondary metabolite IAA in the culture medium. Conversely, IAA production and *ipdC* gene expression increased in the presence of PEG_{6000} and ABA (Table 3) or when the bacterial culture was exposed to white light (75 W) (Fig. 1). Our results support the idea that abiotic stress conditions, or the presence of stress effectors or plant signaling molecules in the culture medium modified the bacterial capacity for growth and produced IAA in a particular way. In this regard, bacterial growth was negatively affected by osmotic stress (PEG) and the presence of ABA. Oxidative stress (H_2O_2) and high temperature (45 °C), for their part, were lethal. On the contrary, saline stress, ACC, and exposure to white light (75 W) stimulated bacterial growth. In the cases of white light and ACC, biomass increase (OD) was not associated with an increase in cell viability (Table 3). In all cases, an increase in *ipdC* gene expression was associated with an increase in the biosynthesis and concentration of IAA in the culture medium. Thus, white light, PEG, and ABA stimulated *ipdC* gene expression, and IAA biosynthesis and concentration, while saline stress, high temperatures, oxidative stress, and ACC negatively affected both parameters.

We extended the experiments above by including increasing concentrations of saline and osmotic stress effectors, such as NaCl (100, 200, 400 mM), PEG_{6000} (2.0, 4.0, 8.0%), and the signaling molecule ABA (0.05, 0.1, 0.2 mM). Results are the difference in percentage between Sp245 and the control treatment (DAC) (Fig. 2). Essentially, the addition of NaCl to the culture medium caused a dose-dependent response at both incubation times, with an increase in bacterial growth (7.4% OD and 6.9% cell viability, Table 3) but a decrease in *ipdC* gene expression (16.6%) and IAA biosynthesis (20.1%). Higher salt concentration and exposure time were associated with the lowest values for *ipdC* gene expression and IAA production (Fig. 2). By contrast, osmotic stress generated by the addition of 2 and 4.0% PEG_{6000} increased *ipdC* gene expression and IAA biosynthesis, but reduced them down to 8.0% after 30 and 120 min of exposure (Fig. 2). In the same conditions, similar effects were observed for Az39 in comparison with Sp245 (data not shown). The addition of ABA to the culture medium produced an increase both in *ipdC* gene expression and IAA biosynthesis (Fig. 2) after 30 and 120 min of incubation. This pattern was dose-dependent, in a similar manner to that obtained with the use of NaCl. No significant differences

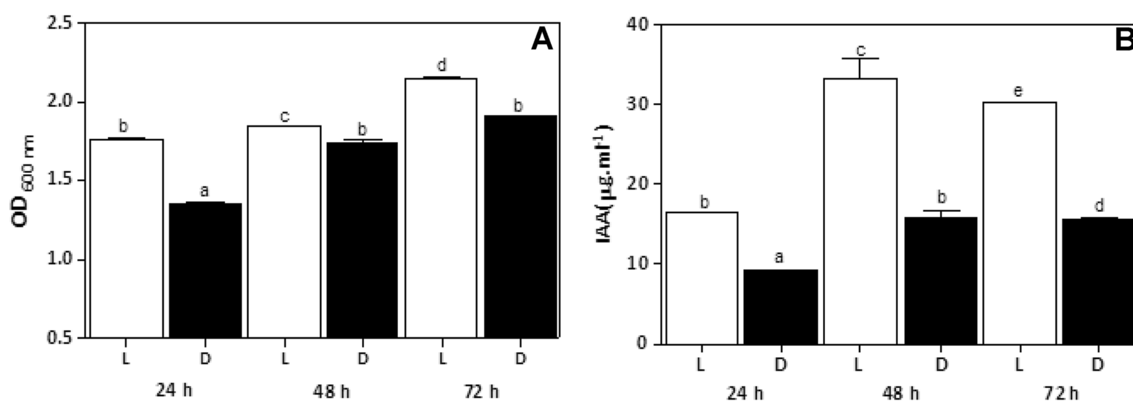

Fig. 1 Bacterial growth as biomass production (**a**) and IAA biosynthesis (**b**) of *A. brasilense* Az39 growing in LB medium, after exposure to daylight (L) (75 W at 22.5 cm from the source) or darkness (D). Treatments were started at exponential growth phase ($OD_{595} \approx 0.4$) and incubated for 24, 48 , and 72 h at 30 °C under static conditions. Columns marked with a different letter differ significantly by Tukey post hoc test at $P < 0.05$

