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Influence of Culture Media on the Antimicrobial Activity of Fluorescents *Pseudomonas* Crude Supernatants Extracts against *Phytophthora colocasiae,* the Causal Agent of the Taro Leaf Blight

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Authors' contributions

This work was carried out in collaboration among all authors. Author NMSA investigated the work, performed methodology, wrote and prepared the original draft and helped in software development of the manuscript. Author KMLB did conceptualization, reviewed and edited the manuscript. Author TNS did data validation, performed methodology and reviewed the manuscript. Author SML did conceptualization, performed methodology, reviewed and edited the manuscript. Author NNRA administered and supervised the work. All authors read and approved the final manuscript.

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ABSTRACT

Crude supernatant extracts of two fluorescent *Pseudomonas* DS15V and DS17R obtained by monoculture under King B (KB), Luria Bertanie (LB), Nutrient Broth Yeast, (NBY), Succinate medium (SM), Tryptic Soy Broth (TSB) and Potato Dextrose Broth (PDB) culture media were evaluated for their antimicrobial potential against *Phytophthora colocasiae* (PC). Co-culture supernatant extracts with PC (PCo) were also tested. Moreover, the thermostability of extracts, inhibition of zoospore potential and *in situ* activity were estimated by the disc leaf test. The results showed that crude supernatant extracts from KB, LB and MS significantly inhibited mycelia growth (80-100%) at concentrations of 1:1 (v/v) and/or 1:2 (v/v) which correspond to Inhibitory Activity Unit (IAU). Furthermore, these supernatant extracts were highly thermosensitive and decreased significantly their antagonistic potential against *P. colocasiae* mycelia at high temperatures (100 and 121°C). On the other side, supernatant extracts obtained from monoculture and co-culture in potato liquid medium (PDB) demonstrated significant activity by IAU values at concentration of 1:3 (v/v) against mycelia growth for both DS15V and DS17R. For the zoospores growth, supernatants from PDB (PCo and PMo) presented high inhibition at low concentrations (1:3 v/v) especially 17Pco which shows 100% inhibition at concentration of 1:4 (v/v). In addition, these extracts were thermoresistant and maintained their antimicrobial potential against *P. colocasiae* without a significant difference. The four supernatants crude extract belong to co-culture on PDB medium (15PCo, 17PMo and 17PCo) used as best extracts on disc leaf test promoted 100% leaf disc prevention against *P. colocasiae* at concentration of 1:1 (v/v) and exhibited it curative effect at the same concentration after 4 days of treatment thereby stabilizing the development of necrosis on leaf disc.

Keywords: Antimicrobial activity; supernatant; Pseudomonas fluorescens; Phytophthora colocasiae; taro.

1. INTRODUCTION

"Taro (*Colocasiae esculenta*) is among major staple food or subsistence food for millions people in developing countries in Asia, Africa, and Central America" [1,2]. "It remains an important crop to many cultural and agricultural traditions worldwide" [3]. "Taro is also an important staple food and source of income in the Cameroon especially in the southern regions, and some neighboring countries. Despite this economic and socio-cultural importance, since 2010, taro has been affected by leaf blight (TLB) disease caused by *Phytophthora colocasiae* Racib. Epidemics of this disease occurred throughout the taro growing-areas during the rainy season, causing up to 100% leaf and corm losses" [4]. "Farmers were also skeptical of the etiology and health consequences, and they abandoned the crop in the field, which led to widespread poverty. Pesticide application for TLB management has been prescribed and applied in many by farmers. Fungicide application remains the fastest and most effective means of TLB management" [5]. "However, poor farmers require alternative control methods because synthetic chemicals are generally considered harmful to the environment, and can result in resistance of the pathogen to fungicides" [6]. This explains why environmental friendly strategies must be put in place as alternative methods. Rhizobacteria are beneficial free-living soil bacteria, which improve plant health or increase yield and have a protective effect against plant pathogens by inducing many mechanisms. Among them, many fluorescent *Pseudomonas* rhizobacteria have been reported to produce secondary metabolites, particularly *P. fluorescens* and its closely related species are well characterized for their ability to produce antimicrobials involved in plant growth promotion [7-10]. In Cameroon, *Pseudomonas* sp. CMR12a showed excellent *in vivo* biocontrol against *R. solani*. Its biocontrol activity has been clearly linked to the production of two cyclic lipopeptides, sessilin and orfamides, including other secondary metabolites such as phenazines and hydrogen cyanide [11]. Besides, our

previous investigations reported that the production of antimicrobial secondary metabolites by fluorescent *Pseudomonas* DS17R against *P. colocasiae* was influenced by abiotic factors such as carbon, nitrogen sources, pH and temperature [12]. In the present study, the objective was to identify suitable culture media for production of antimicrobial substances in free cell culture against *Phytophthora colocasiae*.

2. MATERIALS AND METHODS

2.1 Bacteria Isolates

Bacterial isolates (DS15V and DS17R) identified as fluorescent *Pseudomonas,* isolated from Taro's associated rhizosphere was used in this study as a biocontrol agent.

2.2 Plant Pathogen

Phytophthora colocasiae was isolated from naturally infected Taro's leaves using the methods described by Sameza et al. [2] Pure cultures were maintained on Potato Dextrose Agar (PDA) at 26℃. Sporal suspension of *P. colocasiae* was prepared (5 x 10^5 spore ml^{-1}) following the same guidelines.

