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EXOPOLYSACCHARIDES AND ABIOTIC STRESS TOLERANCE IN BACTERIAL ISOLATES FROM "SABIÁ" NODULES[1]

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ABSTRACT - Several microorganisms produce polysaccharides, deemed to protect the bacteria from several environmental stresses. This paper aims to evaluate the protective effect of exopolyssacharides to different abiotic stresses in bacterial isolates from "sabiá" (*Mimosa caesalpiniifolia*) nodules. 303 fast growing isolates were qualitatively evaluated for exopolyssacharide production and tested *in vitro* for tolerance to two levels of acidity, joint aluminum and acidity, three salinity levels; 11 antibiotics and three herbicides. Most isolates resisted media acidity, acidity with aluminum, salinity, and ampicillin, cefotaxime, gentamicin and vancomycin antibiotics and 2,4D herbicide, while being sensitive to ciprofloxacin, chloramphenicol, streptomycin, kanamycin, nalidixic acid, rifampicin and tetracycline antibiotics and paraquat and glyphosate herbicides. There was no connection between exopolyssacharide production and abiotic stress tolerance.

Keywords: Acidity. Aluminum. Salinity. Antibiotics. Herbicides.

EXOPOLISSACARÍDEOS E TOLERÂNCIA A ESTRESSES ABIÓTICOS EM ISOLADOS BACTERIANOS DE NÒDULOS DE SABIÁ

RESUMO – Muitos microrganismos produzem exopolissacarídeos que podem protegê-los de vários estresses ambientais. Este trabalho visa avaliar o efeito protetor de exopolissacarídeos a diferentes estresses ambientais em isolados bacterianos de nódulos de sabiá (*Mimosa caesalpiniifolia*). 303 isolados de crescimento rápido foram avaliados qualitativamente para a produção de exopolissacarídeos, e testados *in vitro* para tolerância a dois níveis de acidez, acidez e alumínio combinados, três níveis de salinidade, 11 antibióticos e três herbicidas. A maioria dos isolados resistiu à acidez do meio, isolada ou combinada com alumínio, salinidade, e os antibióticos ampicilina, cefotaxima, gentamicina e vancomicina e ao herbicida 2,4D e foram sensíveis aos antibióticos ciproflaxicina, cloranfenicol, estreptomicina, kanamicina, ácido nalidíxico, rifampcina e tetraciclina e aos herbicidas paraquat e glifosato. Não houve conexão entre produção de exopolissacarídeos e tolerância a estresses abióticos.

Palavras chaves: Acidez. Alumínio. Salinidade. Antibióticos. Herbicidas.

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INTRODUCTION

Exopolysaccharides (EPS) are polymers with several pharmaceutical and food industrial applications, but that biologically are considered to protect the microbial cells from environmental stresses such as desiccation, antibiotics, toxic compounds and protozoa and bacteriophage predation (SURESH KUMAR et al., 2007; STAUDT et al., 2012), while enhancing water and nutrient retention, and fixation to surfaces (DOWNIE, 2010; NICOLAUS et al., 2010).

EPS synthesis by rhizobial strains is highly variable in composition and yield under normal conditions (BOMFETI et al., 2011), and different stresses (JANCZAREK; SKORUPSKA, 2011), but generally fast growing rhizobia have high EPS production, which is considered to enhance tolerance to abiotic stress as acid conditions (CUNNINGHAM; MUNNS, 1984), salinity (ELSHEIKH, 1998; NÓBREGA et al., 2004; XAVIER et al., 2007), antibiotics (XAVIER et al., 1998) and pesticides (AHEMAD; KHAN, 2012), enhancing bacterial survival (BOMFETI et al., 2011).

This paper aims to evaluate the relationship between EPS production and "in vitro" tolerance to acidity, acidity with aluminum, salinity, antibiotics and herbicides in "sabiá" (*Mimosa caesalpiniifolia*) nodule bacterial isolates.

MATERIAL AND METHODS

303 bacterial isolates previously obtained according to standard practices from "sabiá" nodules were visually characterized as producing scarce, few, moderate or abundant amounts of EPS, and submitted to "in vitro" evaluation of acidity, acidity+aluminum, salinity, antibiotic and herbicide intrinsic tolerance. For all of the evaluations, initial growth was on 79 media (per litre: 10 g mannitol; 1 mL of 10% K_2HPO_4 solution; 4 mL of 10% KH_2PO_4 solution; 2 mL of 10% $MgSO_4.7H_2O$ solution; 1 mL of 10% NaCl solution; 0,4 g yeast extract; 5 mL of 0,5% bromothymol blue solution in 0,2 N KOH; 15 g agar, pH 6,8) (FRED; WAKSMAN, 1928; Vincent, 1970) and all tests were on 79 media at 28° C.