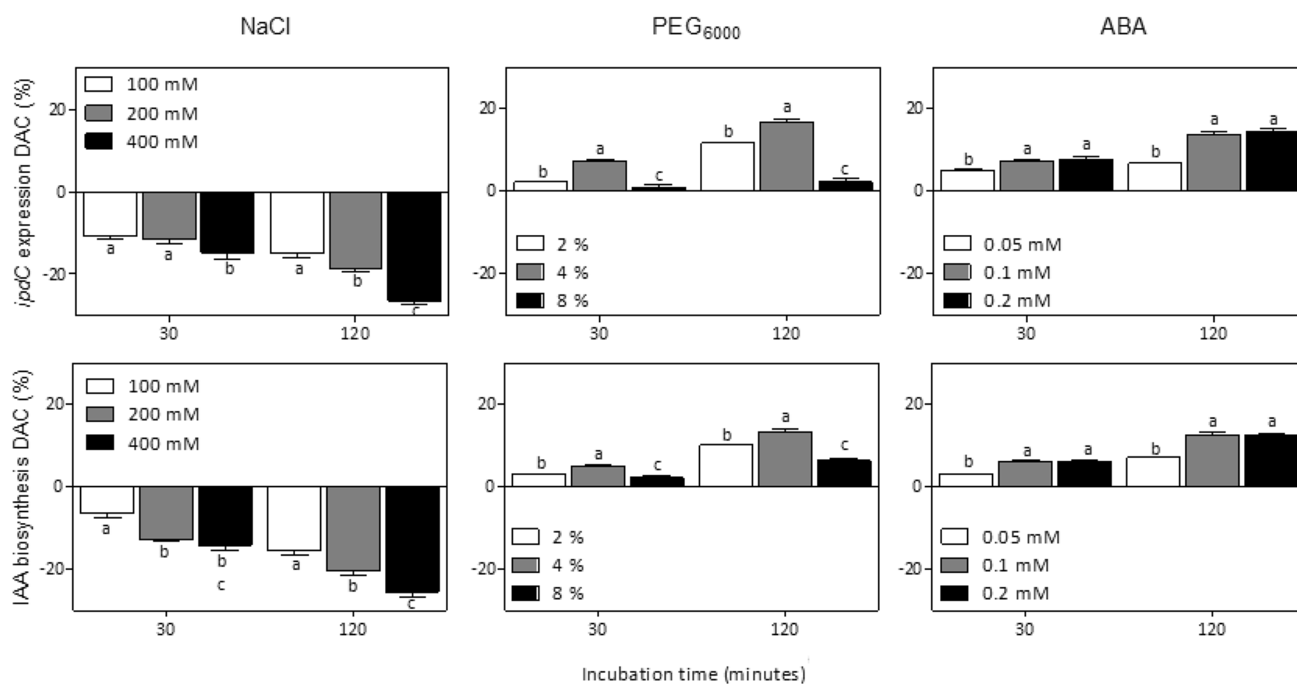

Fig. 2 IAA biosynthesis and *ipdC* gene expression of *A. brasilense* Sp245 (pFAJ64) growing in MMAB medium modified by addition of increasing concentrations of stress effectors. Bars represent the percentage of difference against control (DAC). Cultures were started at exponential growth phase ($OD_{595} \approx 1.0$) and incubated for 2 h at 30 °C with 200 rpm shaking. A similar pattern was found for *A. brasilense* Az39 (data not shown). Columns marked with a different letter differ significantly by Tukey post hoc test at $P < 0.05$

were observed between the results obtained with 0.1 and 0.2 mM ABA, which indicates that 0.1 mM is likely the highest dose with the capacity to generate a response in *A. brasilense* Sp245 and *A. brasilense* Az39.