2.3 Supernatant Crude Extract Preparation for Monoculture of *Pseudomonas* Isolates

This preparation was made following the protocol with some modifications. Five culture media with different nutritional compositions were used in this experiment for extraction of antimicrobial substances. The basic medium was King B (KB), Luria Bertani (LB), succinate medium (MS), Nutrient Broth Yeast (NBY), Tryptic Soy Broth (TSB) and Potatoes Dextrose Broth (PDB). The initial pH of each medium was adjusted to 6.7 [12]. The experiment was carried out in 200 ml Erlenmeyer flasks containing 100 ml of each medium and was inoculated with 2 ml of 18 h fresh cultures of DS15V and DS17R. Inoculated flasks were incubated at 28℃ on a rotary shaker at 110 rpm for seven days. Bacterial growth in each culture medium was measured periodically by spectrophotometer as an increase in optical density at 600 nm. After completion of the incubation period, each of the liquid cultures were centrifuged at 5,000 rpm for 25 min. The cellular pellet was discarded and the supernatant was acidified to pH 2.5 with 6 M HCl. The solution was autoclaved at 121°C, 0.75 atm for 10 min, and then centrifuged again at 3,000 rpm for another 10 min and the precipitate was again discarded. The supernatant was adjusted to a final pH of 7.0 with phosphate buffer (1 M, pH 8). This solution was designated as crude supernatant [13]. The best culture medium for the production of antifungal substances was selected according to the percentage inhibition of radial growth of *P. colocasiae* in the agar plate.

2.4 Supernatant Crude Extract Preparation for Co-cultures *Pseudomonas-Phytophthora colocasiae*

A volume of 1 ml of bacterial culture grown in King B agar for 24 h (containing 10^8 cfu ml^{-1}) and a disc of test fungus (5 mm) from a well-grown fungus colony on PDA plates were inoculated in 50 ml broth of PDB in 250 ml conical flasks at 25°C on a rotary shaker. Broth inoculated only with *P. colocasiae* served as control. The differences in dry weights between the oomycete and the bacterium or the control cultures (without bacterium) were recorded by passing 7 days grown dual cultures through pre-weighed filter paper (Whatman No.1). The filter papers were dried for 24 h at 70°C and weighed. The inhibition of *P. colocasiae* radial growth was calculated via the formula: %I=(Do-Dx)/Do X 100, Do is the growth of the pathogen on the control plate, and Dx the growth in the test plate. All the tests were done in triplicate and the experiment was repeated twice [14].

2.5 Thermosensibility Assay

A 100 ml of crude supernatants were poured in sterilized conical flasks. The flasks were treated as maintained at room temperature (28 ± 2°C) (T0); heated water bath at 50°C (T1); heated water bath at 100°C (T2), and autoclaved at 121°C (T3). Each treatment was submitted for 10 min, 20 min and 30 min, respectively. After treatment, each set of supernatants was amended with molten PDA to get a final ratio of 1:1 (v/v), 1:2 (v/v) and 1:3 (v/v) respectively. The amended medium was inoculated with test fungus, incubated for seven days, and observed for radial growth [15].

2.6 Determination of the Inhibitory Activity Units (IAU) of the Antifungal Substances in the Crude Supernatant

The crude supernatant obtained by liquid culture (in nutrient broth media) of DS15V and DS17R

was sequentially mixed with molten PDA cooled to 45℃ to get final ratio of 1:1 (v/v), 1:2 (v/v), 1:3 (v/v) and 1:4 (v/v); where PDA plates without amended supernatant served as a control. Each of these dilutions was tested against *P. colocasiae* in order to establish the inhibitory activity unit of crude supernatant extract. Radial growth of PC on these amended media was recorded after seven days of incubation at 26℃. The experiment was conducted in a randomized complete design with three replicates. One IAU was defined as the higher dilution of the crude supernatant that inhibits 100% the growth of *P. colocasiae*.

2.7 Leaf Disc Assay

Leaves of taro (var. esculenta) plants (40-days-old) from an experimental field in the University of Dschang, Cameroon were removed, surface sterilized with a 1 % sodium hypochlorite solution for 2 min and rinsed twice with sterile distilled water. Thereafter the leaves were cut into 5 cm diameter discs, dipped in supernatant extracts for 30 min at a range of concentrations 1:1 (v/v), 1:2 (v/v) and 1:3 (v/v), with leaf disc dipped in (Ridomil) (3.3 mg/ml) as standard antimicrobial chemical as control. Ten microliters (10 µl) of zoospore suspension (10^6/ml) were applied to the underside of each leaf disc and incubated at 25 ± 2°C in the dark for 5 days. On the last day, the diameter of necrosis was measured according to the protocol described by Brooks [16]. Each treatment was repeated three times [17].

2.8 Zoospore Inhibition Assay

Assessment of sporangia growth inhibition was carried out using a liquid dilution method in V8 broth (2, 16) at six different concentrations; 1:1 (v/v), 1:2 (v/v), 1:3 (v/v), 1:4 (v/v), 1:5 (v/v), 1:6 (v/v), 1:7 (v/v) and 1:8 (v/v) of crude supernatant extract. The medium was inoculated with 500 µl of microbial suspension from a 10- day-old culture, adjusted to 10^6 cells/ml and incubated at 25 ± 2°C. Each treatment was repeated three times. Germination was recorded after 3 h for zoospores and 24 h for sporangia of *P. colocasiae* as percentage of total zoospores and sporangia germinated under a light microscope using a hemocytometer.

