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MALAGASY TRADITIONAL TREATMENTS OF INFECTIOUS PLANT DISEASES EXERT ANTI-VIRULENCE ACTIVITIES AGAINST *PSEUDOMONAS AERUGINOSA AND RALSTONIA SOLANACEARUM*

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ABSTRACT

Traditional Malagasy farmers have developed a range of biological methods to restrict plant diseases without reliance on external or synthetic inputs. Five common Malagasy traditional practices demonstrated to be efficient against potato crop bacterial disease in experimental fields have been investigated for their antibacterial (*i.e.* bacteriostatic and bactericidal effects) and anti-virulence (*i.e.* anti-quorum sensing and anti-biofilm) activities against two phytopathogens, *Pseudomonas aeruginosa* and *Ralstonia solanacearum.* Results show that polar (methanolic) extracts of recipes exert anti-virulence activities rather than bacteriostatic and/or bactericidal activities. Indeed, three recipes (R5, R7 and R9) reduce the expression of QS-dependent virulence factors whereas only recipe (R5) exhibit anti-biofilm activities without affecting bacterial growth. R4 and R6 were not active, suggesting other bacterial targets and/or other bioactivity properties. Innovative approaches, inspired from ancestral practices, should be considered in the struggle against infectious diseases to limit the overuse of antibiotics for controlling infectious plant diseases and to reduce the overspread of multidrug resistant bacteria.

Keywords: Biofilm, Quorum sensing, *Pseudomonas*, *Ralstonia*

INTRODUCTION

The current global food needs require agriculture practices to cover large crop surfaces with high output. Plant infectious diseases, factors that reduce productivity, are usually controlled by using chemical products such as pesticides or fungicides and, occasionally, antibiotics (**McManus *et al.*, 2002; Stockwell & Duffy, 2012**). The use of such chemical products is controversial, not only for the risk in soil contamination and human intoxication but also for the selection of resistant bacteria strains. In fact, faced with phytopathogenic bacteria, modern agriculture is sorely missing selective antibacterial products with original modes of action that may avoid selective pressure. In a developing country such as Madagascar, traditional farmers have developed a range of methods to restrict plant diseases without reliance on external or synthetic inputs. Although many of their techniques are unknown, poorly understood and documented, they allowed farmers to produce crops with minimal purchased inputs in a difficult tropical environment. Also the active promotion of modern products led to a disaffection towards traditional practices and there is a risk of losing orally transmitted ancestral knowledge. In a recent experimental field study, some Malagasy traditional practices demonstrated curative activity against potato wilt disease due to *Ralstonia solanacearum* (**Rasamiravaka *et al.*, 2017**). Effectiveness of these recipes is presumably explained through either bactericidal effects or disruption of phytopathogen development which limits spreading and colonization within plant host. Indeed, the success of an infection relies on the ability of bacteria to survive, colonize, disseminate through their hosts by using different types of motility, form structured biofilms and diffuse virulence factors (**Wu *et al.*,** 2008).

Relevant phytophatogens that may cause infectious diseases in potato are *Ralstonia solanacearum (*also called *Pseudomonas solanacearum*, responsible of wilt disease*)* and different species of *Pseudomonas* (responsible of bacterial soft rot) (**Mansfield *et al.*, 2012**). Additionally, *P. aeruginosa* is an opportunistic bacterium infecting humans, animals and plants (*Arabidopsis thaliana* (L.) Heynh., *Nicotiana tabacum* L. and *Lactuca sativa* L.) and is a widely used model for studying anti-virulence properties of natural products (**Jimenez *et al.*, 2012; LaSarre & Federle, 2013**). The present study aims to investigate *in vitro* the five (R4, R5, R6, R7 et R9) common Malagasy traditional practices previously demonstrated to be efficient against potato bacterial disease in experimental fields (**Rasamiravaka *et al.*, 2017**) by focusing on their possible anti-bacterial and anti-virulence activities against both phytopathogens *R. solanacearum* and *P. aeruginosa*.

