

1 **Antifungal potential of acetone and ethyl acetate extracts of *Thevetia peruviana* on**  
2 **development of *Phytophthora colocasiae*, causal agent of late blight of taro**  
3 **(*Colocasia esculenta* (L.) Schott) from three Agro-ecological zons of Cameroon.**  
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## ABSTRACT

**Aims:** This study was aimed to evaluate the antifungal activities of acetone and ethyl acetate extracts of *Thevetia peruviana* seeds on the *in vitro* growth of the fungus.

**Study design:** A randomized sample block design containing four treatments (T- = Negative control; T2= Ethyl acetate extract; T3= Acetone extract; T+=Callomil Plus) with three repetitions was used. Plant extracts were used at three concentrations: C1: 12.5 µl/ml; C2: 25 µl/ml and C3: 50 µl/ml; the chemical fungicide at the dose of 12.5 µL/ml.

**Place and Duration of Study:** The study was conducted in the University of Yaoundé 1, Faculty of Sciences, Department of Plant Biology, Laboratory of Phytopathology and Crop Protection, and in the Institute of Agricultural Research for Development (IARD) of Yaoundé, Laboratory of Phytopathology, during the year 2019-2020.

**Methodology:** acetone and ethyl acetate extracts of *T. peruviana* were prepared and used at concentrations of 12.5, 25 and 50 µl/ml. *P. colocasiae* was isolated from infected taro leaf cultivars "Macumba or Ibo coco" located in three different regions: west, Littoral and Centre. The various explants were maintained in pure culture by being placed in V8 agar media. Mycelial fragments of *P. colocasiae* of about 0.8 cm in diameter were cut and placed in sterile Petri dishes containing Potato Dextrose Agar (PDA) medium supplemented with different concentrations of plant extracts and incubated at 23±1°C for seven days for the evaluation of the radial growth.

**Results:** The results obtained showed that the acetone and ethyl acetate extracts have completely inhibited the growth of the strain of West at 25 µ/ml while total inhibition of the pathogen was not obtained with strain of Centre region. The lowest inhibition was obtained with the strain of Littoral region: 93.88 % for acetone extract and 90.78 % for ethyl acetate extract compare to 100 % for west and Centre region at highest concentration.

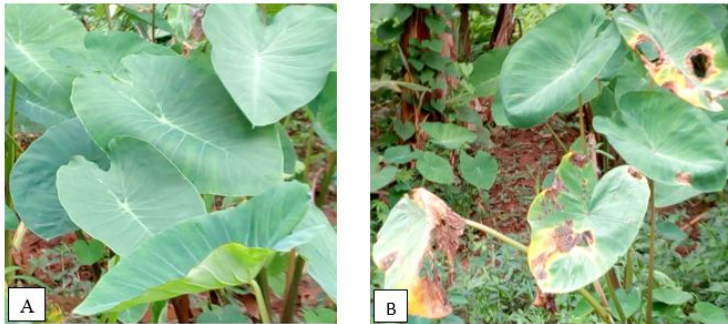
**Conclusion:** The acetone and ethyl acetate extracts at the concentration of 25 µ/ml totally inhibited the *in vitro* radial growth of some strains of *P. colocasiae*. These extracts, which are effective against *P. colocasiae*, may substitute fungicides in the fight against taro leaf blight.

**Keywords:** Extracts of *Thevetia peruviana*, antifungal potential, *Phytophthora colocasiae*, taro

## 1. INTRODUCTION

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11 "Cocoyams (*Colocasia esculenta*) are well adapted food crops across many agro-ecological  
12 zones of sub-Saharan Africa. They rank third in importance, after cassava and yam, among  
13 the root and tuber crops cultivated and consumed in many West and Central Africa  
14 countries. Cocoyams are nutritionally superior to both cassava and yam in the possession of  
15 higher protein, mineral and vitamin contents as well as easily digestible starch" [1]. "Africa in  
16 the last three decades has consistently accounted for an increasing percentage of global  
17 cocoyam production, which currently stands at about 10 million tonnes per annum" [1,2].  
18 "Cocoyam is therefore undoubtedly an important food crop in sub-Saharan Africa (SSA),  
19 particularly in Nigeria, Ghana and Cameroon. Global production is estimated at about 10.64  
20 million tonnes on a cultivated area of 1.67 million hectares" [1,2,3]. "In addition, 77% of  
21 global taro production comes from sub-Saharan Africa" [1]. "However, the increasing