Biotic Stress

Both Sp245 and Az39 strains grew similarly under similar biotic stress conditions (Table 3). The presence of methyl jasmonic acid (MeJA), salicylic acid (SA), and chitosan in the culture medium decreased bacterial growth, assessed both as optical density and cell viability. The most remarkable effect was obtained when adding MeJA. Growth was stimulated in both strains with the addition of *P. savastanoi*- and *F. oxysporum*-filtered supernatants, but the increase was not significant in the specific case of *F. oxysporum* added to Sp245. IAA biosynthesis decreased significantly in the presence of MeJA and *P. savastanoi*-filtered supernatant, whereas it increased in the presence of SA, chitosan, and *F. oxysporum*-filtered supernatant. Surprisingly, *ipdC* gene expression increased under all conditions (Table 3), exceeding 15.0 and 17.0% with MeJA and *P. savastanoi*, respectively, though it was not statistically significant for SA or chitosan (Table 3).

We developed new assays using the same experimental model, but this time in the presence of increasing concentrations of *P. savastanoi*- (Fig. 3a, b) and *F. oxysporum* (Figure S1C)-filtered supernatants, i.e., 2.0, 4.0, and 8.0% (v/v), or with the addition of 0.05, 0.1, and 0.2 mM MeJA (Figure S1D). The graphs show the difference in percentage between Sp245 and the control treatment (DAC) for IAA biosynthesis and *ipdC* gene expression. Similar results were found for Az39 (data not shown). The presence of *F. oxysporum* supernatants induced IAA biosynthesis as well as *ipdC* gene expression in a dose-dependent way (Figure S1C). Finally, there was an induction of the *ipdC* gene with 0.1 mM MeJA, but it was not accompanied by an increase in the production of IAA. With a higher dose (0.2 mM MeJA), both IAA biosynthesis and gene expression were diminished.

Pseudomonas savastanoi Cultures and IAA Biosynthesis

We performed a final assay with the aim of understanding whether bacterial growth and IAA biosynthesis were regulated by the presence of *P. savastanoi*-filtered supernatants obtained from different culture media. Following the experimental model described in "Bacterial and fungal growth and culture conditions," the strains were grown in MMAB, modified by the addition of *P. savastanoi*-filtered supernatant obtained from a culture medium containing alternative energy (C and N) sources. We performed stationary phase cultures of *P. savastanoi* in a protein-hydrolyzed

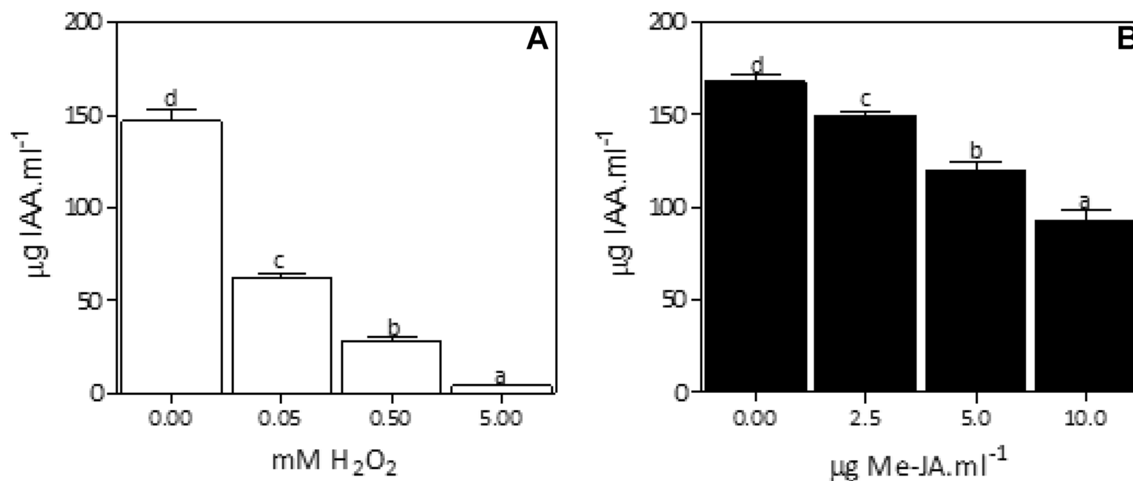

Fig. 3 IAA concentration obtained from 0.1 mM IAA solution modified by addition of increasing concentrations of pure water (control), H_2O_2 (**a**), or MeJA solutions (**b**), incubated for 30 min at 30 °C under shaking conditions. Columns marked with a different letter differ significantly by Tukey post hoc test at $P<0.05$