MATERIAL AND METHODS

Recipe preparation and extraction

In our previous study, 50 practices have been collected from traditional farmers managing rice and potatoes crop (**Rasamiravaka *et al.*, 2017**). These practices were mainly based on the concoction of plant mixtures with cow dung or manure followed by 2-weeks maceration at room temperature (see **Rasamiravaka *et al.* (2013)** for details on collection of material and preparation). Five of these recipes (See Table 1 for composition), shown, in experimental fields, to efficiently treat plants naturally infected with *R. solanacearum* (Figure 1), were selected for the present study. One liter of each recipe was soaked overnight (25 °C, agitation at 50 rpm) in methanol (500 ml), filtered on cellulose and concentrated to dryness under vacuum. Extracts were dissolved in DMSO and stored at -20°C until use. Concoctions of plant mixtures with cow dung or manure without maceration or with one-week maceration were also extracted following the same procedure.

Table 1 Composition of recipes used in this study

N°*	Composition	Quantity (for 20L)
R4	Cow dung	1kg
	Water	sqf
R5	*Agave vivipara* L.	1kg
	Tephrosia purpurea L.	500g
	Cow dung	1kg
	local soap	50g
	Cow dung manure	sqf
R6	*Furcrea foetita* (L.) Haw.	1kg
	Tephrosia purpurea L.	500g
	Cow dung	1kg
	local soap	50g
	Cow dung manure	sqf
R7	*Melia azedarach* L.	1kg
	Agave vivipara L.	1kg
	Cow dung manure	sqf
R9	*Buddleja madagascariensis* Lam	1kg
	Agave vivipara L.	1kg
	Cow dung manure	sqf

*Number attributed according to **Rasamiravaka *et al.* (2017)** nomenclature

Bacterial strains, plasmids and culture conditions

To determine MIC (Minimal Inhibitory Concentration) and MBC (Minimal Bactericidal Concentration), PAO1 was grown on 24-well micro-plates with 1 ml of LB broth in presence of the methanolic extracts at different concentrations (31.25 to 4000 µg/mL) and incubated at 37°C for 24 h. The MIC was defined as the lowest concentration that completely inhibited growth as detected by the naked eye (**Chérigo *et al.*, 2009**). All inhibited growth culture were then sub-cultured onto LB agar plate and incubated at 37°C for 24 h to determine the MBC which was defined as the lowest concentration that yielded negative sub-cultures (**Okusa *et al.*, 2007**). Gentamicin was used as positive control.

Antibacterial assay

P. aeruginosa PAO1 wild-type were grown (37 °C, agitation at 175 rpm) in LB-MOPS broth (50 mM, pH 7.2) supplemented with carbenicillin (300 µg/mL) when required. For the detection of anti-QS activity, two PAO1-derived strains harboring QS-related promoter-*lacZ* fusions (*lasB-lacZ* and *rhlA-lacZ)* and one PAO1-derived strain harboring QS-independent promoter-*lacZ* fusion (*aceA-lacZ*) were used. Descriptions of the used strains and of the experimental procedure for the determination of QS activity were as previously reported by **Rasamiravaka *et al.* (2013)**. *Ralstonia solanacearum* used in this study has been isolated from infected potato plants that present symptoms of bacterial wilt disease (**Rasamiravaka *et al.*, 2017**). Identification of *R. solanacearum* was confirmed by PCR according to DNA 16S amplification protocol (**Kutin *et al.*, 2009; Massart *et al.*, 2005**).

Biofilm formation and quantification

Biofilm formation was performed on 24-well micro-plates and quantified by crystal violet staining protocol as previously described (**Rasamiravaka *et al.*, 2013**). Briefly, an overnight culture of PAO1 was washed twice and diluted in fresh biofilm broth (BB) medium (Na_2HPO_4, 1.25 g/L; $FeSO_4.7H_2O$, 0.0005 g/L; glucose, 0.05 g/L; $(NH_4)_2SO_4$, 0.1 g/L; $MgSO_4.7H_2O$, 0.2 g/L and KH_2PO_4, 0.5 g/L). In 24-well polystyrene plates, 50 µl of the diluted culture were added to 940 µl of BB medium (initial A_{600} of the culture between 0.14 and 0.16) and supplemented with 10 µl of the tested extract, negative control (DMSO, 1% final concentration) or positive control (oleanolic acid, 800 µM final concentration) (**Kiplimo *et al.*, 2011**). The methanolic extracts were tested at 100 µg/mL final concentration then, for active extracts at different concentrations (25 to 300 µg/mL). PAO1 cultures were incubated statically for 24 hours at 37 °C and, after staining by crystal violet, the biofilm was quantified at 590 nm with a SpectraMax M2 device (Molecular Devices).

