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Isolation and identification of phytopathogenic bacteria in vegetable crops in West Africa (Côte D'ivoire)

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Yield losses in food crops due to plant pathogenic bacteria are significant and increasing over the years. The increasing losses caused by bacterial plant pathology are explained by the emerging resistance of bacteria to the chemical agents used in plant protection. Moreover, these chemical agents harm the environment through residue accumulation leading to soil pollution and the perturbation of the soil's inner ecosystem. The most important bacteria causing plant pathology belong to the genera of *Pseudomonas, Ralstonia, Agrobacterium, Xanthomonas, Erwinia, Xylella, Pectobacterium,* and *Dickeya*. However, in Côte d'Ivoire, only the *Ralstonia* species have been identified. Therefore, this study aims to identify plant pathogenic bacteria present in market garden plants in Côte d'Ivoire. Three sites in the cities of Anyama, Abidjan, and Bingerville were selected for the sampling and the detection of *Pseudomonas syringea*, *Erwinia carotovora, Clavibacter michiganensis, Ralstonia solanacerum*, and *Xanthomonas campestris*. The samples consisted of healthy and affected plant leaves and soils. In brief, 70 bacterial strains were isolated and phenotypically identified in this study. Among them, we noticed that 20% were isolated from the leaves and 80% from the soil. Regarding the bacterial species, *C. michiganensis* (37.14%), *E. carotovora* (18.57%), *R. solanacerum* (15.71%), *X. campestris* (14.28%), and *P. syringae* (11.42%) were identified. The molecular identification has confirmed the identification of the 5 plant pathogenic bacteria within all the studied sites. To the researchers' knowledge, this study is the first to describe the identification of *P. syringea*, *E. carotovora, C. michiganensis,* and *X. campestris* isolated in plant crops in Côte d'Ivoire.

Key words: Phytobacteria, vegetable plants, PCR

INTRODUCTION

Vegetables contribute to more than 33% of world agricultural production and employ 800 million people (Kanda et al., 2014). Tomato *(Solanum lycopersicum)*, red pepper *(Capsicum spp.),* and, eggplant *(Solanum*

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melongena) are among the 40 most cultivated plant species in the world (FAO, 2008). In sub-Saharan Africa, market gardening represents an important sector of activity because of the nutritional material produced, but also because of the source of income that it provides (Djiéto-Lordon, 2007). In Côte d'Ivoire, the market gardening of vegetables represents an average of 27% of the gross national product (GDP). In addition, the demand for vegetables such as tomatoes, eggplants, and pepper is increasing due to population growth. Then, farmers are intensifying their production to cover the needs. However, this production faces many biotic constraints such as bacterial diseases causing high losses, especially in tropical climates (Lebeau, 2010). The plant pathogenic diseases led to the decrease in agricultural yield and the increase in the prices of the products on the market. Therefore, there is need to identify bacteria responsible for plant pathogenic diseases in Côte d'Ivoire to propose a solution to overcome the problem. According to their scientific and economic importance in the world, five bacterial species were highlighted (Mansfield et al., 2012). Firstly, *Ralsotonia solanacearum* is responsible for bacterial wilt in tomatoes, eggplants, and potatoes. The bacterium, initially, infects the roots and then, invades the vascular system of the plants (Nakahara et al., 2021). Next, *Pseudomonas syringae* an epiphytic bacterium that survives on weed roots, asymptomatic plants as well as seeds is known to cause symptoms of bacterial speckling (black dot surrounded by a yellow halo) (Canzoniere et al., 2021). *Xanthomonas campestris*, for its part, is responsible for black rot and causes vascular disease in some plants or leaf spots in others (Vicente and Holub, 2013). Following this, *Clavibacter michiganensis* spreads through plant vessels and causes symptoms such as wilting, stem canker, vascular discoloration, and cell habit (Yim et al., 2012). The contaminated seeds by *C. michiganensis* remain the main mean of bacterial propagation. Finally, *Erwinia carotovora* enters the plant through wounds. The plant walls are then degraded and the tissues are macerated by pectolytic enzymes causing soft rotting of stems and fruits (Boumaaza et al., 2018). Regarding the economical and nutritional implications of bacterial plant diseases, this study aimed to detect the presence of cited bacteria that can cause infections in tomato, eggplant, and chili pepper.

MATERIALS AND METHODS

Samples collection

The sampling sites chosen are fields where organic culture is practiced. Eggplant and pepper plant samples were collected from site 1, located in a forest of the city of Anyama and near a waterway. Tomato plant samples were collected from site 2, located in the city of Abidjan. Finally, eggplant and pepper plant samples were also collected on site 3 in the outskirt of Bingerville city (Figure 1). For each plant, samples of diseased and healthy leaves were collected and at the base of each plant, soil samples were also collected. All in all, for each site, 3 soil samples and 3 leaves samples were collected per plant. Soil samples (10 g) were taken at 15cm depth (Popoola et al., 2015). The leaves were cut off at the base of the petiole. Healthy and diseased leaves were collected at the rate of 10 leaves per plant (Gracein et al., 2012). Plant samples collected were carefully bagged, shipped to the laboratory, and processed immediately or stored at 4°C and processed within 48 h.