22 production in the region has depended largely on farming more land rather than increasing  
23 crop yields. This is contrary to the projections of FAO that the 70% growth in global  
24 agricultural production needed to feed an additional 2.3 billion people by 2050 must be  
25 achieved by increasing yields and cropping intensity on existing farmlands, rather than by  
26 increasing the amount of land brought under agricultural production" [1,2]. This could be due  
27 to the enemies of this crop such as diseases that hinder its production. One of the most  
28 important is late blight caused by *Phytophthora colocasiae* [4,5].. It was first described in  
29 Java by Marian Raciborski in 1900 [6]. The disease mainly affects the leaves of the taro (Fig.  
30 1B), and can completely destroy susceptible cultivars in less than 10 days and cause yield  
31 losses in the range of 50 to 100% [4,5,6]This loss of yield has a remarkable impact on  
32 farmers' incomes as well as on the food security of human populations. pH 7 and  
33 temperature of 27°C are the optimal conditions for the pathogen to grow in the field [7,8,9].



34 **Fig.1. (A): colocasia esculenta plants. (B): attacked plants showing symptoms of taro**  
35 **late blight on the upper leaf surface.**  
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37 Control strategies for this pathogen are most often focused on the use of metalaxyl-based  
38 chemical fungicides [10,11]; but due to the problems of residues in groundwater [12], the  
39 development of resistance in the target organism and the danger to man and the  
40 environment, alternative control methods are increasingly being considered. Currently,  
41 considerable efforts are directed towards the exploration of plant extracts with pesticide  
42 potential as alternative or complementary sources to synthetic pesticides. Plant extracts  
43 have the advantage of being not only available to farmers, but also non-toxic and easily  
44 biodegradable and therefore healthy for the environment [13,14]. Several studies have  
45 shown the antifungal effects of plant extracts on *Phytophthora infestans*, the causative agent  
46 of late blight in potatoes, tomatoes and black nightshades [15,16,17], but no information is  
47 available on the effect of seed extracts of plants such as *Thevetia peruviana* on *P.*  
48 *colocasiae* in Cameroon. The seeds, leaves, fruits and roots of the Yellow Oleander  
49 (*Thevetia peruviana*) are considered potential sources of biological compounds active as  
50 insecticides [18,19,20] fungicides [20,21,22] virucides [23,24] and bactericides [25]. Thus,  
51

52 the present work aims to evaluate the antifungal potential of acetone and ethyl acetate  
53 extracts of *Thevetia peruviana* seeds on the *in vitro* development of *P. colocasiae* from three  
54 agro-ecological zones of Cameroon.

55

## 56 **2. MATERIAL AND METHODS**

### 57 **2.1. Plant and chemical material**

58 The plant material consists of the kernels of *Thevetia peruviana* collected in the city of  
59 Yaounde where the tree serves as a house fence; and leaves of *Colocasia esculenta*  
60 collected in peasant plantations located in the locality of: Bafang in the Department of Haut-  
61 Nkam in West (OU123), Penja in the department of Moundou in Littoral (LT122) and Yaoundé  
62 in the department of Mfoundi in Central Cameroon (CE111); and taken to the lab. The  
63 chemical material is a product with the trade name Callomil Plus 72 WP consisting of 12%  
64 metalaxyl and 60% copper oxide; and organic solvents such as acetone and ethyl acetate.

65

### 66 **2.2. Methods**

#### 67 **2.2.1. Preparation of extracts of *Thevetia peruviana* seeds**

68

69 The plant of *Thevetia peruviana* has been identified according to the botanical systematics  
70 key of the species with reference to the recent version of the International Code of Botanical  
71 Nomenclature [26,27]. The mature *T. peruviana* fruits were picked, the stones extracted from  
72 the fruits were crushed and the resulting kernels were dried at room temperature for 3 to 4  
73 weeks in the laboratory and then crushed using a manual mill to obtain a paste. The organic  
74 extract was prepared by maceration of 1 kg of paste in 5 L of solvent for 48 hours and then  
75 filtered. The resulting filtrate was concentrated at 60 °C using a rotary evaporator and the  
76 solvent extract obtained was stored in the refrigerator at 4 °C until use. Doses of extracts of  
77 12.5; 25 and 50 µl/ml were obtained following a progression geometry of reason 2 [28] from a  
78 stock solution of 500 µl/ml.