Acidity tolerance was tested on 79 media, modified to pH 4.5 or 5.5, with 80 g L^{-1} of agar, while for acidity+aluminum, the media was modified to pH 4.5 and 0.5 $cmol_c$ Al L^{-1} with $AlCl_3.6H_2O$. Each Petri dish was inoculated with 20 strains, each strain being added through 7 µL drops of approximately 10^8 cells mL^{-1} bacterial broth. Five days after inoculation, the strains were scored visually as tolerant, if there was colony formation, or otherwise sensitive. Salinity tolerance was evaluated on 79 media with 15, 30 or 45 g L^{-1} if NaCl, with inoculation, growth conditions and evaluated conducted as for acidity. Non-modified 79 media was used as control for all of the above conditions.

Antibiotic resistance was evaluated by disc diffusion in agar (BAUER et al., 1966) for 11 antibiotics (Table 1). Inoculation was by immersion of sterile cotton balls in bacterial broth, followed by swabbing the media surface, which after dry received four antibiotic discs, 6 mm diameter, per Petri dish. Sterile paper discs, also 6 mm diameter, without antibiotic were used as control, and growth was evaluated under identical conditions to the previous tests. Inhibited growth haloes were measured with a caliper, observed after 24 h, and isolates were scored according to Table 1.

Herbicide resistance was evaluated with sterile paper discs, as used for the control treatment above, saturated with commercial products at full concentration glyphosate (Isopropylamine salt - Glyphosate 480 g L^{-1}), paraquat (Paraquat dichloride 200 g L^{-1}) and 2,4D (Amine salt 806 g L^{-1}), after filtration through 0.22 µm seringe filters (model 99722 Techno Plast Products AG). Evaluation was conducted as for antibiotics, with all three herbicides and a control disc in each plate and isolates were scored as resistant when the inhibition halo was ≤ 10 mm.

Table 1. Classes, antibiotics and their inhibition haloes scoring for antibiotic resistance evaluation of 303 bacterial isolates from "sabiá" nodules. Inhibition zones adapted from Wilder et al. (2005)

Class	Antibiotic	Symbol	Content per disc	Action spectra	Inhibition zone (mm)	
					Resistant	Sensitive
β-lactam	Ampicillin	AMP	10 µg	Wide	≤13	≥14
	Cefotaxime	CTX	30 µg	Wide	≤14	≥15
Quinolone	Ciprofloxacin	CIP	5 µg	Wide	≤15	≥16
	Nalidixic acid	NAL	30 µg	Gram negative	≤13	≥14
Phenicol	Chloramphenicol	CLO	30 µg	Wide	≤12	≥13
Aminoglycoside	Streptomycin	EST	10 µg	Wide	≤11	≥12
	Gentamicin	GEN	10 µg	Wide	≤12	≥13
	Kanamycin	KAN	30 µg	Wide	≤13	≥14
Ansamycin	Rifampicin	RIF	30 µg	Wide	≤11	≥12
Tetracycline	Tetracycline	TET	30 µg	Wide	≤14	≥15
Glycopeptides	Vancomycin	VAN	30 µg	Gram positive	≤14	≥15

All data was submitted to χ^2 tests comparing resistance/sensitivity ratios for the EPS groups, for each level of each stress individually.

RESULTS AND DISCUSSION

Most isolates resisted pH 5.5 (97%), 4.5 (95%) and acidity+aluminum (90%), and all of the latter resisted also acidity *per se*, confirming that acidity resistance is necessary for aluminum resistance (SÁ, 2001). These results also agree with Rejili et al. (2009) in which 94% of the *Rhizobium*, *Mesorhizobium* and *Sinorhizobium* isolates from *Lotus* spp in Tunisia were resistant to pH 5.5, as well as to those from Chagas Júnior et al. (2010) in which 87% of the cowpea rhizobia from Amazonian soils resisted to pH 4.5 + 2 $cmol_c$ Al L^{-1}. While Cunningham e Munns (1984) studying then-*Rhizobium* induced acidity tolerance indicate that higher EPS production is linked to increased acidity resistance, this was not confirmed by present work. Over 90% of the scarce or low EPS production isolates were resistant to acidity or acidity+aluminum, with no significant difference on resistance proportion due to EPS production (Table 2). This would indicate that EPS production is not the major reason for this resistance, as seen by Correa e Barneix (1997) studying the cellular mechanism of pH tolerance in *Rhizobium loti* (now *Mesorhizobium loti*).