Pre-treatment

Small pieces of leaves were cut aseptically; the surface of the leaf was sterilized in 70% alcohol and washed in three series of sterile distilled water to remove traces of alcohol. The leaves were suspended in tubes containing 3 ml sterile distilled water for 15 to 20 min (Gracein et al., 2012; Nakahara et al., 2021). One gram of soil sample was weighed and added to a test tube containing 10 ml of physiological water (9% NaCl). Then, the tubes were shaken for 30 min, 50 rpm at room temperature to allow the separation of bacteria from the soil (Popoola et al., 2015 modified).

Isolation and characterization of phytobacteria

Isolation

A serial decimal dilution of the bacterial suspensions obtained after pretreatment was carried out in 9 ml of sterile distilled water when the water contained in the tubes became slightly cloudy. Then, 1 ml of the diluted bacterial cell suspension was poured onto sterilized Petri plates containing nutrient agar King B or YPGA. The inoculated plates were incubated at 28° for 24 to 48 h. Depending on the appearance, coloring, and morphology of the bacterial colony; an isolated colony was picked and plated again on nutrient agar using a Pasteur pipette (Table 1). This step was repeated three times to allow purification of the isolated strain (Chartier, 2005; Amkraz et al., 2010; Gracein et al., 2012; Huynh et al., 2019).

Bacterial detection by polymerase chain reaction

The DNA extraction of isolated bacteria was performed using Qiagen® DNA extraction kit following the manufacturer's recommendations. Fives colonies of bacteria were diluted in 100µl water (Qiagen, 2003).

The PCR analysis was performed to amplify conserved genes for each phytobacterium. The reaction mix contained 1XFirepolMasterMix (SolisBiodyne), 16 µl molecular water, 5 µl of DNA extract, and 0.5 µM specific primers for each bacterium (Table 2). For amplification, initial denaturation was carried out at 95 °C for 10 min, followed by 32 cycles of denaturation at 95°C (30 s), annealing at 54°C (30 s), and elongation at 72°C (1 min). Finally, the elongation of 5 mn was carried out. The amplification program was conducted on Applied Biosystems (9700 *PCR* System thermocycler). *PCR* products were visualized using 1.5% (wt/vol) agarose gel electrophoresis and a 100-bp DNA ladder (Promega).

Bacterial activity tests

Bacterial growth in vitro

The bacterial count makes it possible to know the exponential growth time of the bacteria used. This count is done at different times (0, 2, 4, 6, 8, and 24h). A stock solution of fresh phytobacteria is serially diluted up to 10^{-8} and then plated on King B or LPGA

Figure 1. Sites of the sampling of the study.

Table 1. Isolation of bacteria.

Bacterial diseases	Bacteria causing the infection	Culture center	Temperature and incubation time	Aspects of the colonies	References
Bacterial wilt	*R. solanacearum*	King B	26°C, 48h	Bluish fluorescent	Huynh et al., 2019
Bacterial canker	*C. michiganensis*	LPGA	26°C, 24 - 48h	Cream to yellowish	Amkraz et al., 2010
Bacterial scib	*X. campestri*	LPGA	26°C, 24 - 72h	Pale or yellow	Gracein et al., 2012
Bacterial speck	*P. syringae*	King B	26°C, 24 - 48h	Round white and smooth	Amkraz et al., 2010
Soft rot	*E. carotovora*	King B	26°C, 24h	Convex and bluish	Chartier, 2005

Table 2. Oligonucleotides for molecular identification of bacteria.

Species	Sequences	Lenght (pb)	Target	References
R. solanacearum	ATTACGAGAGCAATCGAAAGATT TCGCTTGACCCTATAACGAGTA	91	16S-23S	Kumar et al., 2017
	GTCGCCGTCAACTCACTTTCC GTCGCCGTCAGCAATGCGGAATCG	280	16S-23S	
C. michiganensis	TCATTGGTCAATTCTGTCTCCC TACTGAGATGTTTCACTTCCCC	271	16S-23S	Yim et al., 2012
X. campestri	AGTTGCAGCAGCTGTTCT ATAGCACGTATTGGCAGGG	304	*rpfH*	Kiran et al.,2019
E. carotovora	TTACCGGACGCCGAGCTGTGGCGT CAGGAAGATCTCGTTATCGCGAGT	435	ARN 16S	Photchanachai et al., 2006
P. syringeae	ACGAGCTGAAGGAAGACA CAGCCTGGTTAGTCTGGTTA	525	HrpZpst	Zaccardell et al., 2005

Table 3. Distribution of isolated strains according to the sites.

Variable	Isolated strains				Positive PCR strains			
	Site 1	Site 2	Site 3	Total	Site 1	Site 2	Site3	Total
Ralstonia sp	4	2	4	10	1	1	2	4
Clavibacter sp	7	10	10	27	1	2	4	7
Pseudomonas sp	6	3	3	12	4	0	0	4
Erwinia sp	4	4	4	12	1	1	0	2
Xanthomonas sp	2	6	1	9	0	2	2	4

agar. The tests were repeated in triplicate. The dishes were incubated at 28°C for 24 h.