79 The extraction yield of each extract was calculated using the formula cited by

80 [29,30]:

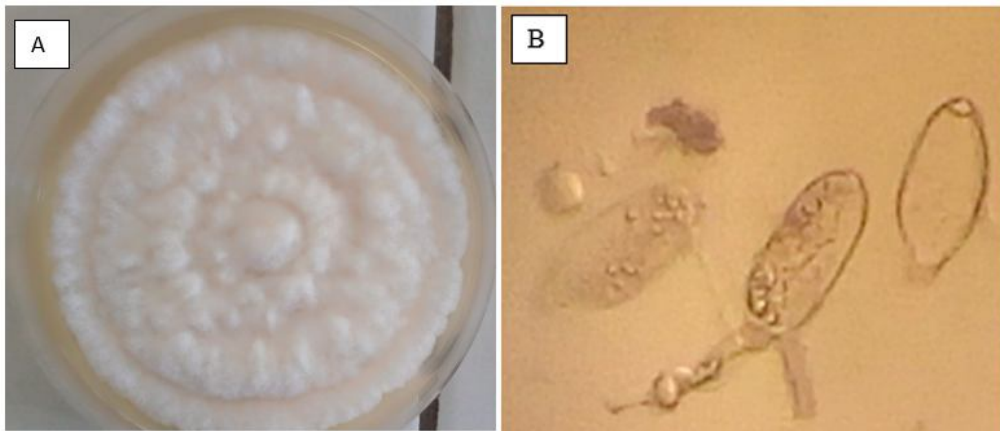
$$81 \quad Rd\% = \frac{\text{Mass of extract}}{\text{Mass of powder}} \times 100$$

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#### 83 **2.2.2. Isolation and purification of *Phytophthora colocasiae***

84 "The infected leaves of the harvested taro variety "Macumba" were cut into fragments of  
85 about 2 cm<sup>2</sup> at the growth front of the pathogen and disinfected superficially in a solution of  
86 5% sodium hypochlorite for 2 minutes. After three rinses with sterilized distilled water (EDS),

87 the fragments were dried on hydrophilic paper and then deposited at the rate of four  
88 fragments in a petri dish poured in the gelled V8 culture medium supplemented with a  
89 solution of antibiotics composed of penicillin (250 mg / l), ampicillin (250 mg / l) and nystatin  
90 (20 mg / l)" [31,32,33]. "After three days of incubation in the laboratory at a temperature of  
91  $23\pm 1^{\circ}\text{C}$ , colonies of the pathogen, visible around the fragments, were taken and  
92 transplanted into new petri dishes containing the PDA culture medium. This process was  
93 repeated several times until pure morphological cultures of the mycelium (not septate) and  
94 fruiting (sporangia) as described" by [34] and [35] were obtained (Fig. 2)  
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**Fig.2.** *Phytophthora colocassiae*: pure culture of mycelium (A) and sporangia (B). (Gr: X20)

100 The isolates obtained are characterized according to morphological criteria such as  
101 pathogenicity and growth rate [36].

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### **2.2.3. In vitro evaluation of the antifungal activity of the crude extracts**

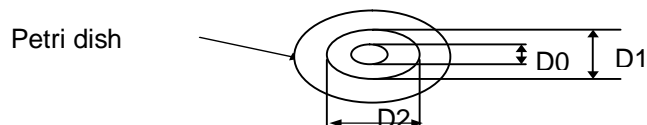
105 The in vitro evaluation of the antifungal activity of the extracts was done at concentrations of  
106 12.5; 25 and 50  $\mu\text{l/ml}$  for the two extracts from the stock solutions of 500  $\mu\text{l/ml}$  for each. A  
107 synthetic fungicide (Callomil Plus 72 WP) was used as a positive control by taking from a  
108 50g sachet, 1g of powder per 5 ml of distilled water. Mycelial explants of *P. colocassiae* about  
109 8 mm in diameter were taken with a cookie cutter from a pure fruiting culture seven days old  
110 and placed in the center of the petri dish containing the media enriched with the different  
111 extracts or **chemical** fungicide. A negative control not supplemented with extract was  
112 developed. Each treatment was repeated 3 times. Incubation was carried out at  $23\pm 1^{\circ}\text{C}$   
113 under a photoperiod of 12/12 for one week. A daily measurement of the radial growth  
114 diameter of each cultured explant was taken and continued until the mycelium filled the  
115 control dishes. The radial (D) growth of the pathogen was assessed by measuring two  
116 perpendicular diameters traced to the back of the petri dish. The average of the two