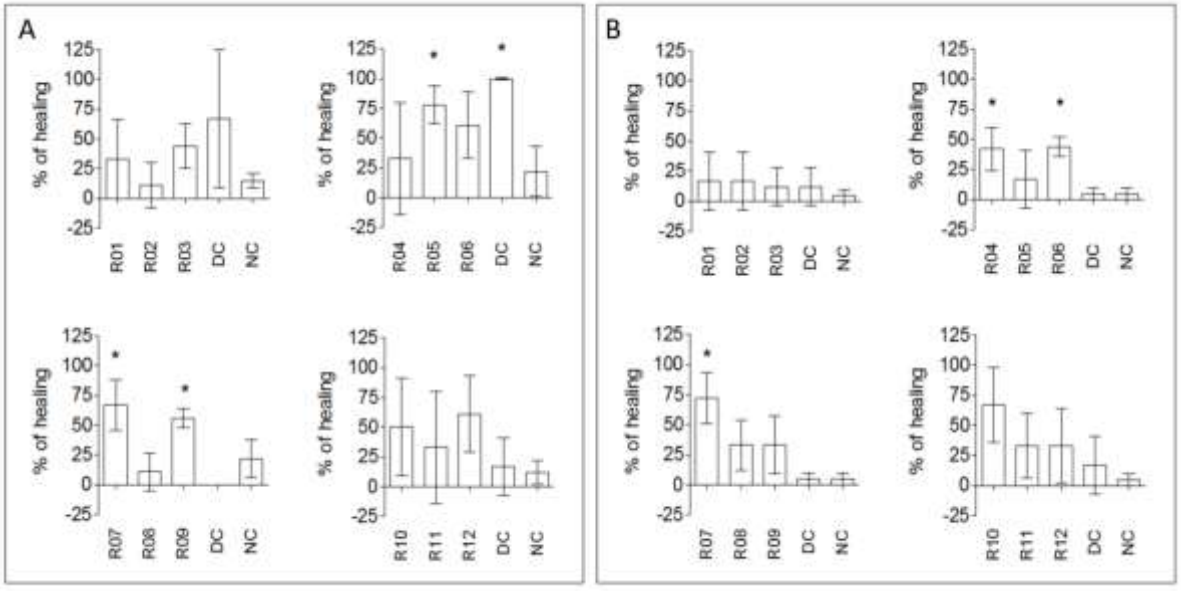

Figure 1 Healing rates observed on experimental parcels of the "Diamondra" (A) and "Meva" (B) potato varieties (**Rasamiravaka *et al.*, 2017**).
Data were recorded throughout the 14 weeks of the vegetation cycle; 3 independent experiments, each on 4 blocks of 15 plantlets (n=12). Dithane control (DC) represents the positive control. Asterisks indicate data that are statistically different compared to negative control (NC) after Dunnett's multiple comparison tests at $P < 0.01$.

Gene expression and β-galactosidase measurements

β-Galactosidase measurements were performed as previously described (**Vandeputte *et al.*, 2010**) to monitor gene expression. Briefly, after growth in liquid LB-MOPS-carbenicillin at 37 °C with agitation (175 rpm) for 18 hours, PAO1 reporter strains were washed twice in fresh LB medium and re-suspended in liquid LB-MOPS-carbenicillin. PAO1 reporter strain inocula (50 µl) were incubated (37°C with agitation at 175 rpm) for 18 hours in 1 ml LB-MOPS-carbenicillin (initial A_{600} of the culture was between 0.020 and 0.025) supplemented with either 10 µL of methanolic extract dissolved in DMSO (100 µg/mL final concentration), 10 µl DMSO (control condition) or 4mM of naringenin (Sigma-Aldrich), a compound known as a QS inhibitor in *P. aeruginosa* (positive control) (**Vandeputte *et al.*, 2011**). After incubation, the bacterial density was assessed by spectrophotometry (A_{600}) and the β-galactosidase was measured with *o*-nitrophenyl-β-D-galactopyranoside as described by **Zhang & Bremer, (1995)**.