Bacterial pathogenicity test in vivo

One-month-old tomato plants were inoculated with a fresh culture of the following bacteria: *R. solanacerum, E. carotovora, X. campestris*, *C. michiganensis,* and *P. syringea*. The bacterial strains used are those that have been confirmed by PCR. Inoculation was done by adding 500µl of fresh bacterial culture to the leaf surface of each plant. The inoculated plants are observed until the appearance of the first symptoms (Table 1, Figure 9). The inoculated plants were re-isolated on agar and compared to the base strains as follows. Leaves from each plant were cut (3 leaves per plant) and placed in tubes containing 2ml of sterile distilled water for 1 hour. Dilutions in physiological water were then made and 10µl spots were deposited on the agar incubated at 28°C for 24 h. All the tests were repeated three times for each bacterium (Kumar et al., 2017 modified).

RESULTS

Collection of samples and bacterial identification

A total of 70 bacteria were isolated. The strains isolated from site 1 are 23 in number, 25 from site 2, and 22 from site 3. Among them, 20% were isolated from the leaves and 80% from the soil. According to the bacterial species, there were 38.57% of *Clavibacter sp*; 17.14% *Erwinia sp*, 14.728% *Ralstonia sp*; 12.85% *Xanthomonas sp,* and 14.42% *Pseudomonas sp.* 36% of the bacterial strains were isolated from site 2, while for sites 1 and 3 we had 33 and 31% of isolated bacteria respectively (Table 3), (Figures 2 and 3). The five investigated bacteria were recovered in the 3 sites.

Quantification of genomic DNA

Genomic DNA was extracted from 70 strains. The amount of nucleic acid was quantified using Nanodrop oneC instruments. Indeed, the highest amounts of DNA were identified in *X. campestris* followed by strains 26 and 15 of *C. michiganensis* then strains 10 and 8 of *Ralstonia*. The highest concentrations are 218, 246, 172, 165.5 and 165.1 µg/ml. The purity of the samples was between 1.4 and 2.1. The table shows the concentration and purity of the PCR-positive strains (Table 4).

Molecular characterization

Genomic amplification by PCR made it possible to obtain 271 bp fragments for *C. michiganensis* representing the 16S-23S gene. Products of 280 bp for *R. solanacearum* and 435 bp for *E. carotovora* were both obtained targeting the 16S-23S gene. *P. syringae* targets the *HrpZpst* gene with a fragment size of 525 bp, while the *X. campestris* (*hrp*) gene has a fragment size of 304 bp. For molecular characterization by PCR, we identified 28.57% positive phytobacteria. According to the bacterial species, there is 35% of *C. michiganensis*, 5% *E. carotovora*, 20% *R. solanacearum*, *X. campestris* and *P. syringae* (Figures 4 to 9).

Bacterial growth curve

The number of colony-forming units (CFU) was evaluated at different dilutions of isolates at different times. The isolates used are the PCR positive isolates. The number of colony-forming units (CFU) was assessed at different dilutions of isolates at different time points. The isolates used are the positive PCR isolates. The highest bacterial growth was observed in *E. carotovora*; followed by one of the *Ralstonia* strains. Two of the strains of *Clavibacter* had similar bacterial growth. They could be the same strain or a neighboring strain. *Clavibacter* strain 18 has much weaker growth than the previous two strains. All like the two strains of *Xanthomonas* and *Erwinia* which could come from distant strains (Figure 10).

Pathogenicity test

Pathogenicity tests showed that the selected strains were able to induce infection in tomato plants. The first symptoms appeared 14 days after inoculation for *R. solanacearum, E. carotovora,* and *X. campestris*. Early symptoms of *R* solanacearum were characterized by yellowing and wilting of leaves. Erwinia induces yellowing

