

First Isolation and Identification of Agriphages in Vegetable Crops in West Africa (Côte d'Ivoire): Potential Uses of Biocontrol in Plants

Adioumani Akoua Emmanuella

Institut de Cote d'Ivoire, Plateforme de Biologie Moléculaire, Côte d'Ivoire. Université Felix Houphouët Boigny, Laboratoire de Biologie Fonctionnelle et Moléculaire, Abidjan, Côte d'Ivoire

Kakou-Ngazon Solange

Institut Pasteur de Cote d'Ivoire, Plateforme de Biologie Moléculaire, Abidjan, Côte d'Ivoire

Thibaud Martin,

Cirad, Université Felix Houphouët Boigny CEA-CCBAD, Abidjan, Côte d'Ivoire

Trebissou Jonhson Noel David

Université Felix Houphouët Boigny, Laboratoire de Biologie Fonctionnelle et Moléculaire, Abidjan, Côte d'Ivoire

Addablah Audrey

Institut Pasteur de Cote d'Ivoire, Plateforme de Biologie Moléculaire, Abidjan, Côte d'Ivoire Université Felix Houphouët Boigny, Laboratoire de Biologie Fonctionnelle et Moléculaire, Abidjan, Côte d'Ivoire

Koudou Aristide

Sina K. Mireille

Aoussi S.

Dosso Mireille

Institut Pasteur de Cote d'Ivoire, Plateforme de Biologie Moléculaire, Abidjan Côte d'Ivoire

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Abstract

Agriphages or bacterial viruses are ubiquitous in the environment. The discovery of virulent phages against phyto bacteria improves crop growth and proposes biopesticide uses for plant diseases. In Africa, many phyto bacteria like *Ralstonia*, *Clavibacter*, and *Xanthomonas* were reported in several regions. The study aims to evaluate the presence of agriphages for the biocontrol of phyto bacteria in Côte d'Ivoire. Leaves and soil samples were collected from healthy and diseased plants, and in three sites located in Anyama, Abidjan, and Bingerville. The pretreatments occur with sterile and physiological water for leaves and soil samples respectively. The isolation of agriphages was done on specific media with *Xanthomonas campestris* as bacterial host. Lytic activity was tested on agar media for five bacteria strains. After DNA extraction using the Qiagen method kit, molecular confirmation of agriphages was done by Random Amplified Polymorphic DNA-PCR. From this study, five (5) agriphages were isolated in soil and leaves in site 2. These agriphages have all been isolated from *Xanthomonas campestris* and have a broad spectrum of lytic activity. Molecular characterization by RAPD-PCR showed that three of these agriphages are DNA phages. The dendrogram showed that phages Φ XanS1, Φ XanS2 have 93% similarities. While Φ XanS1 and Φ XanS2 are 62% similar to Φ XanF1. This study is the first reported agriphages in West Africa and their potential uses against phyto bacteria for biocontrol infection in crops.

Keywords: Agriphages, Vegetable crops, RAPD-PCR, West Africa, phyto bacteria

1. Introduction

Phyto bacteria are responsible for several plant diseases worldwide. These diseases cause losses of economic importance. These losses in current agricultural production are estimated at 1.3 billion tonnes per year. The impact is implicated in the food chain, from production to consumption (Zaczek-Moczydłowska et al., 2020).

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 it provides (Djeto-Lordon, 2007). In Côte d'Ivoire, the market gardening of vegetables represents on average 27% of the gross national product (GDP). Also, the demand for vegetables like tomatoes, eggplants, and bell pepper is increasing due to population growth. The increase in the production of these foodstuffs causes the growth of phyto bacteria in these crops.

The use of chemical phytosanitary products has made it possible to limit the extent of the damage caused by phyto bacteria. However,

phytosanitary products of chemical origin have for the most part proven to be ineffective over time in eliminating bacteria. They not only have adverse effects on human and environmental health (FAO, 2015); but represents a human health hazard for consumers and promotes multi-resistance (Jovana et al., 2014). Many phytosanitary products such as copper also present several environmental risks such as phytotoxicity, negative effects on pollinating insects, bioaccumulation in soil and surface waters as well as the reduction of microbial diversity (Vloek et al., 2018; Wang et al., 2019; Alengebawy et al., 2021).