Statistics

All experiments were performed in six replicates and repeated in three independent assays. The data were statistically analyzed by conducting Dunnett's multiple comparison test (*i.e.* each test was compared with the DMSO-treated cells) and a P-value <0.01 was considered as being significant.

RESULTS

Bactericide and bacteriostatic activities of five Malagasy traditional practices

Five Malagasy traditional recipes were prepared following traditional users' recommendations and polar (methanolic) extracts were investigated for *in vitro* antibacterial activities against *R. solanacearum* and *P. aeruginosa.* The positive control, gentamicin, was selected for its reported effectiveness in controlling various bacterial diseases of vegetable crops caused by *Pseudomonas* and *Ralstonia* species *(***Vidaver, 2002**). Interestingly, both the MICs and MBCs of the five tested methanolic extract were higher than 4000 µg/mL whereas gentamicin-MIC and -MBC were 0.5 - 2 µg/mL and 1 - 4 µg/mL, against *R. solanacearum* and *P. aeruginosa*, respectively (data not shown). As these recipes seem to not exert bactericidal or bacteriostatic activity against both species, they may exert other biological activities which successfully impair plant infections without killing bacterial population.

Reduction of biofilm formation and QS-dependent virulence factor genes expression in *P. aeruginosa* PAO1

The five Malagasy traditional recipes were assessed for their potential to affect biofilm formation, an important process in plant-bacteria interaction (**Bogino *et al.*, 2013**). A concentration of 100 µg/mL has been selected according to optimum solvability in DMSO. As shown in Figure 2, anti-biofilm activity is observed in presence of R5 (52 ± 5 % inhibition) at 100 µg/mL without affecting bacterial growth in both strains as compared to DMSO control, whereas the other recipes (R4, R6, R7 and R9) did not exert any significant anti-biofilm activities. This anti-biofilm activity appears dose-dependent as no activity is observed at 25 µg/mL in both strains (Figure 3). Likewise, the five recipes were assessed for their effect on the expression of QS-related virulence factor genes *(lasB* encoding for elastase and *rhlA* encoding for the precursor of rhamnolipids) in *P. aeruginosa* reporter strains. As shown in Figure 4, R5, R7 and R9 inhibited QS-related *lasB* (45 ± 4 %, 30 ± 4% and 32 ± 3% of inhibition, respectively as compared to DMSO control) and *rhlA* (52 ± 5 %, 34 ± 4% and 33 ± 3% of inhibition, respectively as compared to DMSO control) genes in *P. aeruginosa* PAO1 without affecting the expression of the QS-independent *aceA* gene (a constitutive gene encoding for isocitrate lyase) (**Kretzschmar *et al.*, 2008**) whereas R4 and R6 did not inhibit QS-related *lasB* and *rhlA* genes at all. These results suggest that the R5, R7 and R9 recipes affect the ability of both strains to invade and colonize plant host.