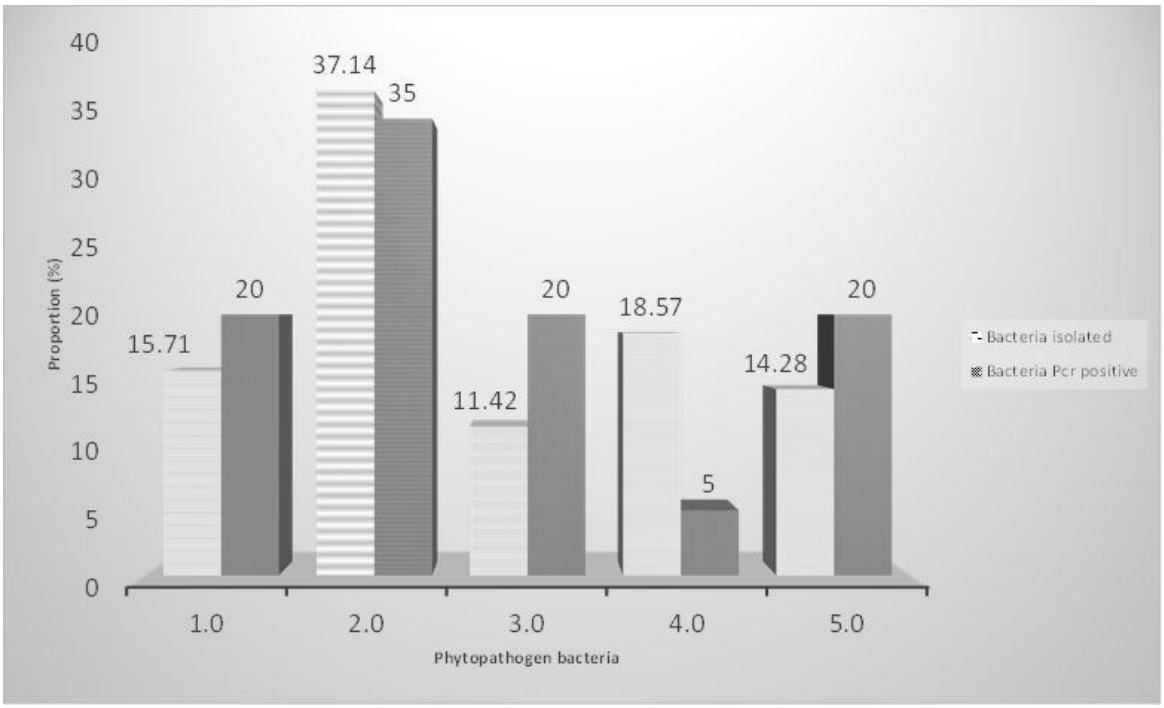

Figure 2. Distribution of bacteria strains.

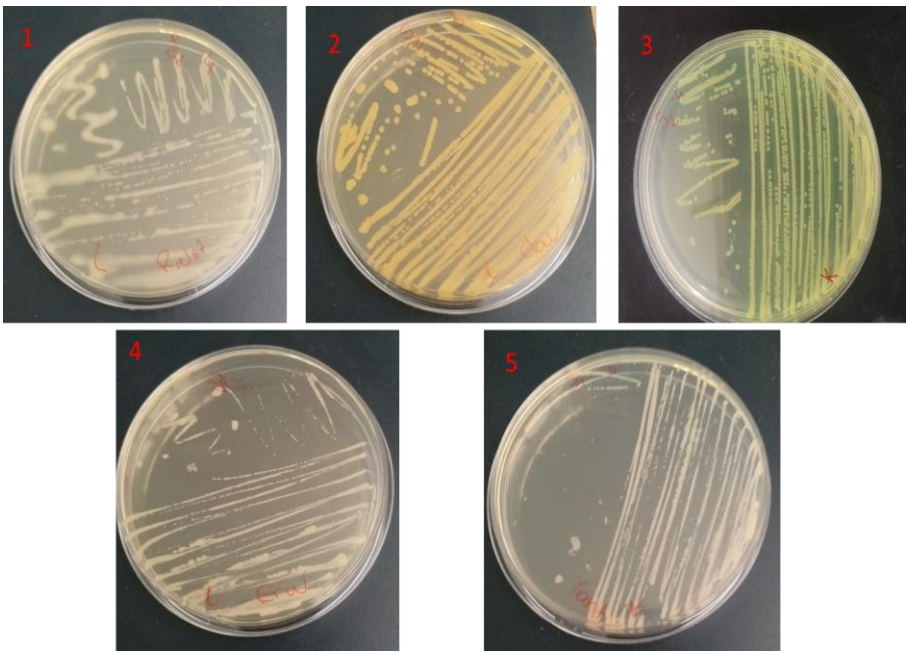

Figure 3. Bacteriological identication of phytopathogen bacteria. 1. *Ralstonia Solanacearum*; 2. *Clavibacter michiganensis*; 3. *Pseudomonas syringeae*; 4. *Erwinia carotovora*; 5. *Xanthomonas campestris*

of the leaves while small yellow to brown spots have been observed on the leaves of plants infected with *X. campestris. C. michiganensis* and *Pseudomonas* appear one month after inoculation in the plant. Symptoms of *C. michiganensis* were manifested by the appearance of a burning color on some leaves as well as their wilting. One

Table 4. ADN concentration.

Isolate	Concentration (ng/µl)	Purity (260/280)	Isolate	Concentration (ng/µl)	Purity (260/280)
Isolate 1	56.6	1.97	Isolate10	165.1	1.94
Isolate 2	163.7	1.79	Isolate11	135.1	2.11
Isolate 3	109.7	1.92	Isolate12	165.5	2
Isolate 4	151.4	1.24	Isolate13	117.7	1.96
Isolate 5	246.7	2.02	Isolate14	33	1.82
Isolate 6	21.4	2	Isolate15	80.2	1.34
Isolate 7	36.3	1.99	Isolate16	218	1.62
Isolate 8	123.7	2.1	Isolate17	16.3	2.09
Isolate 9	68.1	1.96	Isolate18	120.8	2.05