117 perpendicular measurements removed from the diameter of the explant represents the  
118 measure of the radial growth of the fungus. It is obtained by the formula described by [37]  
119

$$D = \frac{D1 + D2}{2} - D0$$

120  
121

122 Where: D0 is the diameter of the explant; D1 and D2 are the culture diameters  
123 measured in both perpendicular directions (Fig. 3).



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125  
126 **Fig.3. Diagram of measurement of mycelial growth in petri dish on V8 medium.**

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128 The percentage of inhibition (I%) due to each extract is evaluated in relation to the mycelial  
129 growth in the control boxes according to the formula developed by [37].

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$$I\% = \frac{D_{to} \text{ mm} - D_{xi} \text{ mm}}{D_{to} \text{ mm}} \times 100$$

131 With I (%): percentage of inhibition; Dto is the average diameter of the control batch and Dxi  
132 is the average diameter of the batches in the presence of the extract.

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#### 135 **2.2.4. Correlation between concentration and inhibition percentages**

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138 "Correlation tests were performed to determine the relationship between the concentrations  
139 used and the inhibition percentages obtained for each extract. In other words, it was a  
140 question of establishing a linear relationship model to predict the percentage of inhibition  
141 from the concentrations of each extract, for each type of fungus, and for each stage of life. In  
142 each case, the correlation coefficient was determined in order to provide information on the  
143 degree of linear dependence between the two variables.

144 In this case if a < 0 then the relationship is inversely proportional and the correlation is  
145 negative. If a > 0 then the relationship is positive; if r between 0.7 and 1 then the correlation  
146 is perfect and positive; if r between -0.7 and -1 then the correlation is perfect and negative; if  
147 r < 0.7 then the correlation is positive but imperfect; if r > -0.7 then the correlation is negative  
and imperfect" [38].

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#### 149 **2.2.5. Fungicidal or fungistatic activity of extracts and chemical fungicide**

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151 “At the end of each test, the mycelium explants from the boxes where the growth was  
152 completely inhibited, were taken and deposited aseptically on the culture medium containing  
153 no extract. After 7 days of waiting, depending on whether or not the fungus has resumed  
154 growth, the starting extract was identified as fungistatic or fungicidal respectively” [39].

155

156

### 157 **2.2.6. Statistical analysis**

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159 The inhibition percentages of the radial growth of the pathogen were transformed  
160 into probits and the values obtained were regressed on the logarithm of the  
161 concentration of plant extracts. The efficacy of the extracts was evaluated on the  
162 basis of the inhibiting concentration value of 50% (CMI<sub>50</sub>) and 90% (CMI<sub>90</sub>)  
163 determined after 8 days of growth according to the formula developed by [40].  
164 Inhibition percentage data were subjected to analysis of variance using R analysis  
165 software version 5.1.0 and means separated by Duncan's multiple test at the 5%  
166 probability threshold.

## 167 **3. RESULTS AND DISCUSSION**

### 168 **3.1. Results**

#### 169 **3.1.1 Extraction yield**

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171 The yield, volume, colour and appearance of the different extracts obtained varied  
172 depending on the extraction solvent used. Extraction with ethyl acetate yielded slightly  
173 higher (28.5%) than acetone (23.3%). The ethyl acetate extract has an oily appearance and  
174 pale yellow color while the acetone extract has a viscous appearance and brown color  
175 (Table 1).