medium (trypticase soybean broth, named Ps-TSB) or a glycerol medium (named Ps-GB), and compared them with non-inoculated TSB and GB medium. In Sp245 and Az39, expression of the *ipdC* gene significantly increased but IAA biosynthesis decreased with the addition of *P. savastanoi* supernatants (Ps-TSB and P-G) (Figure S2). Likewise, there was a significant decrease of both parameters with the addition of non-inoculated TSB medium. In summary, Sp245 and Az39 had the same behavior under similar experimental conditions. Our results show that in the presence of *P. savastanoi* supernatants, *ipdC* gene expression increased while IAA biosynthesis was reduced. These results indicate that either a compound produced by *P. savastanoi* in TSB or GB medium, or a compound present in TSB medium is able to regulate IAA metabolism in *A. brasilense*. The inability of *Azospirillum* to produce IAA after the addition of TSB (control treatment) was attributed to the presence of free amino acids that inhibit bacterial production of IAA, even when the *ipdC* gene is expressed. This effect was consistently observed by Rivera et al. (personal communication, 2018) for the amino acid L-phenylalanine (regulated by *ipdC* at the level of phenyl acetic acid production). On the other hand, the presence of L-valine, L-serine, and L-leucine, among others, reduced IAA accumulation in the culture medium due to a reduction in bacterial growth or IAA biosynthesis (Rivera et al. personal communication, 2018).

Chemical Interference of H_2O_2 and MeJA with the IAA Molecule

To determine if there was any chemical interference of H_2O_2 or MeJA with the IAA molecule, IAA concentration was measured in non-inoculated MMAB culture medium modified by the addition of 10 μg/mL IAA and increasing concentrations of H_2O_2 and MeJA. The presence of these molecules in the culture medium resulted in a dose-dependent reduction of IAA concentration after different incubation times (Fig. 3).

Discussion

In this study, we focused on the ability of *A. brasilense* to grow and regulate auxin biosynthesis in the presence of a wide range of stress conditions, stress effectors, and plant signaling molecules. We analyzed bacterial growth and IAA biosynthesis in the model strain *A. brasilense* Sp245 and the agriculturally used strain *A. brasilense* Az39, under optimal growth conditions, in MMAB minimal medium with or without addition of the amino acid L-tryptophan as a precursor of IAA biosynthesis. The application of L-Trp did not have any effects on biomass or cell viability (Table 2). A significant increase (21.4%) in *ipdC* gene expression was determined for Sp245 in comparison with Az39, even though this last strain was able to produce and accumulate amounts of IAA similar to those accumulated by Sp245. Like many other bacteria, *Azospirillum* spp. require L-Trp to synthesize IAA [34]. In batch culture conditions *A. brasilense* Sp245 (pFAJ64) grows normally, but produces increasing concentrations of IAA in MMAB supplemented with increasing concentrations of L-Trp as a precursor. In these experiments, IAA biosynthesis coincided with *ipdC* gene expression [24]. In agreement with this, an increase in IAA concentration in the culture medium is preceded by *ipdC* gene expression in a "feed forward" regulation mechanism [36].

We analyzed growth and IAA biosynthesis in *A. brasilense* under saline stress conditions imposed by the addition of NaCl and Na_2SO_4 to the culture medium. Growth of *A. brasilense* Sp245 increased in the presence of 100 mM NaCl and Na_2SO_4, while both IAA concentration and *ipdC* gene expression significantly decreased. Additionally, IAA concentration and *ipdC* gene expression showed a dose-dependent decrease (100, 200, and 400 mM NaCl) (Table 3; Fig. 2). Similar results were obtained by Spaepen [32] for IAA biosynthesis, and by Tripathi et al. [35] at the level of biological nitrogen fixation. In this regard, Nabti et al. [22] measured growth and IAA production in both halophytic and non-halophytic strains of *A. brasilense*. The halophytic strain NH of *A. brasilense* showed optimal growth and a better capacity to produce IAA with 200 mM NaCl, but both parameters decreased with 1.7 mM NaCl. The non-halotolerant strain *A. brasilense* Sp7 showed a significant decrease in IAA biosynthesis with 300 mM NaCl. Madkour et al. [19] reported that growth of *A. brasilense* Sp7 was significantly inhibited by 0.5 M NaCl, but strains SBR, SHS6, and SLM5 showed greater tolerance to the salt at the same concentration. Thus, our results confirm previous reports about the capacity of *Azospirillum* sp. to regulate saline stress under moderate concentrations of sodium salts. Nevertheless, from the point of view of the plant–microbe interaction, this behavior should be understood as a bacterial capacity to respond to the saline condition in coordination with the plant, through the reduction of IAA biosynthesis and the consequent promotion of root growth.