Faced with these drawbacks, the use of environmentally friendly phytosanitary products becomes essential. And the use of phytosanitary products of biological origin such as agricultural production, the use of resistant plant varieties, or biopesticides not only makes it possible to defeat pests while respecting nature. Microbial biopesticides are ecologically much more compatible than chemical products and have increased specificity towards the pathogens against which they are directed. Among the biopesticides, we have agriphages.

Agriphages are ubiquitous in the environment, so humans, animals, and plants are exposed to them daily without any danger. Agriphages persist in an environment when their bacterial hosts are accessible to them. Frequently, the phage has a narrow host range, restricted to a well-defined bacterial species. The discovery of virulent phages against certain phytopathogenic bacteria would help improve crop growth (Buttimer et al., 2017).

The elaboration of the phage cocktail allows increasing in their efficiency. The isolation of phages is less expensive and easier for tropical countries. However, in West Africa, No agriphage has been reported and their use is unknown. This study aims to evaluate the presence of agriphages to control some phyto bacteria in Côte d'Ivoire.

2. Material and Methods

2.1 Sites of study

The sampling sites are all biological fields. The site 1 was located in a forest area in the city of Anyama. Soils, eggplant leaves, and chili samples were collected for this study site 2. The site 2 was located in an urban area (Abidjan City), for the sampling of soil and tomato leaves in this site. The site 3 was located in a peri-urban area of the city of Bingerville. Soil, tomato, and eggplant leaf samples were collected at site 3 (*Figures 1 & 2*).



Figure 1: Sampling sites of the study.
A: Field for sampling in Anyama (Site 1).
B: Field for sampling in Abidjan (Site 2)
C: Field for sampling in Bingerville (Site 3)