176

177 **Table 1: Extraction yield (%) and characteristics of extracts for 1kg of seeds.**

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Extract with	Yield	Characteristics
Ethyl acetate (EAE)	28,5	Oily and pale yellow in colour
Acetone (EAc)	23,3	Brown and viscous

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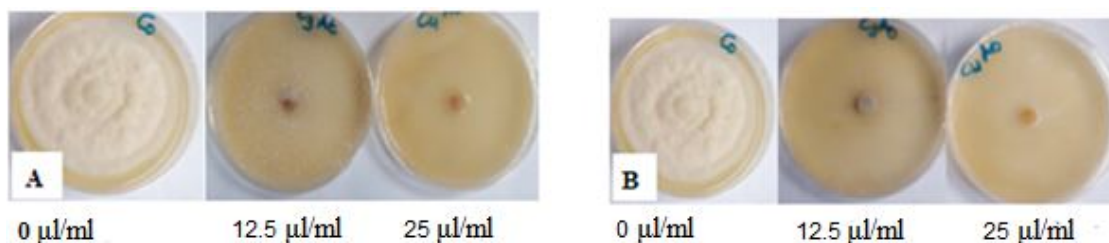
#### 180 **3.1.2 Effect of *Thevetia peruviana* extracts on the *in vitro* growth of *P. colocasiae***

181

182 The seed extracts tested significantly inhibited the radial growth of *P. colocasiae*.  
183 The diameter of the fungal colony that received the high concentrations of the extracts was  
184 very small and zero at the highest concentrations. Total inhibition was achieved at a  
185 concentration of 25 µl/ml for acetone and ethyl acetate extract. On the other hand, in the

186 control boxes, the growth of *P. colocasiae* was significantly higher compared to the different  
187 concentrations of the extracts tested (Fig. 4).

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**Fig.4** In vitro inhibitory activity of *Thevetia peruviana* extracts on the radial growth of the *P. colocasiae* strain (OU123) after 8 days of incubation on PDA medium; A: ethyl acetate extract, B: acetone extract.

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The EAc showed inhibition on the growth of *P. colocasiae* strains. With the CE111 strain we had the lowest percentage of inhibition, 93.88% at the highest (C3) dose, (Fig. 5) compared to 100% for OU123 and LT122 (Fig. 5). Inhibition was proportional to concentration. EAc was found to be effective in the same way as Callomil at dose C3 with 100% growth inhibition on both strains compared to control ( $P>0.05$ ). The OU123 strain was more sensitive to the extract at the 12.5  $\mu\text{l/ml}$  dose with 100% reduction in mycelial growth.

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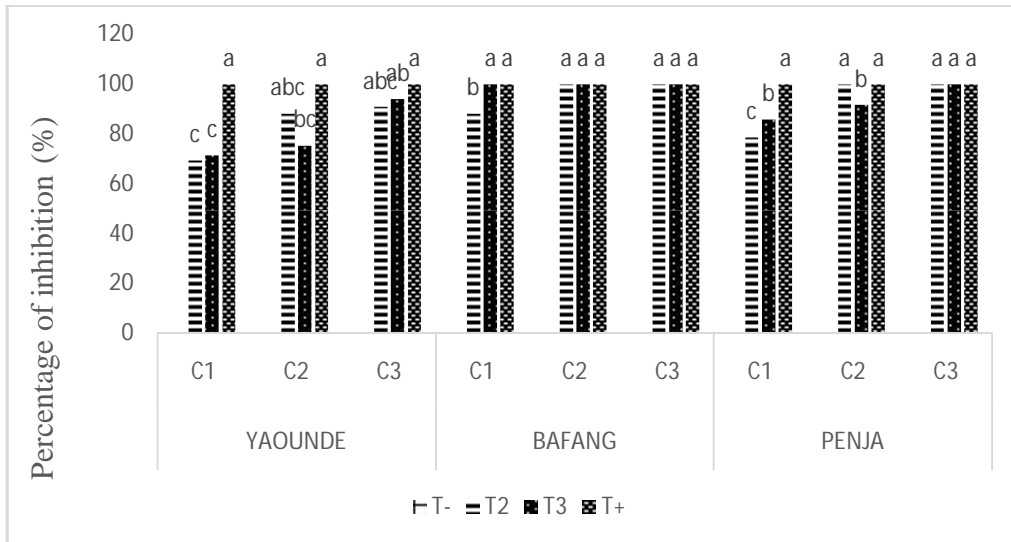
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#### **3.1.4 Effect of EAE on the growth of *P. colocasiae* strains**

The EAE showed inhibition on the growth of *P. colocasiae* strains. With the CE111 strain we had the lowest percentage of inhibition, 90.78% at the highest dose (C3), (Fig. 5) compared to 100% for OU123 and LT122 (Fig. 5). Inhibition was also proportional to concentration. EAE was found to be effective in the same way as Callomil at dose C3 with 100% growth inhibition on two strains compared to the control ( $P<0.05$ ). The OU123 strain was more sensitive to the extract at the smallest dose of 12.5  $\mu\text{l/ml}$  with more than 88% reduction in mycelial growth.