Table 2. Resistance of 303 isolates from "sabiá" nodules to abiotic stresses in function of EPS production. R and S indicate resistance and sensitivity.

	Stress		Total (percent)	EPS Production				$Pr>\chi^2$
				Scarce	Few	Moderate	Abundant	
Acidity and Acidity+Al	pH 5,5	R	295 (97%)	56 (100%)	94 (96%)	86 (95%)	59 (100%)	0,1681
		S	8 (3%)	0 (0%)	4 (4%)	4 (4%)	0 (0%)	
	pH 4,5	R	287 (95%)	52 (93%)	94 (96%)	84 (93%)	57 (97%)	0,6968
		S	16 (5%)	4 (7%)	4 (4%)	6 (7%)	2 (3%)	
	pH 4,5+Al	R	274 (90%)	48 (86%)	91 (93%)	80 (89%)	55 (93%)	0,4099
		S	29 (10%)	8 (14%)	7 (7%)	10 (11%)	4 (7%)	
Salinity (gL^{-1} of NaCl)	30	R	230 (76%)	50 (89%)	68 (69%)	64 (71%)	48 (81%)	0,0199(1)
		S	73 (24%)	6 (11%)	30 (31%)	26 (29%)	11 (19%)	
	45	R	199 (66%)	44 (79%)	63 (64%)	54 (60%)	38 (64%)	0,1361
		S	104 (34%)	12 (21%)	35 (36%)	36 (40%)	21 (36%)	
Antibiotics	AMP	R	215 (71%)	33 (59%)	69 (70%)	48 (53%)	45 (76%)	0,0134(1)
		S	88 (29%)	23 (41%)	29 (30%)	42 (47%)	14 (24%)	
	CIP	R	106 (35%)	9 (16%)	28 (29%)	21 (23%)	15 (25%)	0,3719
		S	197 (65%)	47 (84%)	70 (71%)	69 (77%)	44 (75%)	
	CLO	R	122 (40%)	8 (14%)	29 (30%)	19 (21%)	17 (29%)	0,1259
		S	181 (60%)	48 (86%)	69 (70%)	71 (79%)	42 (71%)	
	CTX	R	216 (71%)	32 (57%)	72 (73%)	52 (58%)	47 (80%)	0,0072(1)
		S	87 (29%)	24 (43%)	26 (27%)	38 (42%)	12 (20%)	
	EST	R	116 (38%)	10 (18%)	29 (30%)	21 (23%)	19 (32%)	0,2517
		S	187 (62%)	46 (82%)	69 (70%)	69 (77%)	40 (68%)	
	GEN	R	213 (70%)	3 (5%)	19 (19%)	16 (18%)	8 (14%)	0,1052
		S	90 (30%)	53 (95%)	79 (81%)	74 (82%)	51 (86%)	
	KAN	R	106 (35%)	4 (7%)	20 (20%)	16 (18%)	12 (20%)	0,1630
		S	197 (65%)	52 (93%)	78 (80%)	74 (82%)	47 (80%)	
	NAL	R	117 (39%)	16 (29%)	39 (40%)	29 (32%)	22 (37%)	0,4845
		S	186 (61%)	40 (71%)	59 (60%)	61 (68%)	37 (63%)	
	RIF	R	53 (17%)	4 (7%)	24 (24%)	19 (21%)	4 (7%)	0,0040(1)
		S	250 (83%)	52 (93%)	74 (76%)	71 (79%)	55 (93%)	
	TET	R	108 (36%)	13 (23%)	45 (46%)	29 (32%)	9 (15%)	0,0004(1)
		S	195 (64%)	43 (77%)	53 (54%)	61 (68%)	50 (85%)	
	VAN	R	165 (54%)	20 (36%)	42 (43%)	39 (43%)	39 (66%)	0,0055(1)
		S	138 (46%)	36 (64%)	56 (57%)	51 (57%)	20 (34%)	
Herbicides	2,4D	R	161 (53%)	22 (39%)	59 (60%)	45 (50%)	33 (56%)	0,0800
		S	142 (47%)	34 (61%)	39 (40%)	45 (50%)	26 (44%)	
	GLI	R	51 (17%)	6 (11%)	22 (22%)	12 (13%)	11 (19%)	0,2009
		S	252 (83%)	50 (89%)	76 (78%)	78 (87%)	48 (81%)	
	PAR	R	50 (17%)	7 (12%)	19 (19%)	17 (19%)	9 (15%)	0,6736
		S	253 (83%)	49 (88%)	79 (81%)	73 (81%)	50 (85%)	