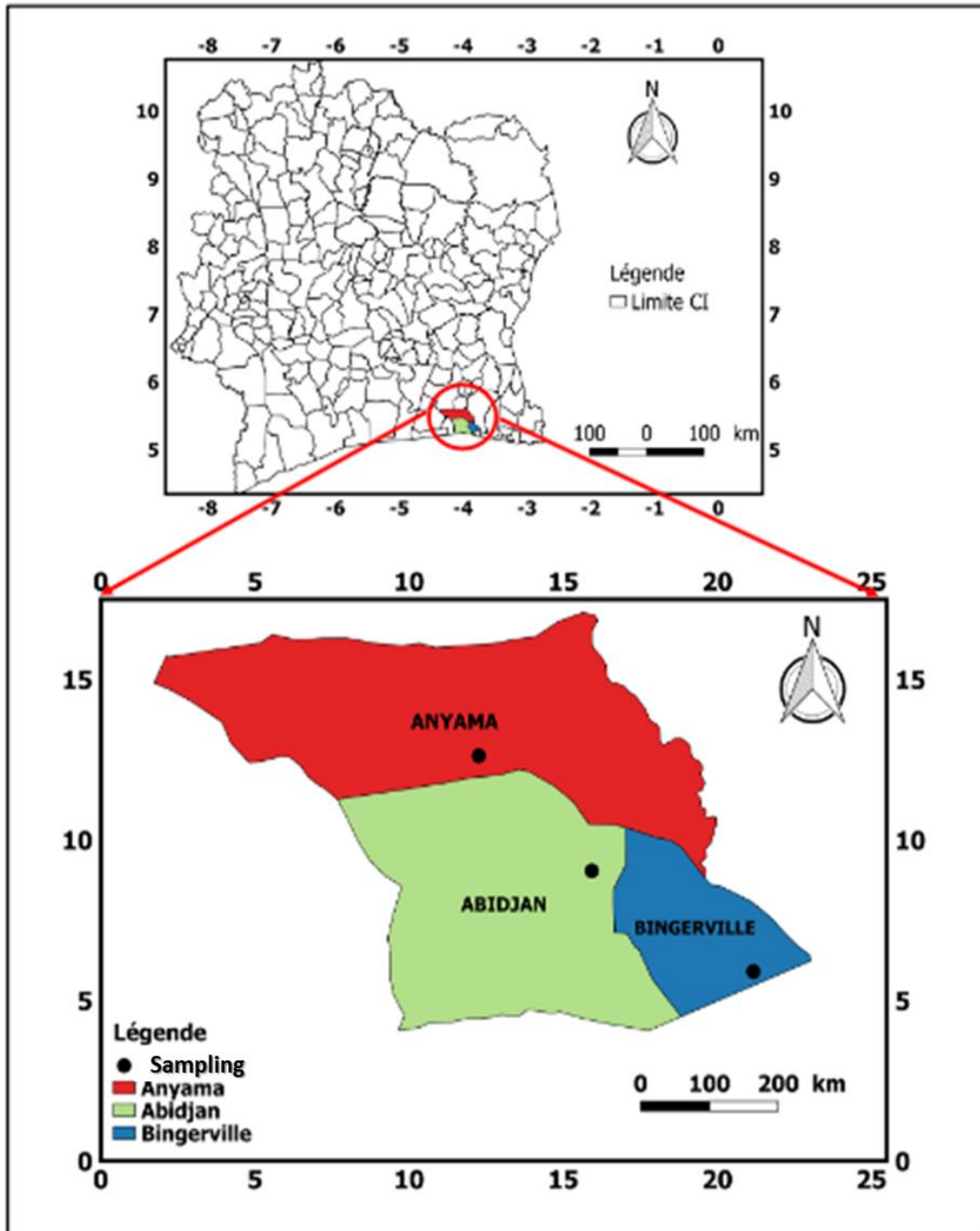


Figure 2. Geographical location of sampling sites (Source: CNTIG, 2022 QGIS 3.10)

2.2 Collection of samples

Soils and leaves (6) samples were collected from three biological fields around Abidjan city. Eggplants, tomatoes, and peppers leaves were collected at this site. Soil samples were taken from the base of each plant. Soil samples (10 g) were taken at the depth of 15cm (Popoola et al., 2015). The leaves were cut off at the base of the petiole (Gracelin et al., 2012).

2.3 Bacterial strains and culture conditions

The bacteria strains used in this study were obtained from the Pasteur Institute Côte d'Ivoire (PICI). The strains were characterized in a previous study (Adioumani et al., 2022). Phages were isolated from bacteria PCR positive in this study. The bacteria strains used for phage isolation were listed in Table 1. Phages were tested with the bacterial strains *Ralstonia solanaceum*, *Clavibacter michiganensis*, *Pseudomonas syringea*, *Erwinia carotovora*, and *Xanthomonas campestris*. All the bacteria were grown at 28 °C in liquid Luria Bertani medium (LB) and solid media (King B & LPGA). One hundred microliters of fresh culture were added to 5 ml of LB Broth and incubated at 28° C for 18-24 hours.

2.4 Pre-treatment and phage isolation

The soil samples were treated as follows: 1g of soil was added to 9 ml of physiological water. The solution was mixed for 30 minutes at room temperature to allow the separation of bacteria from the soil. Then the tubes were stored for 30 minutes at room temperature (Popoola et al., 2015). The leaf samples were sterilized with 70% alcohol and washed three times. The small pieces of leaves were placed in tubes containing 3 ml of sterile distilled water for 15 to 20 minutes (Gracelin et al., 2012). When the water became slightly turbid due to the oozing of bacterial cells from the cut ends of the diseased tissue, 10 ml of supernatant were mixed with 100 µl of host bacteria and incubated at 28° C for enrichment. The tubes were incubated at room temperature for two weeks. After enrichment, the supernatant was centrifuged (12500 rpm, 10 minutes at 4°C). The supernatant was filtered with a 0.45-µm filter. The filtrate was added with a double agar assay. One hundred microliters of exponential bacterial culture were added to 5 mL of soft agar (TSB, 0.7% agar) and 10 µl of the filtrate were added in solid TSB/agar and incubated at 28°C for 24 hours (Twest & Kropinski, 2009). Lytic plaques that displayed unique morphology were isolated three times using the streaking method to assure the isolation of unique phage clones. Phage propagation was performed by suspending a lytic plaque in 1 ml SM buffer (10 mM Tris-HCl, pH 7.5; 100 mM NaCl; 10 mM MgSO₄), and added to 10 ml of bacteria solution and incubated overnight at 28°C (Flores et al., 2020).