2.9 Statistical Analysis

Results were expressed as means ± standard deviation (SD). Data were analyzed with analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS version 21.0) software for Windows and Seaborn (Jupyterlab 3.0.14) for Mac Os. The significance of the difference between means was determined at $p < 0.05$ using Newman-Keuls and Mann-Whitney-Wilcoxon tests.

3. RESULTS

3.1 Effect of Culture Media on the Growth of DS15V and DS17R

The growth of DS17R and DS15V (Fig. 1) in KB, LB, MS, PMo and PCo different culture media show great microbial biomass after 96 h compared to NYB and TSB culture media which various curves stabilized after 48 h of growth.

3.2 Antimicrobial Activity of DS15V and DS17R crude Supernatant Extracts on Mycelia Growth of *P. colocasiae*

The tables and figures below presented effects of five liquid culture media KB, LB, MS, NYB and TSB on the growth of *P. fluorescens* DS15V and DS17R respectively and the effects of monoculture (PMo) and co culture (PCo) with *P. colocasiae* in PDB medium. DS15V (Table 1) show that KB and LB liquid culture media extracts show 100% mycelia inhibition at 1:1 (v/v) which represents the Inhibitory Activity Unit (IAU). Whereas PMo and PCo culture extracts show 100% inhibition of *P. colocasiae* mycelia corresponding to an IAU of 1:3 (v/v). This could explain the inhibitory potential at low concentrations of these liquid culture media and their IAU values. The same results are observed in Table 2 where only KB, LB, MS, PMo and PCo culture extracts showed 100% of mycelia inhibition with IAU values of 1:1 (v/v), 1:1 (v/v), 1:2 (v/v), 1:2 (v/v) and 1:3 (v/v) respectively. In addition, the inhibitory activity of the extracts was characterized by the decrease in mycelial growth depending on the concentration of the different crude supernatant (Fig. 2).

Given to each nutritional condition, supernatant crude extracts tested against *P. colocasiae* reveal that the antagonistic potential depends on culture media composition. Depend on consecutive dilution of supernatant, we found that the antagonistic potential of each culture supernatant extracts is significantly linked to the concentrations. For the three isolates, monocultures (PMo) and co cultures (PCo) supernatant extracts from PDB medium presented best IAU at low concentration.

3.3 Thermostability of Crude Supernatant Extracts at Different Temperature

Based on the previous results, the most active crude supernatant extracts were selected to evaluate their thermo stability. Figs. 3 and 4 present the effects of heating on 17PMo, 17PCo, 15PMo, 15PCo, 15LB, 17KB supernatants under 50, 100 and 121°C during 10, 20 and 30 minutes respectively. These figures show that increase of temperature did not affect significantly the antagonistic potential of 17PMo, 17PCo, 15PMo, 15PCo against *P. colocasiae*. Non-the-less, for 15LB and 17KB supernatant extracts, the antimicrobial activity decreases significantly at higher temperatures precisely between 100°C to 121°C. These results show that the antimicrobial metabolites produced in PDB are thermoresistant whereas nutrient media are thermo sensitive at high temperatures.

Table 3 shows the effects of heating on supernatants inhibitory activity at three different concentrations. At 121°C for 30 min, the Pseudomonads supernatants revealed inhibitory activity against *P. colocasiae* mycelia at 1:3 (v/v) for both 17PCo and 15PCo respectively. This demonstrated that IAU was conserved for co-culture and decreased at 1:2 (v/v) for monoculture supernatant (17PMo).

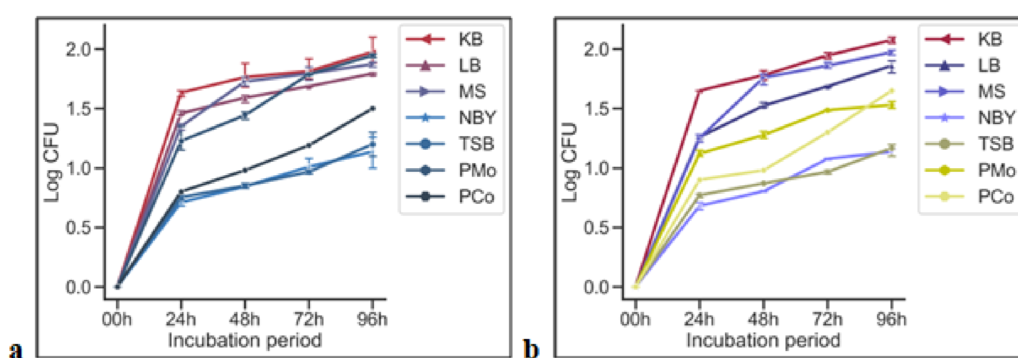

Fig. 1. Growth rate of DS15V (a) and DS17R (b) under KB, LB, MS, NBY, TSB, PDB (PMo) culture media by monoculture and co-culture with *P. colocasiae* (PCo)