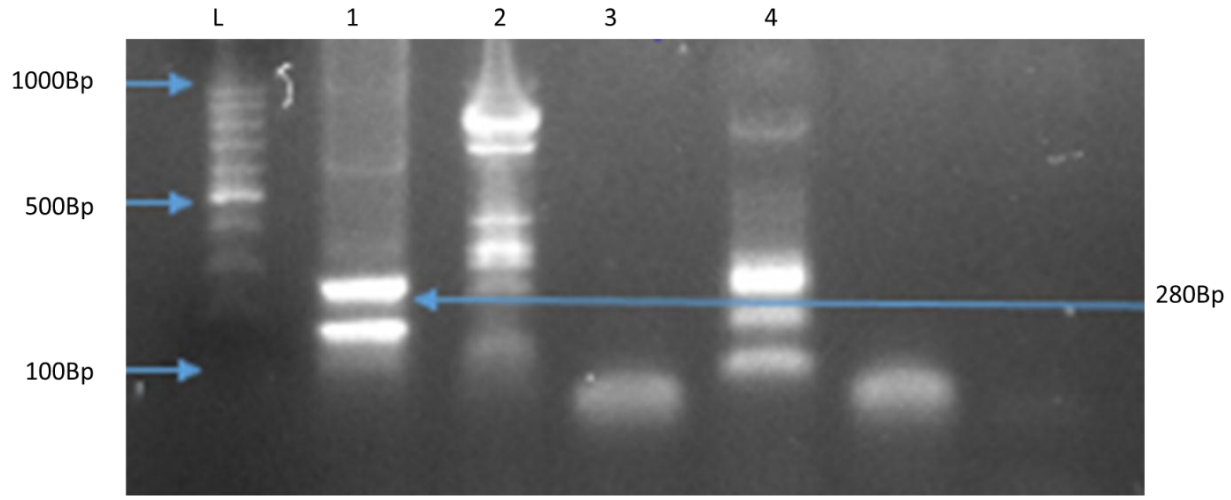

Figure 4. Molecular identification of *Ralstonia solanacerum.* L: Ladder, 1,2,3,4: Sample of tomato.

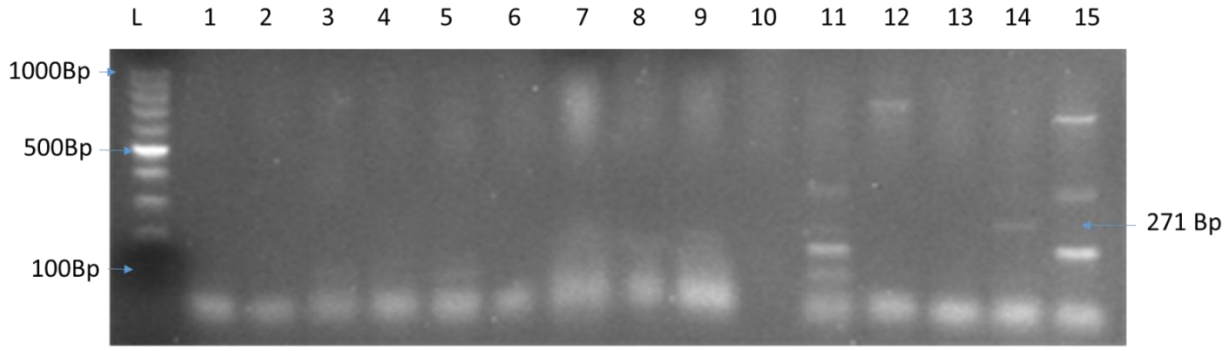

Figure 5. Molecular identification of *Clavibacter michiganensis*. L: Ladder, 1,2,3,4,5,6,7: Sample of eggplant; 8,9, 10,11, 12, 13, 14, 15: Sample of tomato.

appearance of leaves infected by *Pseudomonas* was characterized by the appearance of yellowish to brown spots on the leaves. The bacterial count of each strain was assessed weekly after inoculation. The bacteria concentrations obtained were listed in Figures 10 and 11. The figure showed that from 0 to 4 days, the bacterial

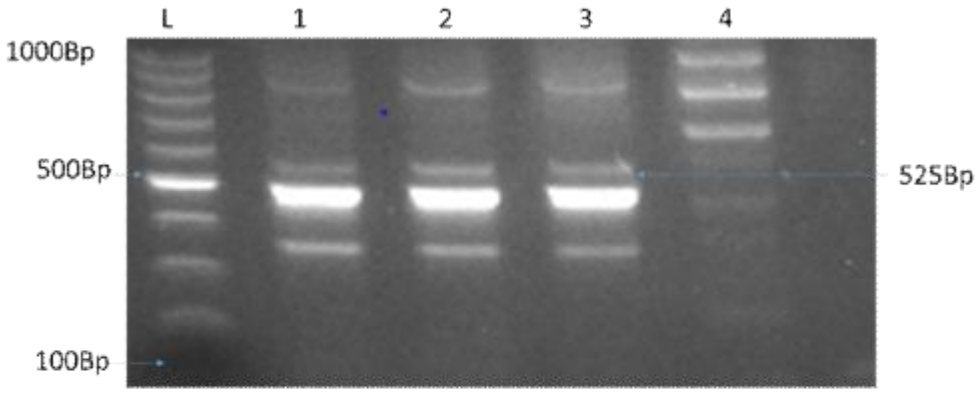

Figure 6. Molecular identification of *Pseudomonas syringea*. L: Ladder; 1.2.3.4: Sample of tomato.