(1) Significant effect of EPS production on resistance

All isolates resisted 15 g L^{-1} of NaCl, while 76 and 66% of them resisted 30 and 45 g L^{-1}, respectively (Table 2), although rhizobial strains generally tolerate up to 30 g L^{-1} of NaCl (NÓBREGA et al., 2004; XAVIER et al., 2007) while 11 of 40 Portuguese *Sinorhizobium* strains resisted up to 80 g L^{-1} of NaCl on a media with soil extract (FARELEIRA et al., 2007). Gauri et al. (2011) working with *Trifolium alexandrinum* fast-growth rhizobial isolates in India, found that 85% of the isolates resisted to 40 g NaCl L^{-1} 11,6% resisted to 50 g NaCl L^{-1} While fast growing rhizobia are generally considered more tolerant

to saline media due to the usually higher EPS production (ELSHEIKH, 1998), most isolates with scarce (79%) or low (64%) EPS production tolerated 45 g L^{-1} of NaCl (Table 2), without any significant effect of EPS production on resistance, while the significant effect observed for 30 g L^{-1} of NaCl was due to a higher proportion of tolerant strains in the scarce and low EPS production groups, than with moderate or abundant EPS production groups, again denying that EPS production is a major indicator of tolerance to abiotic stresses.

While 13% of the isolates resisted all the antibiotics, 8% were sensitive to all of them, with results highly variable overall (Table 2). Resistant isolates were the majority for ampicillin, cefotaxime, gentamicin and vancomycin (71; 71; 70 and 54%, respectively), and sensitive to ciprofloxacin, chloramphenicol, streptomycin, kanamycin, nalidixic acid, rifampicin and tetracycline (65; 60; 62; 65; 61; 83 and 64 % respectively). So the isolates were resistant to the antibiotics in the order ampicillin = cefotaxima > gentamicin > vancomycin > chloramphenicol > nalidixic acid > streptomycin > tetracycline > ciprofloxaxin = kanamycin > rifampicin. High resistance incidence to ampicillin, gentamicin and vancomycin were also found for *Rhizobium* and *Bradyrhizobium* strains (HOSNEY et al., 2006; FLORENTINO et al., 2010), while chloramphenicol, streptomycin, kanamycin, nalidixic acid, rifampicin and tetracycline also induced higher sensitivity (HOSNEY et al., 2006; FLORENTINO et al., 2010). Gauri et al. (2011) studying fast-growth rhizobial isolates from *Trifolium alexandrinum* also found that most of the 85 isolates were resistant to ampicillin, gentamicin and vancomycin and sensitive to rifampicin and tetracycline, but found high resistance to chloramphenicol, streptomycin, kanamycin and nalidixic acid, while Maâtallah et al. (2002) found that most rhizobial isolates from chickpea were sensitive to ampicillin, chloramphenicol, streptomycin and kanamycin and resistant to nalidixic acid, showing that probably antibiotic resistance is a species linked trait, and not growth rate related characteristic. Suresh Kumar et al. (2007) indication that EPS may physically protect against antibiotics notwithstanding, the only significant effect observed was an increase in sensitivity to ampicillin, cefotaxime, rifampicin, tetracycline and vancomycin among the isolates with higher EPS production.

Only 8% of the isolates were resistant to all herbicides, while 42% were sensitive to all three and the only herbicide where resistance was prevalent was 2,4D (Table 2). This correlates to *Bradyrhizobium* known sensitivity to glyphosate (SANTOS et al., 2004; JACQUES et al., 2010). For example, glyphosate effects on metabolism of phenolic compounds in *Rhizobium* from *Cajanus cajan* negatively affects rhizobial by inhibition or repression of the action of some enzymes (SHENDE; PATIL, 2013) although Malty et al. (2006) have not found glyphosate to affect *Bradyrhizobium elkanii* and *B. japonicum* strains, as for paraquat in *Rhizobium tropici* strains (SANTOS et al., 2006), and paraquat at 1272 ppm alto prevented rizobial growth in *Rhizobium* from - (VEENA et al., 2012). While there was no significant effect for EPS production, most isolates with moderate to high EPS production were sensitive to paraquat, glyphosate or all three herbicides. There was a significant increase, though, in the 2,4D resistant strain proportion with higher EPS productions (Table 2).

CONCLUSION

EPS production is not the major factor in *in vitro* nodule bacteria resistance to acidity, aluminum, salinity, antibiotic and herbicides stresses.

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