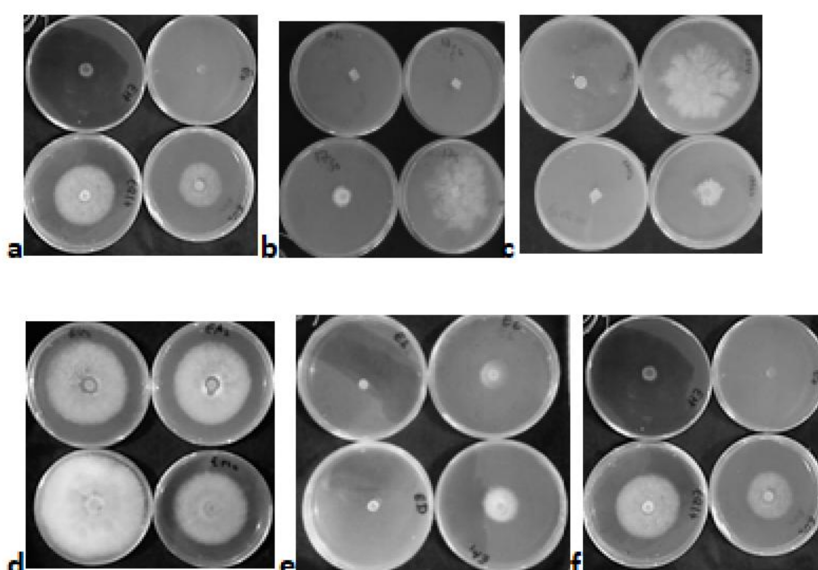

Fig. 2. Mycelia inhibition of *P. colocasiae* by DS15V and DS17R co culture crude supernatant extracts at 1:1 (v/v), 1:2 (v/v), 1:3 (v/v) and 1:4 (v/v) respectively. 17PMo (a), 17PCo (b), 15PCo (c), 15NBY (d), 15KB (e), 17KB (f)

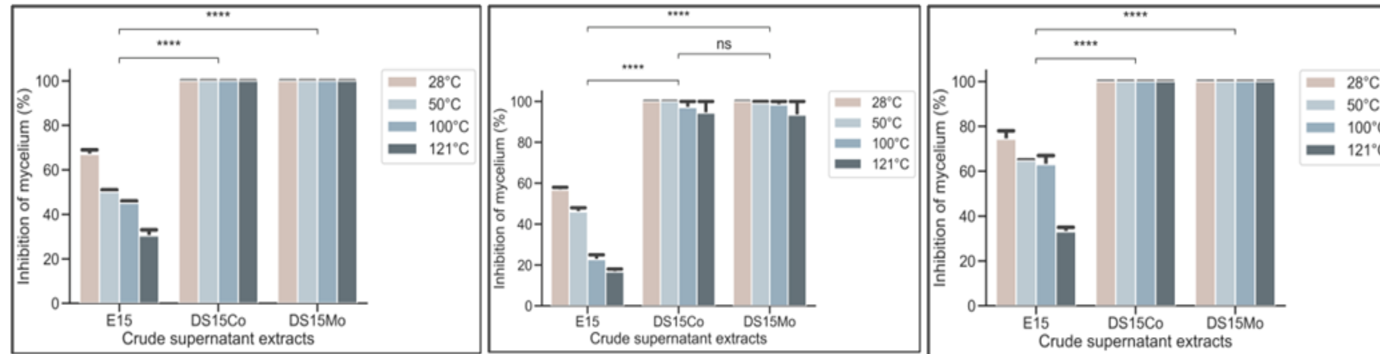

Fig. 3. Mycelia inhibition of crude supernatants extracts of DS15V heated at 28°C, 50°C, 100°C and 121°C for 10 min, 20 min and 30 min respectively

*E15= Monoculture supernatant extracts belong to KB medium. DS15Mo and DS15Co= Monoculture and co-culture supernatant extracts belong to PDB medium. ns: non-significative 0.05 < p <= 1; *: 0.01 < p <= 0.05; **: 0.001< p <= 0.01;***: 0.0001 < p <= 0.001;****: p <= 0.0001 according to the Mann-Whitney-Wilcoxon*

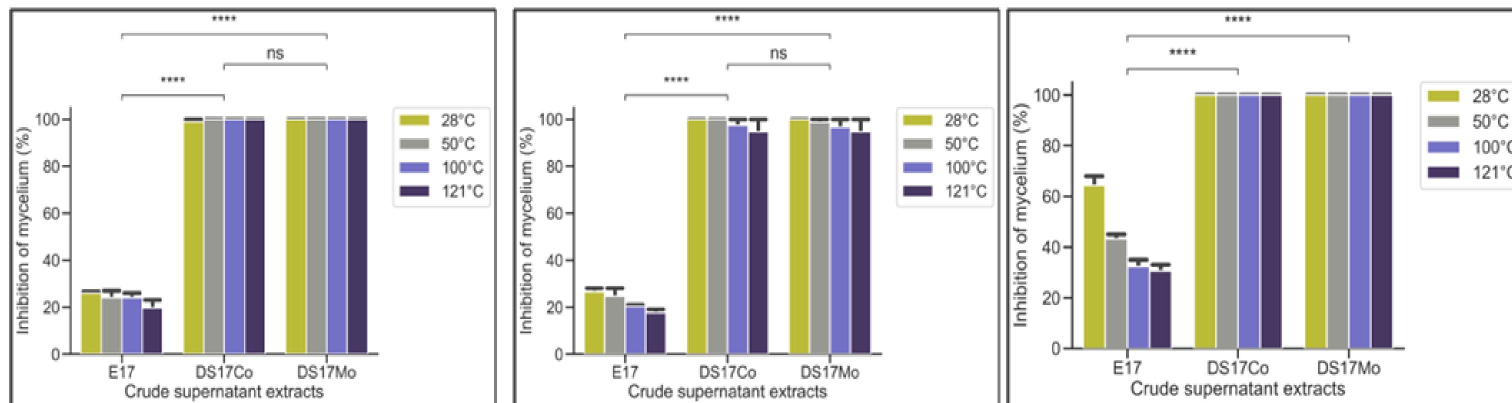

Fig. 4. Mycelia inhibition of crude supernatants extracts of DS17R at 28°C, 50°C, 100°C and 121°C for 10 min, 20 min and 30 min respectively