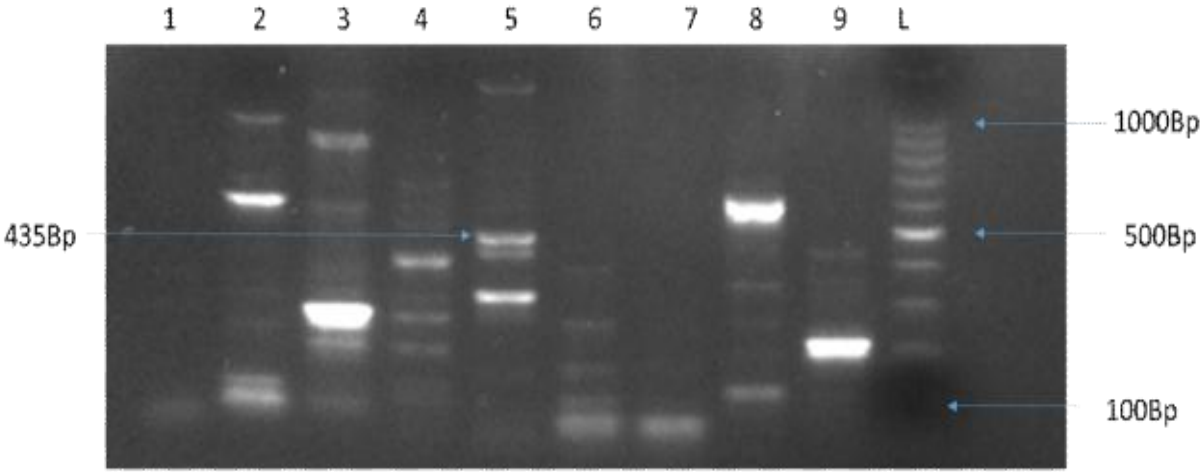

Figure 7. Molecular identification of *Erwinia carotovora*. L: Ladder (Promega 100pb); 1.2.3.4: Bacteria sample of tomato from site 1.2.3.

concentration of all bacteria was almost zero. Between 14 and 10 days, the concentration of bacteria increased slightly. From day 10, bacterial growth of *Clavibacter; Xanthomonas,* and *Erwinia* on leaves increased exponentially. As for *Ralstonia* and *Pseudomonas*, growth remained low throughout the experiment.

DISCUSSION

This study confirms the presence of phytopathogenic bacteria in leaf and soil samples from the sites. A collection of 70 bacterial strains was isolated. The bacteria isolated from site 1 are 33%. The results corroborate those of Kumar et al, 2017 in their study which shows that the rhizospheric bacteria were a mixture of antagonists and neutral and mutualistic pathogens. Site 1's cultivation system was organic and chemical-free. The constituent elements of the rhizosphere have therefore been preserved. And there is a close connection between subsoil microbes and aboveground components of the plant ecosystem (Kumar et al., 2017). Previously used as a household waste dump 36% of the phytobacteria were isolated from site 2. Indeed, this waste was the growth biotope of several microorganisms of bacterial, viral, and parasitic origin. And Cissé's study demonstrated that a biotope where contamination is high presents a high risk for cultivated plants (Cissé, 1997). *C. michiganensis* responsible for bacterial canker, was the most isolated phytobacteria on the three sites with 38.57% confirmation. The results coincide with those of Ftayeh and Nandi who state that *Clavibacter* is a devastating emerging disease for crops. It is a serious disease in the world and new epidemics have recently been reported in several countries (Ftayeh et al., 2011) hence its strong presence in these soils has

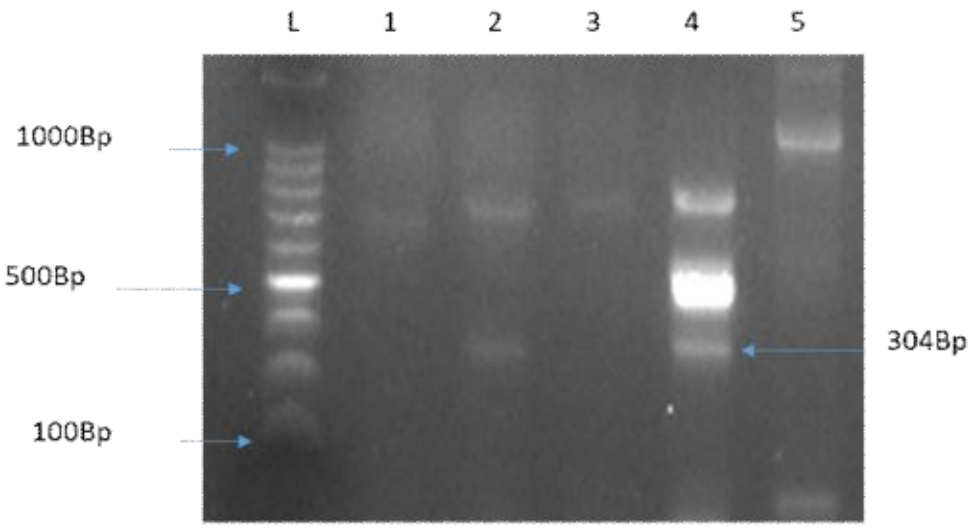

Figure 8. Molecular identification of *Xanthomonas campestris*. L: Ladder; 1.2: Sample of eggplant; 3.4: Sample of tomato.