2.5 Lytic activity (Virulence) test

The virulence test was done by the double layer method. The bottom LB agar layer at 1.5 % was first spread on the Petri dishes. Once dry, an upper LB agar layer at 0.7 % containing 100 µl of fresh bacteria were inoculated. Five microliters of phage filtrate were spotted on LB agar (Twist & Kropinski, 2009).

2.6 Characterization of agriphages

2.6.1 DNA Phage extraction

DNA extraction was performed using Qiagen® DNA extraction kit following the manufacturer's recommendations. To lyse the capsid of the phage, 1 µl of DNase 1 (1U/µl) was added to a tube previously containing 450 µl of phage solution. The mixture was incubated at 37°C for 90 minutes. After this incubation, 20 µl of EDTA (20 nM) and 1.25 µl of Proteinase K (20 mg/ml) are added and incubated at 56° C for 90 minutes. After lysis, 200 µl of the solution were added to a tube containing 200 µl of buffer AL and then vortexed. The tubes were then incubated at 70°C for 10 minutes. After incubation, 200 µl of ethanol (99.9%) were added and mixed by vortexing and centrifuged (10 000 rpm, 1 minute). The column is placed in a new collecting tube and 500 µl of buffer AW1 were added and centrifuged (10 000 rpm, 1 minute). After washing, the column was placed in a new sterile 1.5 ml microtube and 50 µl of elution buffer was added and incubated at room temperature for 1 minute following centrifugation (10000 rpm, 1 minute) (Qiagen, 2003 ; Jakociūnė & Moodley, 2018)

2.6.2 Nucleic acid Absorbance assay

The Nanodrop was used to evaluate the DNA concentration of isolated agriphages. 1 µl of DNA extract was placed on the base of the device, and the lecture of absorbance at 260 nm was done. The assay was carried out according to the manufacturer's method (OZYME).

2.6.3 Random Amplification of polymorphic DNA

The RAPD- PCR was performed by using oligonucleotides for random amplification of phages (Gutierrez et al., 2011; Addablah et al., 2021). The PCR was performed with a modification of Kumari's method. (Kumari *et al.*, 2009). PCR mixture (50 µl) consisted of 2.0 µl of phage DNA, 10.0 µl 5X Reaction Buffer, 3.0 µl MgCl₂ (25 mM), 1.0 µl dNTPs (25 mM) (Promega, Wisconsin, USA), 1.0 µl primer (10 µM), 0.5 µl GoTaq G2 Flexi DNA polymerase (5 U/µl) (Promega, Wisconsin, USA. Primers P1 (5'-CCGCAGCCAA-3') and P2 (5'- AACGGGCAGA-3') were used. The PCR was done with the following parameters: initial denaturation (95 °C for 10 min), followed by 32 cycles of denaturation (95°C for 30 secondes),

hybridization (54°C for 30 seconds), and elongation (72°C for 1 minute). Finally, the elongation was done at 72°C for 10 minutes. The 9700 PCR System thermocycler was used for all PCR reactions. PCR products were visualized using agarose 2% (wt/vol) gel electrophoresis. Amplicon sizes were estimated by comparison to a 100-bp DNA ladder (Promega).

After amplification, 15µL of amplicons were running in an agarose gel (1.5%) for migration in (1X) Tris Borate EDTA buffer for 45 minutes, 150 Volts. At the end of the migration, the bands are revealed under UV light using the Gel Doc EZ Imager machine (Bio-Rad, 2018).

3. Results

3.1 Agriphage's collection

Five agriphages were isolated from soil and leaves samples, and collected in three biological sites (*Figure 3*). These agriphages were from site 2 (Abidjan), while site 1 and site 3 have not detected phages. The bacteriophages were called Φ XanS1, Φ XanS2, Φ XanS3, Φ XanF1 and Φ XanF4. Two agriphages Φ XanF1, Φ XanF4 were isolated from the leaves, and three agriphages Φ XanS1, Φ XanS2, Φ XanS3 were isolated from soils (*Figures 4 & 5*). All agriphages were isolated from a single bacterium host *Xanthomonas campestris*.