*E17= Monoculture supernatant extracts belong to KB medium. DS17Mo and DS17Co= Monoculture and co-culture supernatant extracts belong to PDB medium. ns: non-significative 0.05 < p <= 1; *: 0.01 < p <= 0.05; **: 0.001< p <= 0.01;***: 0.0001 < p <= 0.001;****: p <= 0.0001 according to the Mann-Whitney-Wilcoxon*

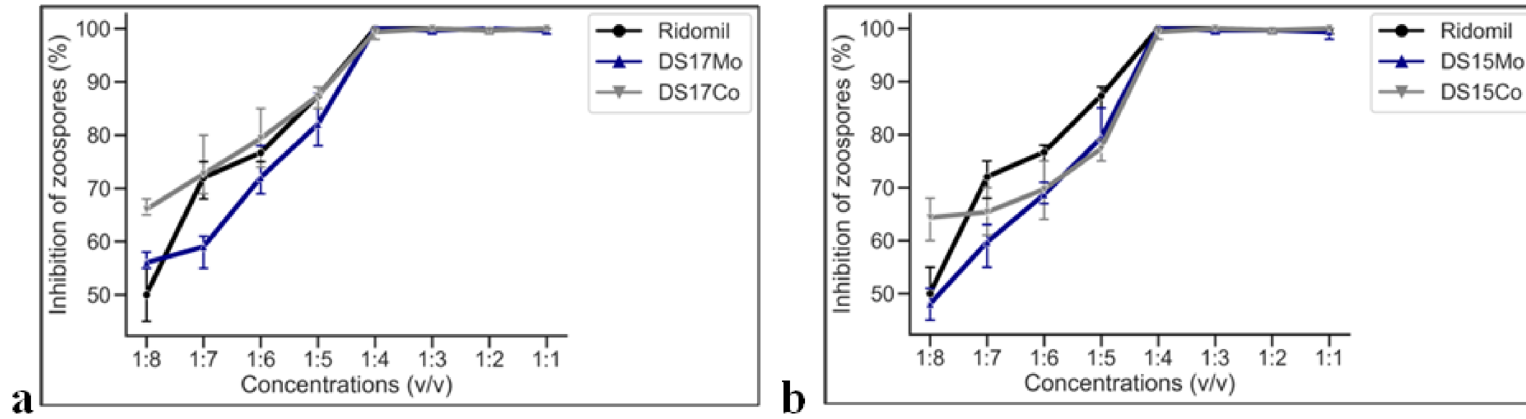

Fig. 5. Inhibition of *P. colocasiae* zoospore by supernatant extracts of DS17R (a) and DS15V (b) respectively. DS17Mo and DS17Co= Monoculture and co-culture supernatant extracts belong to PDB medium

Table 1. Inhibition of mycelia growth percentages of *P. colocasiae* by monoculture and co culture supernatant extracts of DS15V

Isolate DS15V	Inhibition of mycelia growth (%)				
	Concentrations	1 :1 (v/v)	1 :2 (v/v)	1 :3 (v/v)	1 :4 (v/v)
	S15KB	100±0.00[a]	95.83±2.15[a]	53.33±4.22[c]	0,00±0.00[d]
	S15LB	100±0.00[a]	87.08±3.50[a b]	68.75±6.92[b]	39.58±2.05[c]
Monoculture supernatants	S15MS	82.08±1.45[b]	64.16±5.86[d]	39.58±5.36[d]	2.08±0.85[d]
	S15NBY	76.25±3.80[c]	72.91±3.41[c]	37.91±3.33[d]	0.00±0.00[d]
	S15TSB	80.00±0.50[b]	55.41±4.02[e]	30.83±1.49[e]	0.83±0.21[d]
	15PMo	100±0.00[a]	100±0.00[a]	100±0.00[a]	87.50±0.85[a]
Co-culture supernatant	15PCo	100±0.00[a]	100±0.00[a]	100±0.00[a]	82.91±1.90[b]

S15KB, S15LB, S15MS, S15NBY, S15TSB, 15PMo are crude supernatants extracts of DS15V belong to KB, LB, MS, NBY, TSB and PDB broth media by monoculture; whereas 15PCo =supernatant extract obtained by co-culture with P. colocasiae in PDB culture medium. Values are mean of three replicates. Means followed by the same letter in the same column are not significantly different (p<0.05) according to the Newman-Keuls test

Table 2. Inhibition of mycelia growth percentages of *P. colocasiae* by monoculture and co culture supernatant extracts of DS17R