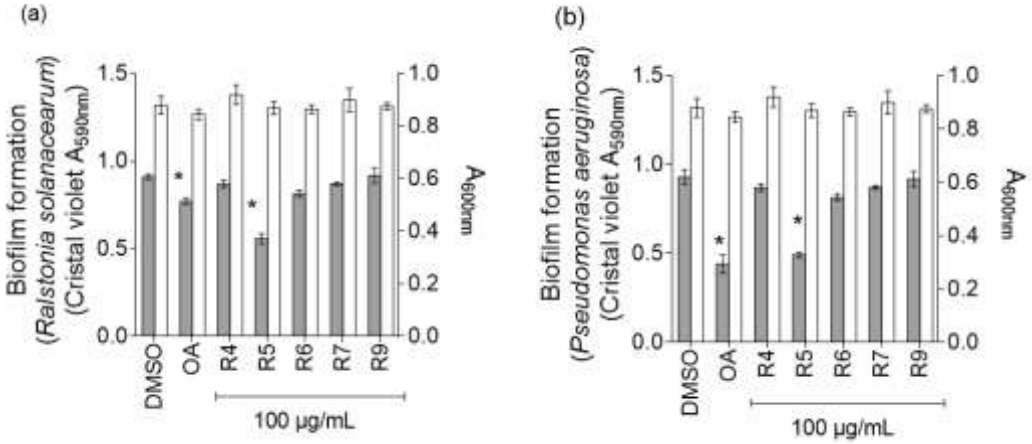

Figure 2 Screening of five recipes extract (R4–R9) on the formation of biofilms (grey bar) and growth turbidity (clear bar) of (a) *Ralstonia solanacearum*; (b) *Pseudomonas aeruginosa* PAO1.
R. solanacearum and *P. aeruginosa* PAO1 were grown statically in minimal medium supplemented with DMSO 1%, oleanolic acid 800 µM (OA) or methanolic extract after incubation without agitation at 37°C for 24 h. The biofilm formation was quantified by crystal violet staining and A_{590nm} measurement. The cell density of the bacteria was assessed as A_{600nm}. All experiments were performed in triplicate with three independent assays. Error bars represent the standard error of the mean and the asterisks indicate samples that are significantly different from DMSO control (Dunnett's multiple comparison test, $p \leq 0.01$).

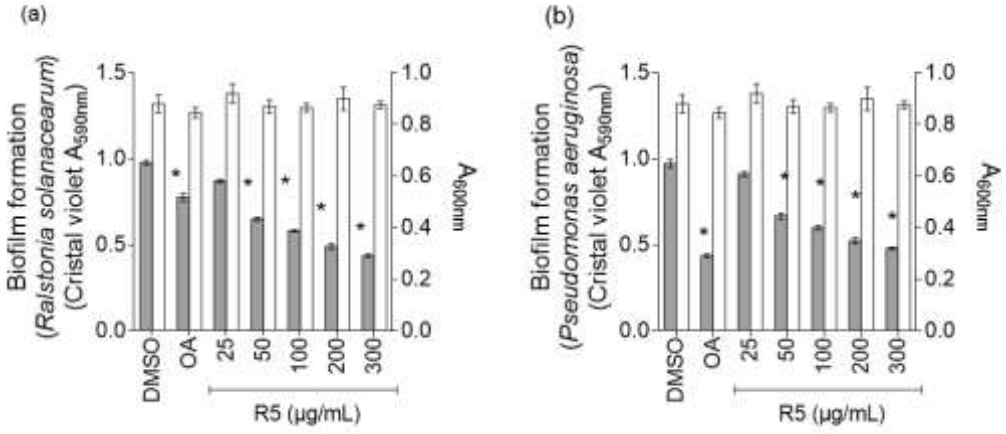

Figure 3 Dose-dependent activity of the R5 recipe on the formation of biofilms (grey bar) and growth turbidity (clear bar) of (a) *Ralstonia solanacearum*; (b) *Pseudomonas aeruginosa* PAO1.
R. solanacearum and *P. aeruginosa* PAO1 were grown statically in minimal medium supplemented with DMSO 1%, oleanolic acid 800 µM (OA) or different concentrations of R5 (from 50 to 300 µg/mL) after incubation without agitation at 37°C for 24 h. Biofilm formation was quantified by crystal violet staining and measured as A_{590nm}. The cell density of the bacteria was assessed as A_{600nm}. All experiments were performed in triplicate with three independent assays. Error bars represent the standard error of the mean and the asterisks indicate samples that are significantly different from DMSO control condition (Dunnett's multiple comparison test, $p \leq 0.01$).