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**Fig.5. Effect of extracts on the growth of *P. colocasiae* strains**

For each strain, the assigned values of the same letter do not differ significantly according to the Newman-Keuls test.

T-= Negative control; T2= Ethyl acetate; T3= Acetone; T+=Fungicide  
T- (0 µl/ml); C1=12.5 µl/ml; C2=25 µl/ml; C3=50 µl/ml; T+ (12.5 µl/ml I)

The fungi tested showed different behaviors towards the extracts and depending on the doses. For strain CE111, EAE was found to be fungistatic at C2 and fungicidal at C3 while EAc was found to be fungistatic at both doses. However, with the OU123 strain, EAE and EAc were found to be fungicidal at both doses. For LT122, EAE and EAc were found to be fungistatic at C2 and fungicidal at C3 (Table 2).

**Table 2. Fungicidal or fungistatic activity of extracts and synthetic fungicide**

Species	Isolates	Extracts	Concentrations	Effect
			C2 (25 µl/ml)	Fungistatic

<i>P. colocasiae</i>	CE111	EAE	C3 (50 µl/ml)	Fungicidal
		EAc	C2 (25 µl/ml)	Fungistatic
			C3 (50 µl/ml)	Fungistatic
	OU123	Callomil	C1 (12,5 µl/ml)	Fungicidal
		EAE	C2 (25 µl/ml)	Fungicidal
			C3 (50 µl/ml)	Fungicidal
	LT122	EAc	C3(5025 µl/ml)	Fungicidal
			C2 (25 µl/ml)	Fungistatic
		EAE	C3(5025 µl/ml)	Fungicidal
			C2 (25 µl/ml)	Fungistatic
		Callomil	C3 (50 µl/ml)	Fungicidal
			C1 (12,5 µl/ml)	Fungicidal

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### 3.1.6 Correlation test between ethyl acetate extract concentrations and inhibition

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#### percentages of *Phytophthora colocasiae* growth

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The equations obtained with the EAE showed increasing linear relationships. Indeed, all

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regression lines obtained with the strains showed positive slopes. The correlation

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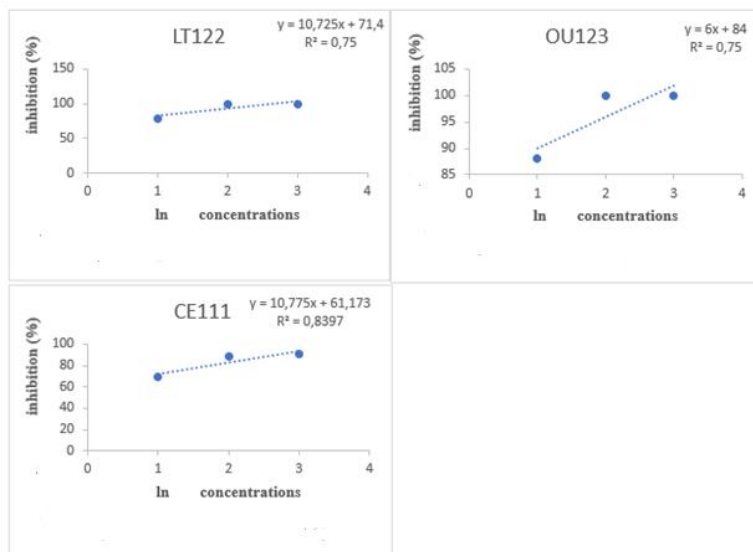
coefficients were all between 0.7 and 1. The strains OU123, CE111 and LT122 showed

240

respectively the following coefficients:  $r = 0.75$ ,  $r = 0.84$  and  $r = 0.75$  which is a perfect and

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positive correlation (Fig. 6).