Isolate DS17R	Inhibition of mycelia growth (%)				
Concentrations		1 :1 (v/v)	1 :2 (v/v)	1 :3 (v/v)	1 :4 (v/v)
	S17KB	100±0.00[a]	91.5±2.33[b]	82.91±2.10[b]	0.00±0.00[e]
	S17LB	100±0.00[a]	85.41±2.22[c]	65.00±2.65[d]	32.08±2.22[b]
Monocultures supernatants	S17MS	100±0.00[a]	100±0.00[a]	75.41±3.92[c]	42.50±6.35[a]
	S17NBY	82.50±2.03[b]	66.66±1.75[d]	38.75±3.35[e]	33.33±2.16[b]
	S17TSB	100±0.00[a]	70.00±0.00[d]	40.42±6.52[e]	6.25±1.44[d]
	17PMo	100±0.00[a]	100±0.00[a]	81.25±1.25[b]	42.50±4.01[a]
Co-culture supernatant	17PCo	100±0.00[a]	100±0.00[a]	100±0.00[a]	35.83±6.95[b]

S17KB, S17LB, S17MS, S17NBY, S17TSB, 17PMo are crude supernatants extracts of DS17R belong to KB, LB, MS, NBY, TSB and PDB broth media by monoculture; whereas 17PCo =supernatant extract obtained by co-culture with P. colocasiae in PDB culture medium. Values are mean of three replicates. Means followed by the same letter in the same column are not significantly different (p<0.05) according to the Newman-Keuls test

Table 3. Inhibition of mycelia growth of *P. colocasiae* by monoculture and co culture supernatant extracts of DS15V and DS17R heated at 121°C during 30min

Temperature 121°C (30 min)				
Isolates	Supernatants Extracts	Concentrations (UA)		
		1:1 (v/v)	1:2 (v/v)	1:3 (v/v)
Inhibition of mycelium (%)				
DS15V	S15LB	59.45±1.15[d]	51.13±4.56[b]	30.14±5.64[d]
	15PMo	100 ± 0.00[a]	100 ± 0.00[a]	100 ± 0.00[a]
	15PCo	100 ± 0.00[a]	100 ± 0.00[a]	100 ± 0.00[a]
DS17R	S17KB	72.94±3.62[b]	56.40±2.24[b]	38.66±3.27[c]
	17PMo	100 ± 0.00[a]	100 ± 0.00[a]	94.50±2.25[a]
	17PCo	100 ± 0.00[a]	100 ± 0.00[a]	100 ± 0.00[a]

Values are mean of three replicates. Means followed by the same letter in the same column are not significantly different (p<0.05) according to the Newman-Keuls test

Table 4. Preventive effects of DS15V and DS17R co culture supernatant extract (15Co and 17PCo) against *P. colocasiae* on taro leaf discs

Preventive effects				
Extracts	**15PCoT**		**17PCoT**	
	Necrosis diameter			
Concentrations (UA)	**Ø (mm)**	**I (%)**	**Ø (mm)**	**I (%)**
0 :0 (v/v)	45.50±0.00	0.00±0.00	45.50±0.00	0.00±0.00
1 :1 (v/v)	0.00±0.00	100±0.00^{a}	0.00±0.00	100±0.00^{a}
1 :2 (v/v)	9.82±1.37	78.41±0.98^{b}	6.18±3.77	86.41±4.55^{b}
1 :3 (v/v)	20.05±2.38	55.92±1.03^{c}	15.24±5.21	66.50±5.76^{c}
Ridomil (3.3 mg/mL)	0.00±0.00	100±0.00^{a}	0.00±0.00	100±0.00^{a}

Values are mean of three replicates. Means followed by the same letter in the same column are not significantly different (p<0.05) according to the Newman-Keuls test. Ø=diameter; I=inhibition. 15PCoT, 17PCoT= co-culture supernatants extracts obtained by heating at 121°C at 30 min of DS15V and DS17R respectively

Table 5. Curative effects of DS15V and DS17R co culture supernatant extract (15Co and 17PCo) against *Phytophthora colocasiae* on taro leaf discs

Curative effects				
Extracts	**15P CoT**		**17P CoT**	
		Necrosis diameter		
Concentrations (UA)	**Ø (mm)**	**I (%)**	**Ø (mm)**	**I (%)**
0 :0 (v/v)	45.50±0.00	0.00±0.00	45.50±0.00	0.00±0.00
1 :1 (v/v)	0.00±0.00	100±0.00^{a}	0.00±0.00	100±0.00^{a}
1 :2 (v/v)	7.77±1.03	82.41±2.25^{b}	15.22±1.02	66.54±2.96^{b}
1 :3 (v/v)	10.07±2.76	77.85±5.78^{bc}	20.35±0.50	55.27±4.63^{c}
Ridomil (3.3 mg/mL)	0.00±0.00	100±0.00^{a}	0.00±0.00	100±0.00^{a}

Values are mean of three replicates. Means followed by the same letter in the same column are not significantly different (p<0.05) according to the Newman-Keuls test. Ø=diameter; I=inhibition. 15PCoT, 17PCoT= co-culture Supernatants extracts obtained by heating at 121°C at 30 min of DS15V and DS17R respectively

3.4 Effects of Supernatant Crude Extract on *P. colocasiae* Zoospore Viability

The Fig. 5 presented the effects of the crude supernatant extracts against *Phytophthora colocasiae z*oospores at different concentrations. After 24 hours, 15PCo showed 100% inhibition of zoospores at 1:3 (v/v) whereas, three other extracts (15PMo and 17PMo) presented their IAU values at 1:2 (v/v). However, only 17PCo supernatant extract inhibited 100% of zoospores at 1:4 (v/v) and make him most potent supernatant extract against zoospore. These results also demonstrated that extracts obtained by co culture (15PCo and 17PCo) with *P. colocasiae* indicate the best inhibition of zoospores activity. The percentages of zoospore germination also decrease from high to less concentration for all crude supernatant extracts.