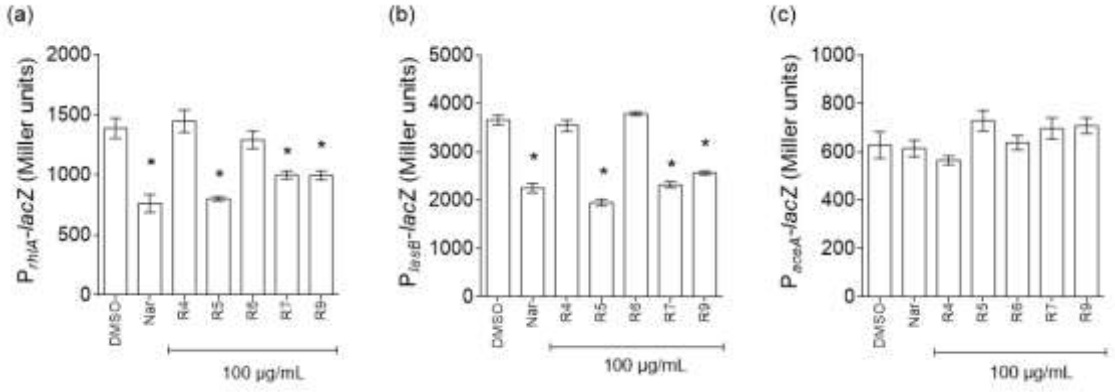

Figure 4. Effect of five recipes extracts on QS-dependent (*lasB* and *rhlA)* genes and QS-independent *aceA* gene expression in *P. aeruginosa* PAO1.
Effect of five recipe extracts at 100 µg/mL on (a) *rhlA*, (b) *lasB* and (c) *aceA* expression following 18 h of growth. Naringenin (Nar 4mM) was used as positive control. Gene expression was measured as the β-galactosidase activity of the *lacZ* gene fusions and expressed in Miller units. All experiments were performed in triplicate with three independent assays. Error bars represent the standard errors of the means and asterisks indicate samples that are significantly different from control condition (DMSO) (Dunnett's multiple comparison tests; $p \leq 0.01$).

Alteration of QS systems in *P. aeruginosa* PAO1

Since R5, R7 and R9 reduce the expression of QS-regulated *lasB* and *rhlA* genes, we assessed the effect of these 3 recipes on *P. aeruginosa* PAO1 QS systems by measuring the expression of the AHL synthetase genes *lasI* and *rhlI* and the QS regulator genes *lasR* and *rhlR*. As shown in Figure 5, the R5 and R9 extracts significantly reduced ($P < 0.01$) the expression of the AHL synthetase genes *rhlI* (33 ± 2 % and 40 ± 5 % of inhibition, respectively) and *lasI* (25 ± 5 % and 30 ± 5 % of inhibition, respectively). Similarly, R9 significantly reduced the expression of the QS regulator genes *rhlR* (29 ± 3 % of inhibition) and *lasR* gene (19 ± 3 % of inhibition). The R7 extract does not exert any inhibition on the expression of the *las* and *rhl* genes. These results demonstrate that the three recipes impaired differently the QS system, suggesting different targets in QS system modulation.

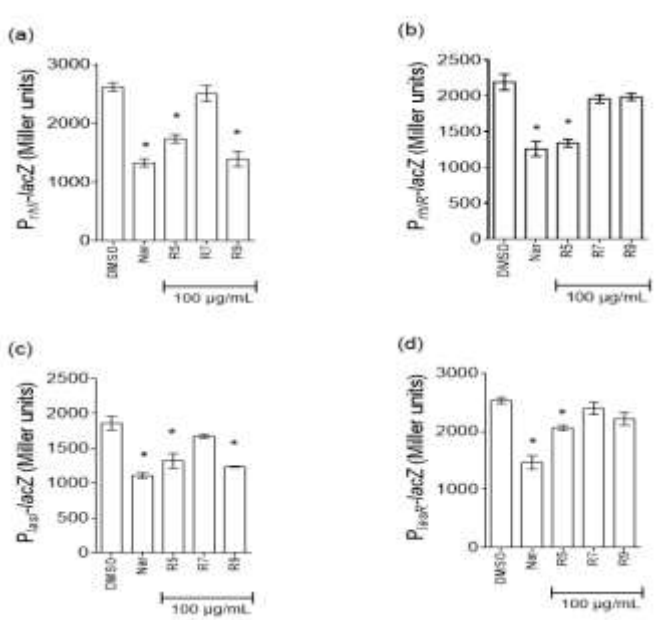

Figure 5 Effect of R5, R7 and R9 extracts on *Pseudomonas aeruginosa* quorum sensing (QS) regulator genes (a: *lasI*; b: *lasR*; c: *rhlI*; d: *rhlR*). The R5, R7 and R9 extracts were tested at 100 µg/mL. DMSO (1% [vol/vol], final concentration) was used as solvent control and naringenin (Nar: 4 mM, final concentration) as QS inhibitory control. *Significance at $P < 0.01$. All experiments were performed in six biological replicates.