Figure 9. Tomato plants inoculated with phytobacteria. A1-A2: Plant without bacteria (negative culture); B1: Tomato plants inoculated with *Xanthomonas campestris* after 3 days; B2: Tomato plants inoculated with *Ralstonia solanacerum* after 3 days; C1: Tomato plants inoculated with Xanthomonas campestris after 15 days; C2: Tomato plants inoculated with Ralstonia solanacerum after 15 days; E: *Erwinia carotovora;* R: *Ralstonia solanacerum;* C: *Clavibacter michiganensis;* X: *Xanthomonas campestris;* Cfu: Colony forming unity.

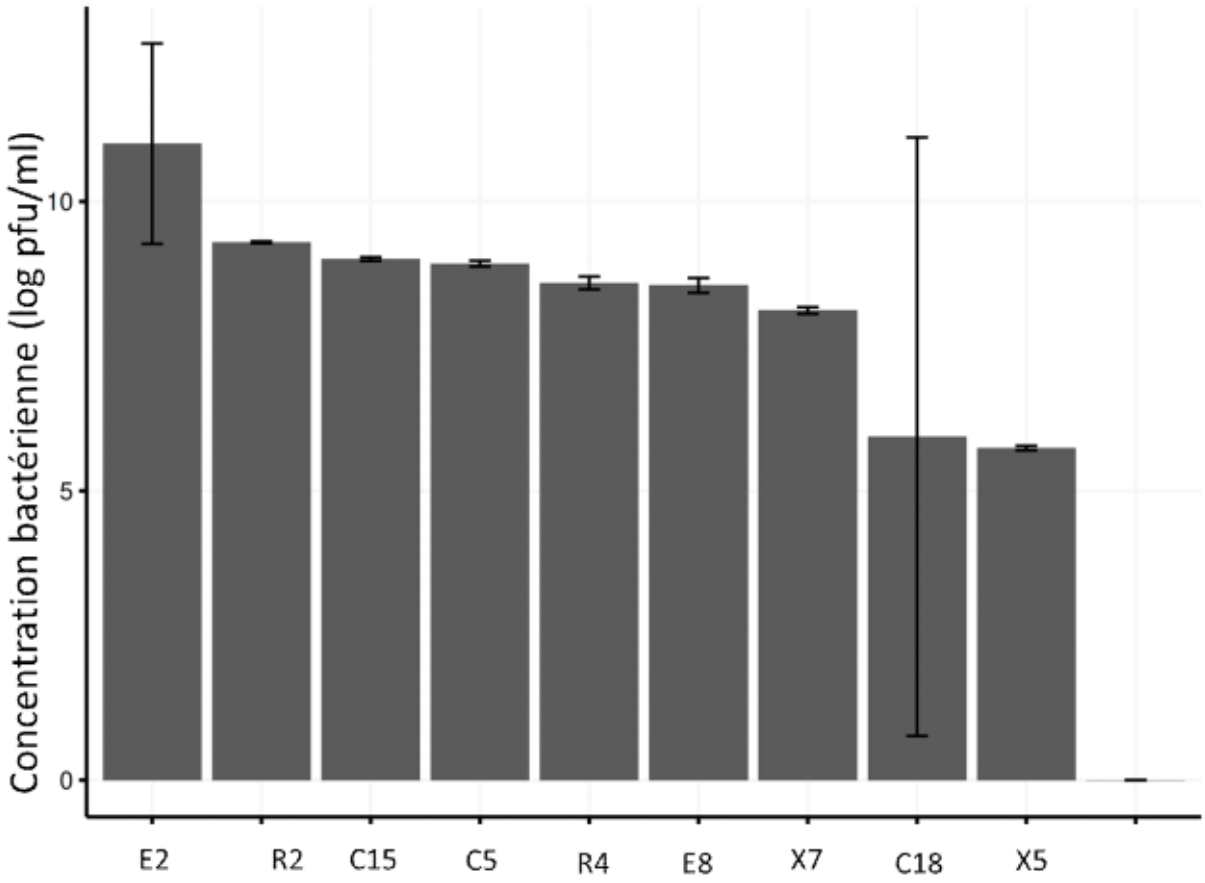

Figure 10. Bacterial concentration of plant pathogens.

caused significant crop losses. It is also considered one of the most devastating plant diseases of crops (Nandi et al., 2018). It is followed by *E carotovora* preferentially isolated on site 2 at 17.14%. The results are contrary to the results of a previous study by Benada which showed that *Erwinia sp* is a bacterium that preferentially infects fruits and potatoes (Benada et al., 2019). Damaged fruits and potatoes deposited on the plot of site 2 formerly used as a dump could be the cause of the appearance of this bacterium. And the insect vectors par excellence have allowed the spread of this bacterium in plants (MHMED, 2019).

One of the most important bacteria in peppers and crucifers is *Xanthomonas campestris*. It is seed-borne and can survive in weeds and plant debris for months. These matrices are a biotope for surviving *Xanthomonas* (Laala et al., 2021). *Xanthomonas campestris* was isolated with a percentage of 12.85% in tomatoes. A previous study isolated xanthomas in rice and it was *Xanthomonas oryzae pv. Oryzicole* in Ivory Coast (Diallo et al., 2010). However, strains of *Xanthomonas campestris* have just been isolated for the first time in Côte d'Ivoire. At the level of molecular characterization, 50% of *Pseudomonas* isolates were PCR positive. Although the hrp gene is unknown in virulence and pathogenicity, this region is essential for the production of symptoms in the host (Zaccardelli et al., 2005). It is considered a stable gene using 16S-DNA. In Côte d'Ivoire in seven agroforestry regions, the study by Guessan (Guessan et al., 2012) described a high prevalence of 87% for *Ralstonia* phylotype I. In this study, 36% of *Ralstonia solanacerum* were confirmed by PCR.