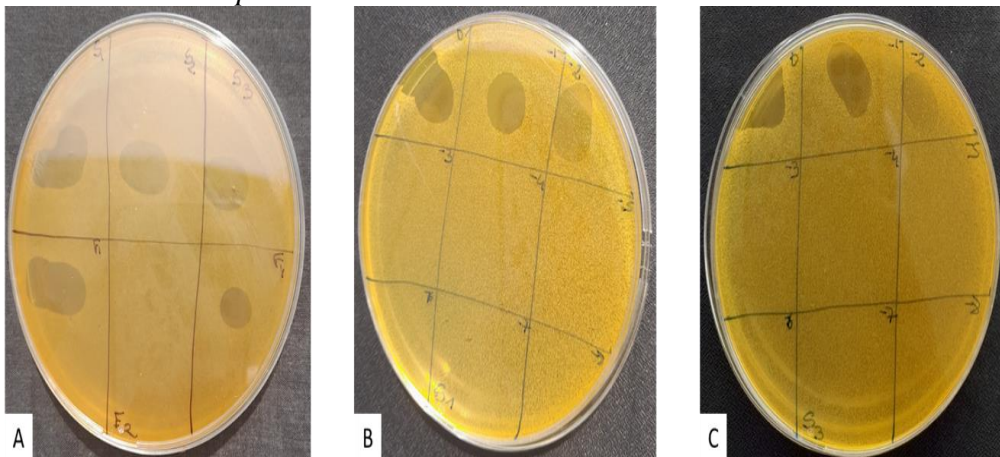


Figure 3. Lysis plaque on nutrient agar.

A: Lysis plaque on five isolated agriphages against *Xanthomonas campestris*; B: Lysis plaque of phage Φ XanS1 ; C: Lysis plaque of phage Φ XanS3