Influence of recipes preparation on bioactivity

The preparation of recipes is based on the concoction of plant mixture with cow dung or manure followed by 2 weeks maceration at room temperature. As frequently suggested by interviewed traditional users (**Rasamiravaka *et al.*, 2017**), the duration of recipe maceration would be a quite important factor. Therefore, we assessed the anti-QS effects of effective recipes R5, R7 and R9 extracted after 3 distinct maceration durations: *(i)* at the date of mixing herbs and dung, which means no maceration; *(ii)* at 7th day of maceration; and *(iii)* at 14th day of maceration. All extracts were tested at 100 µg/mL final concentration. As shown in Figure 6, the 3 recipes extracted at the beginning of maceration do not reduce *lasB* and *rhlA* gene expression. However, the R5 and R7 extracted at 7th day after maceration reduce the expression of genes *lasB* (30 ± 5 % and 25 ± 3 % of inhibition, respectively) and *rhlA* (33 ± 5 % and 30 ± 4 % of inhibition, respectively) contrarily to R9 which is not active. Finally, the expression of genes *lasB* and *rhlA* gene is reduced by the three recipes extracted at 14th day of maceration, similarly to inhibitions recorded in Figure 4. Regarding the anti-biofilm effects of recipes R5 against *R. solanacearum* and *P. aeruginosa*, only the recipe R5 extracted at 14th day of maceration is effective (30 ± 5 % and 25 ± 3 % of inhibition, respectively; Figure 7). These results suggest a biotransformation process that generates active compounds inside recipe mixture. Moreover, the generation of anti-biofilm activity (Figure 7) needs more maceration time compared to anti-QS activities (Figure 5) suggesting a more complex biosynthesis pathway of active compound(s).

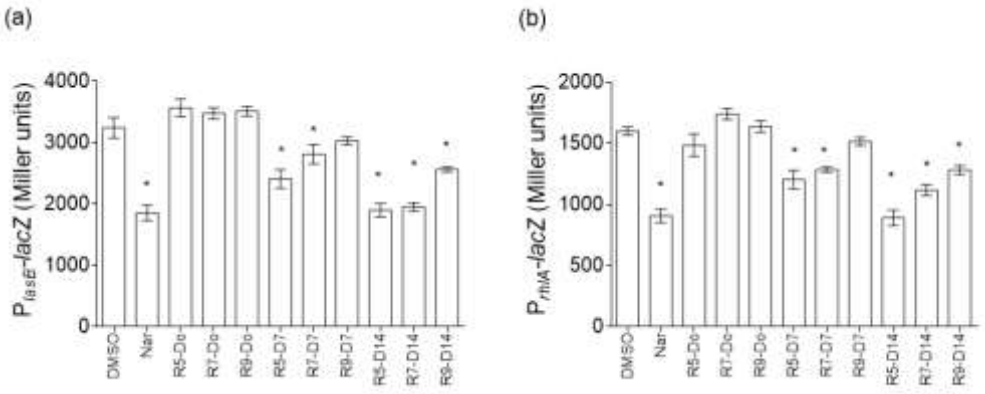

Figure 6. Effect of R5, R7 and R9 recipes with different times of maceration (D0, D7 and D14 of maceration) on the expression of QS-dependent (*lasB* and *rhlA)* genes in *P. aeruginosa* PAO1.
Effect of R5, R7 and R9 extracts on (a) *lasB* and (b) *rhlA* expression following 18 h of growth. Naringenin (Nar 4mM) was used as positive control. The gene expression was measured as the β-galactosidase activity of the *lacZ* gene fusions and expressed in Miller units. All experiments were performed in triplicate with three independent assays. Error bars represent the standard errors of the means and asterisks indicate samples that are significantly different from control condition (DMSO) (Dunnett's multiple comparison test; $p \leq 0.01$).