When Sp245 and Az39 were exposed to osmotic stress conditions by the addition of increasing concentrations of PEG_{6000} to the culture medium at exponential growth phase, there was a decrease in bacterial growth but an increase in IAA biosynthesis and *ipdC* gene expression (Table 3; Fig. 2). Similarly, another study noted that when Az39 grew in the presence of 15 mM PEG_{6000}, bacterial biomass and cell viability were reduced around 37.0% in relation with the control treatment [9]. These authors showed that *A. brasilense* Az39 was able to adapt and grow in the presence of a moderate water deficit, though with significantly decreased viability. According to other reports, *A. brasilense* Sp245 tolerated exposure to 20.0% PEG_{8000} in a chemically defined OAB medium without an appreciable decrease in growth, measured as the number of viable cells [11]. From the point of view of the plant–microbe interaction model, the increase in microbial IAA biosynthesis under osmotic stress conditions could be understood as an adaptive mechanism through which the microbe coordinates its physiological response with the plant by increasing root growth via IAA homeostasis.

The exposure of *A. brasilense* Sp245 and Az39 to high temperature (45 °C) was lethal and significantly affected bacterial growth, *ipdC* gene expression, and consequently the biosynthesis of IAA. *A. brasilense* Sp245 grows when exposed to temperatures between 20 and 38 °C, but IAA biosynthesis is cell density-dependent, with the highest concentrations obtained with a maximum OD at 30 °C in MMAB supplemented with L-Trp [24].

Azospirillum brasilense Az39 and Sp245 were exposed to the presence of the ethylene precursor ACC in the culture medium. Some rhizobacteria containing the enzyme ACC deaminase interfere positively with plant response to abiotic stress through the breakdown of the plant-hormone precursor ACC [30]. However, in our experiments, addition of ACC to the culture medium increased growth (measured as biomass) in Az39 and Sp245 in the absence of an ACC deaminase. The addition of ACC reduced *ipdC* gene expression and IAA biosynthesis in *A. brasilense*. ACC might have a toxic effect on the microorganism, inducing the production of EPS or other molecules with the capacity to increase optical density but not cell number, and decreasing the production of secondary metabolites such as IAA (Table 3).

Azospirillum brasilense Az39 and Sp245 were exposed to ABA in the culture medium. These strains are able to produce ABA in a chemically defined medium as part of their normal metabolism [26] but the production is induced in the presence of NaCl [10]. In our experiments, the increase in IAA biosynthesis due to ABA addition probably occurred because of a direct regulation of the *ipdC* gene promoter. Even though auxin is the key hormone for lateral root formation and root emergence, many other regulators participate in the determination of root architecture.

Azospirillum brasilense Az39 and Sp245 were exposed to oxidative stress by the presence of H_2O_2. Growth of *A. brasilense* as well as IAA biosynthesis and *ipdC* gene expression was severely affected in both strains in the presence of this effector in the culture medium. IAA concentration was limited due to an interference in bacterial growth, a reduction in *ipdC* gene expression, and the direct oxidation of IAA by H_2O_2 due to the presence of O_2 [29]. Molina et al. [21] showed that H_2O_2 affected IAA biosynthesis in *Azospirillum* sp. and the stability of the molecule by direct oxidation. In previous reports, both H_2O_2 and Paraquat drastically affected the growth of *A. brasilense* Sp7 [6].