The isolates of *C. michiganensis* (21.42%) are Pcr positive. ITS region sought for molecular confirmation of Clavibacter is a relatively variable area. Yim et al. (2012) showed a difference of more than 50% between the ITS regions of tomato and pepper crops. And a diversity of genetic variation exists between Clavibacter populations in several countries (De Leon et al., 2009). Our pathogenicity test results showed that *R. solanacerum*; *E. carotovora* and *X. campestris* induce symptoms on tomato plants. The first symptoms appeared after 14 days. And according to the literature the first symptoms appear after an infection time of 14 days (Iiyama et al., 2021). These bacteria are preferentially present in the rhizosphere. They, therefore, infect the plant through the roots and invade the vascular system. The bacteria grow and produce extracellular polysaccharides which cause

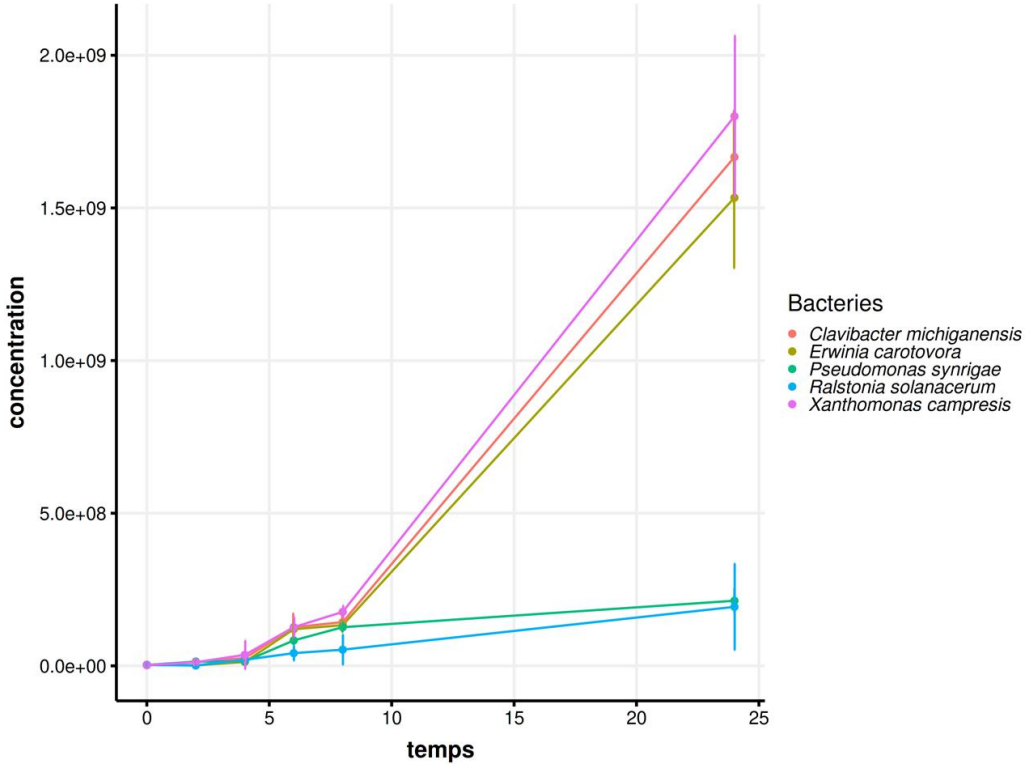

Figure 11. Concentration of phytobacteria.

obstruction of the first symptoms on the plant and the death of the latter (Nakahara et al., 2021). The *Ralstonia* strains used induced infection in tomato plants. It could therefore be race 3. Indeed, according to a study by (Kumar et al., 2017), the strains capable of infecting Solanaceae are part of race 3.

After one month of infection, all plants infected with *C. michiganensis* and *P. syringea* showed symptoms. These results are consistent with those of Yim et al. (2012) which show 25 days after inoculation, the plants show symptoms and 80% of the plants die. This study confirmed the presence of phytobacteria in plants. 70 strains of phytopathogenic bacteria were isolated in three sites (rural, semi-rural, and urban sites). And 20 strains have been confirmed by molecular diagnosis. *E. carotovora* strains *X. campestris C. michiganensis* and *P. syringea* were isolated for the first time in this study. *C. michiganensis* is the most predominant strain in the tomato and eggplant plant. *E. carotovora* and *R. solanacerum* are abundant at all sites. These strains induced in vivo infection tests with major leaf symptoms. Isolated bacteria are responsible for various infections resulting in huge production losses. To compensate for these losses, the use of biopesticides such as agriphages is a godsend, especially since their isolation is inexpensive. The isolation of agriphages would then be a considerable alternative to reduce production losses due to phytopathogens.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests

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