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**Fig.6. Regression lines of growth of *Phytophthora colocasiae* strains after treatment with ethyl acetate extract.**

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### 3.1.6 Correlation test between acetone extract concentrations and *phytophthora*

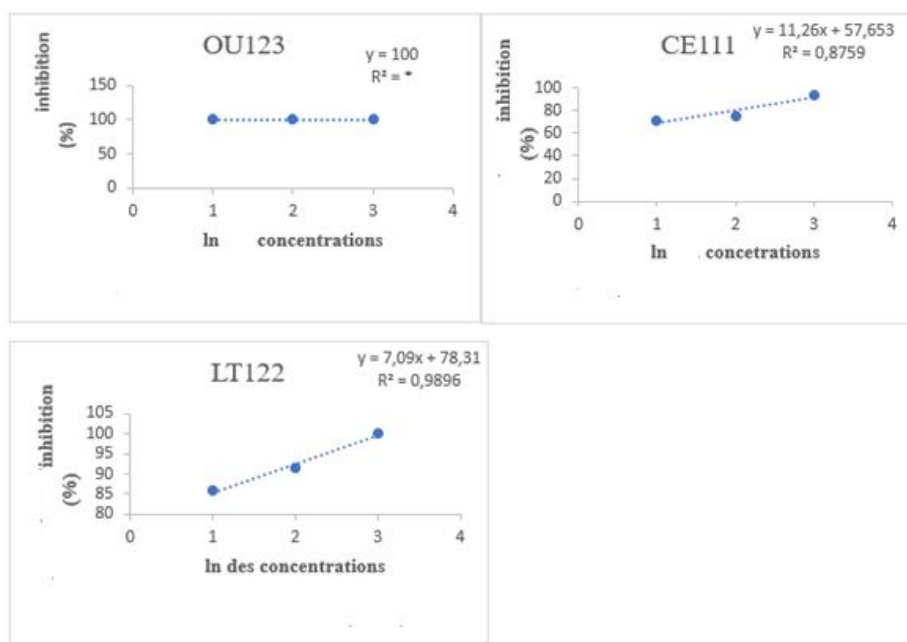
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#### *colocasiae* growth inhibition percentages

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249 *P. colocasiae* strains behaved differently with acetone extract. A strong positive correlation  
 250 was obtained with some strains, the correlation coefficient  $r$  was greater than 0.7; this is the  
 251 case with CE111:  $r = 0.88$ , LT122 :  $r = 0.9$ , a positive and perfect correlation (Fig. 7). With  
 252 the OU123 strain ( $y=100$ ) the equation obtained showed a constant linear relationship which  
 253 highlights an absence of correlation ( $r=0$ ). The lines obtained with isolates CE111 and LT122  
 254 showed positive slopes, respectively  $y=11.26x + 57.7$  and  $y=7.09x + 78.31$ .  
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256  
 257

258 **Fig.7. Regression lines of growth of *Phytophthora colocasiae* strains after treatment**  
 259 **with acetone extract.**

260 **3.1.7 Minimal Inhibitory Concentrations of the Different Extracts**

261 The MICs of the growth of *P. colocassiae* strains varied by extract. MICs<sub>90</sub> are higher with  
 262 EAE and range from 15 to 90  $\mu$ l/ml. The MICs obtained with EAc range from 11.25 to 47.83  
 263  $\mu$ l/ml. The smallest CMI<sub>90</sub> (11.25) was obtained with EAc on strain OU123. However, no  
 264 CMI<sub>50</sub> was obtained with the two extracts (Table 3).

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269 **Table 3: CMI<sub>50</sub> and CMI<sub>90</sub> ( $\mu$ l/ml) of the mycelial growth of *P. colocassiae* in the**  
 270 **presence of *T. peruviana* extracts.**

271

		Isolats		
		OU123	LT122	CE111
EAE	CMI <sub>90</sub>	15	22.5	90
	CMI <sub>50</sub>	*	*	*
EAc	CMI <sub>90</sub>	11.25 <sup>a</sup>	20	47.83
	CMI <sub>50</sub>	*	*	*

272 \* Represents values that are not set to be at zero statistically

273 <sup>a</sup> smaller concentration inhibiting 90% mycelial growth.