Regarding the incidence of light on growth, we considered daylight (60 W) an abiotic stress effector, since it was growth-inhibitory for *A. brasilense* Az39, Sp245, and Sp7 under direct exposure in an agar plate [21]. In our experiments, the growth of *A. brasilense* Az39 was promoted in terms of biomass production (OD_{595}), but no differences were registered in terms of cell number. Under daylight exposure (60 W), IAA production significantly increased in comparison with the control treatment in absence of light.

The results presented in this section have been summarized in Fig. 4, so that the response of *A. brasilense* Sp245

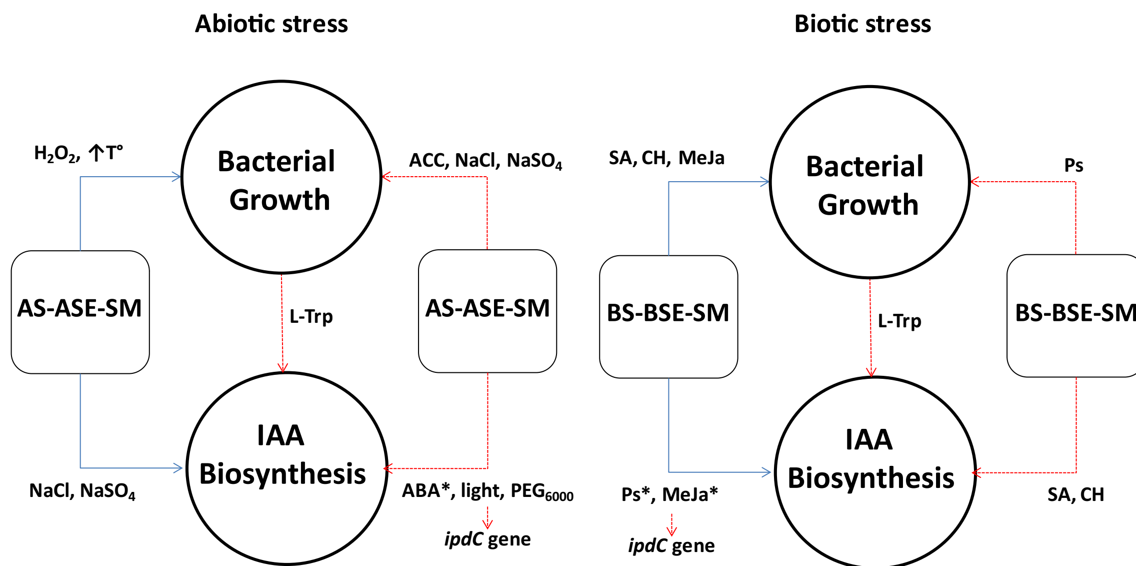

Fig. 4 Schematic model of the biotic and abiotic stress conditions, stress effectors, and related plant signaling molecules regulating the growth and IAA biosynthesis of *A. brasilense*. References: dotted line, induction; continuous line, repression. *AS* abiotic stress, *ASE* abiotic stress effector, *BS* biotic stress, *BSE* biotic stress effector, and *SM* signaling molecule

and Az39 under abiotic stress conditions, stress effectors, or plant signaling molecules may be seen at a glance.

We analyzed the response of *A. brasilense* under biotic stress conditions or the presence of stress effectors or plant signaling molecules in the culture medium. For instance, we observed the ability of *A. brasilense* to regulate IAA biosynthesis in the presence of filtered supernatants of the pathogenic fungus *F. oxysporum. F. oxysporum* supernatants increased growth, *ipdC* gene expression, and IAA biosynthesis in *Azospirillum* sp., probably due to the presence of fungal metabolites in the supernatant that the bacterium used as nutrient sources, or due to a signal molecule that induced bacterial IAA biosynthesis.