3.5 Prevention Effects of Crude Supernatant on Taro Leaf Disc

Preliminary screening of best extracts based to previous results permit to select 17PMo, 17PCo and 15PCo for prevention and curative effects. The prevention effects on taro leaf disc shows 100% prevention for the four supernatants extracts at 1:1 (v/v) (Table 4). This effect against *P. colocasiae* is significantly reduced at low concentrations and decrease to 9 at 46% prevention. Besides we observed that, the severity of necrosis depends on the concentration. At low concentration the disc showed high level of infection compared to their increased concentrations.

3.6 Curative Effects of Crude Supernatant on Taro Leaf Disc

The reduction rate of necrosis after infection on disc leaf of Taro by supernatant showed that after four days of incubation, 17PCo, 17PMo and 15PCo stabilize significantly the evolution of leaf disc necrosis at concentration of 1:1 (v/v) (Table 5). The formation of a clear halo around the necrosis area attests the *in-situ* mechanism of antagonism activity of these crude supernatant extracts. At the lower concentrations, the rate of leaf disc necrosis increases but compared to negative control (0:0 v/v), the severity is not the same and the necrosis diameter is significantly different. This observation proves that, these crude supernatant extracts when pulverized on leaf disc after 2 days of incubation period, significantly reduce the rate of necrosis development or totally stabilize it.

4. DISCUSSION

"Pseudomonas fluorescens comprises a genus of species capable of utilizing a wide range of organic and inorganic compounds and of living under diverse environmental conditions. In general, the productivity of microbial metabolites is closely related to the fermentation process used" [18,19]. "The changes in nutrients and their concentrations have different effects on the accumulation of different metabolites, which are controlled by intracellular effectors. Where, the carbon and nitrogen sources can dramatically influence antibiotic formation. Production of antibiotics, hydrogen cyanide, lytic exoenzymes, cyclic lipopeptides, competition for nutrients and niches, competition for iron mediated by siderophores, competition for carbon, and induced systemic resistance are the most commonly reported biocontrol mechanisms by fluorescent *Pseudomonas* spp" [20-22]. In this study, the observed activity of crude supernatant extracts is cross- linked to nutritional elements present in growth media. The liquid media KB and LB potentiate the activity of DS15V supernatants at the same concentration. This result proves that yeast extract as nitrogen source in the LB liquid media has similar effect as peptone in the basic medium (KB). The same effect was observed for KB, LB, MS and TSB supernatants extracts of DS17R. The different crude supernatant extracts belong to these liquid media conserve the same IAU at 1:1(v/v) except MS which showed 100% inhibition of mycelia growth of *P. colocasiae* at 1:2 (v/v). Based on these results, KB, LB and MS stimulate production of secondary metabolite against *P. colocasiae* mycelium and production of antimicrobials in supernatant could be impaired by sucrose in the growth medium, whereas peptone, yeast extract and NH_4Cl increase production of metabolites (DS15V and DS17R). A similar result was seen by [18] where pyrrolnitrin production was eliminated by glucose in the growth medium, whereas phenazine production was enhanced. This could also explain the difference in antagonist potential observed within the same isolate. Besides, the negative effects of sucrose in some previous studies demonstrated that, this sugar impaired the production of 2,4-DAPG, PRN and phenazine [23,24]. "The importance of nutrient status to pyoluteorin production is corroborated by the observation that pyrrolquinoline quinone, a cofactor required by glucose and alcohol dehydrogenases, represses pyoluteorin production confirm that pyoluteorin production is linked to the physiological status of the cell" [25]. "Regulation of 2,4-DAPG and PLT production in *Pf* CHA0 involves a molecular balance in which each antibiotic induces the expression of its own biosynthetic genes while repressing the expression of the biosynthetic genes of the other antibiotic" [26,27]. "Antibiotics production by biocontrol agents in liquid culture can be affected by variations in the fermentation environment, often resulting in variations in antibiotic production" [28]. Supernatant extracts from monoculture and co-culture of the two *Pseudomonas* isolates in PDB medium inhibited 100% mycelia growth at 1:3 (v/v). This significant increase activity of the supernatant extracts in PDB medium confirms positive effect of glucose for the production of antimicrobial secondary metabolites among *Pseudomonas* genus, especially DS15V and DS17R *Pseudomonas fluorescens*. The previous observation also give evidence that, the co-culture of *Pseudomonas* with *P. colocasiae* could increase the metabolic process of both bacteria and fungi, thereby increase the production of secondary metabolites responsible of the high activity observed for 15PCo and 17PCo. These results could also well explain how glucose could respond and induce antibiotic production when associated or not to other nutrient variations. In the most of case, phosphate ion impairs the positive effect of glucose when combined in the same medium and decrease the production of antibiotic production of 2,4-DAPG, PRN and phenazine. Therefore, glucose without phosphate ion such as under PDB medium condition could freely exert his positive effect.