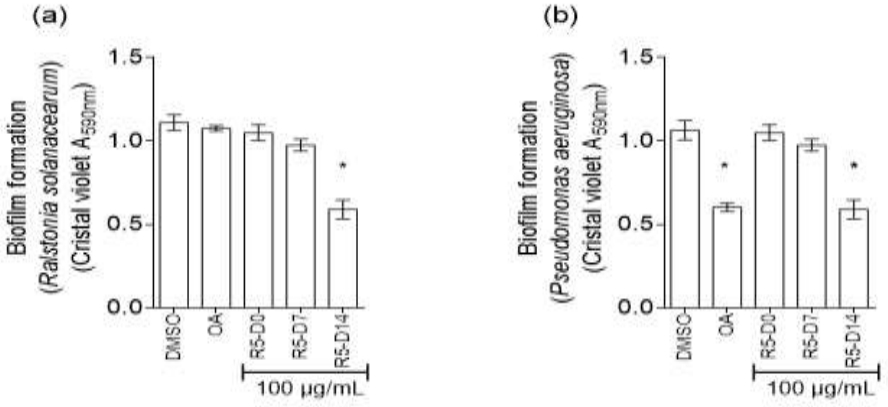

Figure 7. Biofilm formation in the presence of the R5 extract with different durations of maceration (D0, D7 and D14 of maceration). (a) Effect of R5 recipe on biofilm formation of *Ralstonia solanacearum*. (b) Effect of R5 recipe on biofilm formation of *P. aeruginosa* PAO1. *R. solanacearum* and *P. aeruginosa* PAO1 were grown in minimal medium supplemented with DMSO 1%, oleanolic acid 800 µM (OA) or R5 recipes (extracted at D0, D7 and D14) after incubation without agitation at 37°C for 24 h. The biofilm formation was quantified by crystal violet staining and A_{590nm} measurement. The cell density of the bacteria was assessed as A_{600nm}. All experiments were performed in triplicate with three independent assays. Error bars represent the standard error of the mean and the asterisks indicate samples that are significantly different from DMSO control (Dunnett's multiple comparison test, $p \leq 0.01$).

DISCUSSION

This study is the first *in vitro* investigation of the antimicrobial activities of Malagasy traditional crop treatments which highlights that polar extracts of these recipes exert anti-virulence rather than anti-bactericidal activities. Indeed, R5 inhibits biofilm formation in both strains and inhibits QS system in *P. aeruginosa* whereas R7 and R9 exert only anti-QS activities. Interestingly, QS inhibition phenotypes (Figure 4) in the 3 recipes are significantly different, suggesting the presence of either different active compound or similar compounds in different proportions. Although R4 and R6 were not active, they may contain active compounds which do not target the tested strains. Indeed, other bacteria species can be responsible of soft rot such as *Pectobacterium* spp. (**Marquez-Villavicencio *et al.*, 2011**), arguing that investigation should be extended to other phytopathogens. Likewise, *R. solanacearum* reporter strains should be generated to comprehend if active recipe would also affect virulence factors expression and quorum sensing mechanisms in *R. solanacearum* as it does for *P. aeruginosa*. Regarding the importance of maceration time, one hypothesis is that observed activities are the result of compounds newly generated during maceration period through a biotransformation process. Such biotransformation is possible as one of the major components of recipe is dung considered as a "complex bacterial mixture" that can presumably generate secondary metabolites during bacterial growth. One can assume that components of recipes do not contain the bioactive compound(s) in significant levels at the beginning of the maceration process. Moreover, the components of each recipe did not exert any anti-QS nor anti-biofilm activity when tested individually (data not shown). The challenge will be to isolate bioactive compound(s) and to identify the role of each recipe's component (Table 1) in this putative biotransformation process. One relevant point is to clarify in which way all components are necessarily needed to generate bioactivity. This point is important to optimize and standardize the use of such recipes in the struggle against plant bacterial infection. In that, one difficulty is the presence of dung that represents a highly complex bacterial mixture, its composition being highly dependent on the food composition of local cows.

CONCLUSION

The present study reports anti-virulence properties of traditional practices used to treat infectious plant diseases. This approach may be considered as an alternative approach compared to the use of expensive modern treatments, often ineffective on bacterial infections.
Innovative approaches, inspired from ancestral practices, should be considered in the struggle against infectious diseases to limit the overuse of antibiotics for controlling infectious plant diseases and to reduce the overspread of multidrug resistant bacteria. Combining the best of traditional agriculture methods with the best of modern agriculture should go a long way towards sustaining agriculture in the coming decennia.

Conflict of interest: The author confirms that this article content has no conflict of interest

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