In the presence of methyl jasmonic acid (MeJA) and *P. savastanoi*-filtered supernatant, IAA biosynthesis decreased in *A. brasilense* even when *ipdC* gene expression increased. Jasmonic acid and its methyl ester (MeJA) are known for their participation in plant growth and stress tolerance and other physiological processes in plants (see review Ahmad et al. [1]). In our experimental conditions, biomass production and cell viability in *A. brasilense* were affected negatively by the presence of 0.1 mM MeJA, and there was also a decrease in IAA concentration in the culture medium (Table 3). These results allow us to consider two possible explanations: (1) the delay in bacterial growth delayed IAA biosynthesis, or (2) the MeJA interacted directly with the IAA molecule and decreased its concentration in the culture medium (Fig. 3). In the presence of *P. savastanoi*-filtered supernatant, biomass (OD_{595}) and cell viability increased concomitantly with *ipdC* gene expression, although IAA biosynthesis was diminished (Table 3; Figs. 3, 4). The presence of several free amino acids in the TSB culture medium (not fully depleted by *P. savastanoi*) modified the capacity of *A. brasilense* to synthesize IAA, even in the presence of L-Trp, because they are toxic and delay bacterial growth or directly modify IAA biosynthesis. The addition of L-phenylalanine to an MMAB culture medium containing L-Trp decreased IAA biosynthesis but increased *ipdC* gene expression. Both L-Phe and L-Trp are precursors for PAA and IAA, respectively, and are able to increase *ipdC* gene expression, but IPDC has a higher affinity for PAA [31]. In our experiments, the addition of MeJA or *P. savastanoi* to the culture medium of Sp245 and Az39 strains reduced IAA concentration but increased *ipdC* gene expression.

The increase in *ipdC* gene expression is linked to an increase in cell density in *A. brasilense*, and this expression is upregulated by IAA [28]. The increase in *ipdC* gene expression is normally accompanied by an increase in IAA biosynthesis [36], and this might be the major cause for the increase in IAA observed under our experimental conditions with the addition of salicylic acid (SA) or chitosan (CH) to the culture medium. However, the presence of such molecules reduced biomass production (OD_{595}) and cell viability (Table 3). In our experiments, *Azospirillum* sp. was able to produce IAA in the presence of SA and CH, independently of bacterial growth (Table 3). We observed that in the presence of CH, both cell viability and biomass production (OD_{595}) decreased, but IAA biosynthesis and *ipdC* gene expression increased (Table 3). In order to offer a simplified overview of the response of *A. brasilense* Sp245 and Az39 under biotic stress conditions, stress effectors, or

plant signaling molecules, the results from this section have been summarized in Fig. 4.

On the basis of the results presented, we propose two alternative models to explain growth and IAA biosynthesis in *A. brasilense* under abiotic and biotic stress conditions and the presence of stress effectors or plant signaling molecules in the same environment as the bacterium (Fig. 4). Our findings suggest that in most cases, positive or negative regulation of the *ipdC* gene under abiotic stress conditions correlates with IAA concentration in the culture medium. Thus, ABA and PEG were able to increase IAA biosynthesis due to the induction of the *ipdC* gene, and this event was independent of bacterial growth. On the other hand, daylight increased IAA biosynthesis in a bacterial growth-dependent way. Sodium salts reduced *ipdC* gene expression and IAA biosynthesis, but they stimulated growth in *A. brasilense*. Other stress effectors such as H_2O_2 and high temperatures decreased IAA biosynthesis and *ipdC* gene expression, because they were lethal for *A. brasilense*. Additionally, there was a direct interaction between IAA and H_2O_2 with a consequent reduction in the phytohormone concentration in the culture medium. Biotic stress conditions affected *ipdC* gene expression positively, but this promotion was not related with an increase in IAA concentration in the culture medium. Both *P. savastanoi* and MeJA treatments were able to decrease IAA concentration in the culture medium in spite of an increase in gene expression. In the case of *P. savastanoi*, this was because of the presence of L-phenylalanine. With MeJA, it was due to its direct interaction with IAA in the culture medium. Moreover, *P. savastanoi*-filtered supernatants induced growth in *A. brasilense* but MeJA decreased it, possibly due to a toxicity effect. *F. oxysporum* supernatants increased bacterial growth and IAA biosynthesis, because the fungus has the capacity to release specific metabolites into the culture medium, which are used by *Azospirillum* sp. to grow or produce biomass and increase IAA biosynthesis. In contrast to *P. savastanoi* and MeJA, both SA and CH were able to increase IAA biosynthesis with decreased bacterial growth, probably as a general stress response.

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