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### 3.2 Discussion

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This work was based on the evaluation of the antifungal potential of the ethyl acetate and acetone extract of *T. peruviana* seeds on *phytophthora colocassiae* strains responsible of taro late blight.

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The extraction of 1000 g of the seeds of *T. peruviana* produced different yields. These yields ranged from 28.5% with ethyl acetate to 23.3% with acetone. This variation can be attributed to the nature of the solvent. These results are different from those obtained by [41].who, after extraction, using the same amounts of *T. peruviana* seed paste with the same solvent volumes, obtained a yield of 33.16% with ethyl acetate and 9.43% with acetone. Indeed, [42] and Smallfield [43] report that “environmental conditions, harvest period and age of plant material can influence extraction yields”.

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EAE and EAc significantly inhibited the growth of *P. colocassiae* strains compared to the control. These extracts would contain substances that inhibit or reduce the growth of the fungus. Indeed, [44,45] reported that “plant extracts from a number of plants contain compounds such as tannins, flavonoids and alkaloids that have fungicidal properties. The different concentrations of extracts significantly influenced the radial growth of the fungus; high concentrations being more inhibitory. This reduction in growth was more pronounced with acetone extract than with ethyl acetate extract”. The effectiveness of these extracts on the growth of *P. colocassiae* could be explained by the presence in these extracts of bioactive molecules revealed by phytochemical screening, such as essential oils, saponifiable oils, coumarins, sterols, alkaloids, triterpenes, tannins, sugars, phenols, saponins and anthocyanins. All these molecules have antifungal properties, as demonstrated by [46].

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300

The results obtained show that some strains have been shown to be more resistant to certain extracts used compared to others, which would be due to the nature of the specificity they would present at the membrane level. In general, antifungals can be contact: acting at the level of the fungus membrane or systemic: acting inside the cell [47,48]. In both cases,

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304 specific membrane or intracellular receptors may be essential for the expression of the  
305 biological activity of the antifungal. Some chemical constituents have the ability to recognize  
306 sites of action in the pathogen, others do not. They would thus act through a concentration  
307 effect and once fixed on their receptors, would elicit responses such as inhibition of general  
308 metabolism (fungistatic effect) or alteration of the plasma membrane of the fungus and  
309 inhibition of respiration (fungicidal effect) [49,50,51]. Callomil Plus 72 WP was very effective  
310 against *P. colocassiae* at all doses with inhibition percentages of around 100% on strain  
311 growth. Its effectiveness would be due to the presence of copper oxide major active  
312 ingredient (60%), which is known for its action on cellular respiration. This result is similar to  
313 those of [52,53] who showed in vitro the efficacy of Callomil on strains of *P. colocassiae*. The  
314 low MIC values obtained with the acetone and ethyl acetate extract highlight the  
315 effectiveness and fungicidal properties of these different extracts on the growth of the fungus  
316 tested. These results are similar with those of [54,55] who showed that low MIC values of  
317 *Callistemon viminalis* and *T. peruviana* extracts respectively inhibit the development of *P.*  
318 *colocasiae*.

319

#### 320 **4. CONCLUSION**

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322 The study showed that *T. peruviana* extracts inhibited *in vitro* the radial growth of *P.*  
323 *colocasiae*. These extracts have been shown to be active on *P. colocassiae* and may  
324 therefore be an alternative for the fight against taro late blight. Despite the fact that these  
325 crude extracts exhibit activity that is comparable to the reference fungicide (Callomil Plus 72  
326 WP), they still include a variety of distinct chemicals that, after being purified, would be more  
327 effective than the chemical fungicide.

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329

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335

336 **COMPETING INTERESTS**

337

338 Authors have declared that no competing interests exist.

339

340 **AUTHORS' CONTRIBUTIONS**

341

342 This work was carried out in collaboration among all authors. Author CES designed the  
343 study, wrote the protocol and wrote the first draft of the manuscript. Author ZA designed the  
344 study and reviewed all drafts of the manuscript. Authors WKT, HB and DNM managed the  
345 experimental process and identified the fungal strains. Authors PZN, WKT and DTD  
346 performed the statistical analysis and reviewed all the drafts of the manuscript. Authors MT,  
347 AKN, SBM And AH managed the literature searches. All authors read and approved the final  
348 manuscript.

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