The heating treatment of the crude extract in co-culture with *P. colocasiae* showed the same antagonistic activity among 15PMo, 15PCo, 17PMo and 17PCo supernatant extracts without heating. Whereas high temperature revealed negative effects on E15 and E17 supernatants belong to basic medium (KB). Thus, inhibitory substances in PDB are thermo-resistant and certain antimicrobial substances induced by KB medium are sensible at high temperature for reason while the antimicrobial activity decreases. These results also demonstrated that antifungal substances present in the supernatant could be antimicrobial peptides or non-proteinaceous substances. The proteinaceous compound, optimally produced at pH 6.5, showed the highest rate of production within 72 h of incubation (the synthesis began during the early exponential phase of growth, log 4.8 CFU/ml) [15] is resistant to pasteurization, is stable at 15°C for 45 days and maintains more than 50% of the maximum inhibitory activity in the temperature range of 5°C to 10°C. Non-the-less, the thermostability at 121°C attests that non-proteinaceous substances could be present in high amounts and responsible for the activity. The same observations were obtained with some microorganisms. These results are in agreement with [29] who reported that "pyrrolnitrin, produced by *Enterobacter agglomerans* was resistant to heat treatment" [30] stated that "*B. cepacia* strain CF-66 produced a thermostable antifungal compound CF66I, proteolytic enzymes and organic solvents". The antimicrobial activity of iturin, an antibiotic substance produced by *Bacillus subtilis,* was also reported to be resistant to boiling and to autoclaving at 121°C [13]. Several studies [31-33] showed that cyclic lipopeptides could be antimicrobial substances responsible of the given activities and similar findings were reported by Abdel-Mawgoud for *Pseudomonas aeruginosa* isolate Bs20 which revealed excellent stabilities at high temperatures (heating at 100°C for 1 h and autoclaving at 121°C for 10 min). The reports of earlier studies [34] support the finding of the present study, which shows that glucose is preferred as the carbon source for the rhamnolipid production by *Pseudomonas fluorescens* MFS03. These results also explain why, glucose in high amounts in PDB media extracts are more thermostable and conserve the antifungal potential against *P. colocasiae*. The glucose also shows the high biosurfactant yield in recent studies like rhamnolipid [35]. "The non-proteinogenic amino acids identified as secondary metabolites of pseudomonads all displayed some type of selective antimicrobial properties *in vitro* tests. FVG and MVG, for example, inhibit the growth of Erwinia amylovora, the causative agent of fire blight, a disease of roseaceous orchard crops [36]. "MVG also inhibits growth of *Acanthamoeba castellanii"* [36] and *Bacillus* sp. 1283B [37]. Similarly, 3-methylarginine inhibits the growth of *P. syringae* pv. glycinia, the causative agent of bacterial leaf blight [38].

The sporangium is the primary reproductive unit of *P. colocasiae* which requires free water to germinate. It is convenient to take this as the starting point for the life cycle and form an important spread process during Taro Leaf Blight development. The evaluation of oospore germination percentage values demonstrated 100% inhibition for 14PCo and 15PCo *Pseudomonas* crude supernatant extracts totally exhibited the growth at concentrations of 1:3 (v/v) and 1:4 (v/v) for 17PCo extract. These IAU obtained against oospore germination confirm the antifungal potential observed *in vitro.* The supernatant inhibition potential might be due to the presence of high antimicrobial substances produced which deteriorate the membrane integrity of the germinating process by inhibition of germ tube formation that infect the leaves [2].

Several studies have found no correlation between *in vitro* antibiosis and biocontrol. For example, in a recent report where comparative *in* vitro and *in vivo* studies were conducted on *P. corrugata* against fungal attack causing wilting in micro propagated tea, it was observed that the isolates of *P. corrugata* inhibited the fungi only *in vivo* experiments. The leaf disc assay based on these results uses discs (60 mm diameter) from the youngest fully expanded taro leaf. The data revealed that 15PCo and 17PCo crude supernatant extracts significantly stabilized necrosis rate induced by *P. colocasiae* at 1:1 (v/v) in the curative leaf disc test, and 100% prevention was also observed against TLB at the same concentrations. Besides, the fairly effective in inhibiting the growth of *P. colocasiae* in the disc leaf can be closely linked to the amount of inhibitory substances. This compound also impaired disease progression by inhibiting sporangium production and zoospore motility. This could also explain the formation of clear areas around treated leaf discs. Sporulation, zoospore motility, zoospore germination, and infection all are important steps in the disease cycle. They are particularly significant for disease, and the observed curative and

preventive effect of these extracts testify to the quantity of antibiotic produced. Similarly, phenazines and CLPs have been shown to play a role in the biocontrol capability of Pseudomonas spp. CMR12a isolated in Cameroon [39-41].

5. CONCLUSION

Overall, the findings of this study revealed that the antimicrobial activity of DS15V and DS17R *Pseudomonas fluorescens* crude supernatant extracts varies by culture media. Supernatant extracts belong to King B and PDB (15PMo, 15PCo 17PMo and 17PCo) exhibited strong antimicrobial activity against *P. colocasiae* mycelia and growth of zoospores Furthermore, monoculture and co-culture crude supernatant extract from PDB are thermo-resistant at very low concentration (1:3 v/v) whereas supernatants belong to KB were sensible at high temperature. Besides, these extracts showed 100% curative and preventive effects at 1:1 (v/v) by drawn-out and stabilizing leaf disc necrosis development after 4 days. These results prove that easy formulations like crude supernatants of fluorescent *Pseudomonas* DS15V and DS17R could be used as bioinoculants in the field against the TLB, even though the efficiency as biocontrol agents must first be confirmed under field conditions.

DATA AVAILABILITY

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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