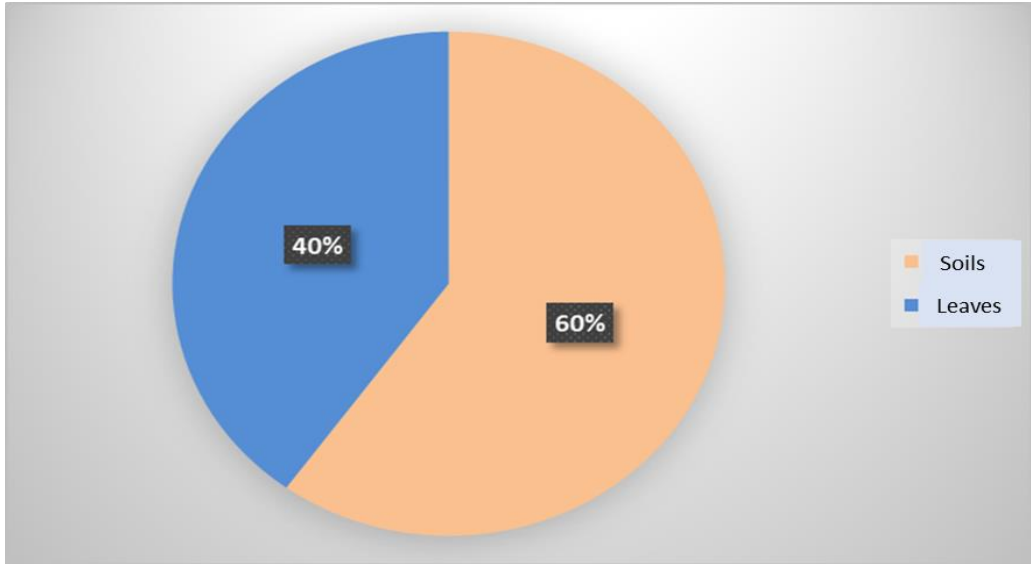


Figure 4. Distribution of agriphages according to the type of sample

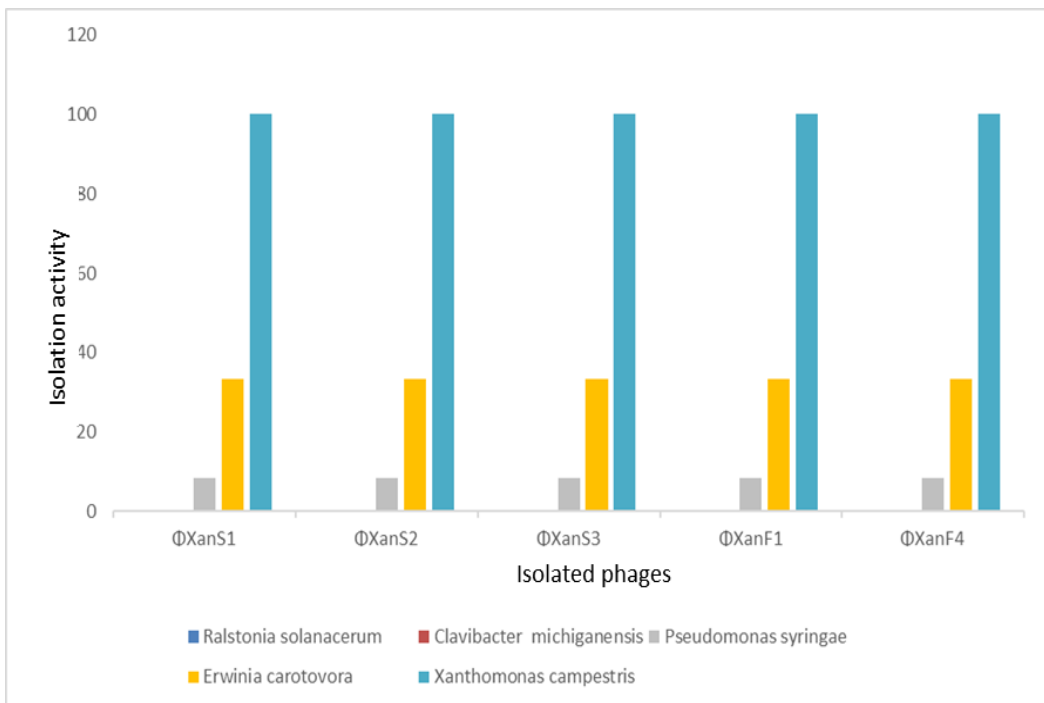


Figure 5. Distribution of the activity of the isolated phages according to the bacteria

3.2 Lytic activity of agriphages

Five bacterial strains were tested with agriphages for the sensibility or resistance effects. 3 bacterial strains (*Xanthomonas*, *Erwinia*, and *Pseudomonas*) were tested positive for lytic activity against all isolated phages. 60% (3/5) virulence was observed in this study for the agriphages (Table 2). *Ralstonia* and *Clavibacter* strains have shown no sensibility against agriphages.

Table 2. Phagogram of agriphages against bacterial species

| Phages | Bacterial strains tested | | | | | % of virulence |
|--------|-------------------------------|---------------------------|-----------------------------|------------------------------|----------------------------------|----------------|
| | <i>Xanthomonas campestris</i> | <i>Erwinia carotovora</i> | <i>Pseudomonas syringae</i> | <i>Ralstonia solanacerum</i> | <i>Clavibacter michiganensis</i> | |
| ΦXanS1 | + | + | + | - | - | 60% |
| ΦXanS2 | + | + | + | - | - | 60% |
| ΦXanS3 | + | + | + | - | - | 60% |
| ΦXanF1 | + | + | + | - | - | 60% |
| ΦXanF4 | + | + | + | - | - | 60% |
| Total | 5/5 | 5/5 | 5/5 | 0/5 | 0/5 | |

3.3 Molecular diversity of agriphages

Random amplification of polymorphic DNA (RAPD-PCR) shows the genetic diversity of the isolated agriphages. The genomic profile of the phages showed that tree isolated agriphages are DNA phages. From the P1 primer amplification, the profiles showed that phages ΦXanS1, ΦXanS2, ΦXanF1 are DNA phages with some similar profiles (Figure 6A).

From the P2 primer, the results showed that the profiles were not identical. This confirms that the agriphages ΦXanS1, ΦXanS2, ΦXanF1 are different from others but they could be genetically very similar phages (Figure 6B). Indeed, the phages ΦXanS1, ΦXanS2, and ΦXanF1 showed more or less similar profiles with approximately the same number of bands for each agriphage. Phages ΦXanS1, ΦXanS2 phages are closely related with 93% similarity by Pearson similarity analysis. While ΦXanS1 and ΦXanS2 are 62% similar to phage ΦXanF1 (Figure 7).

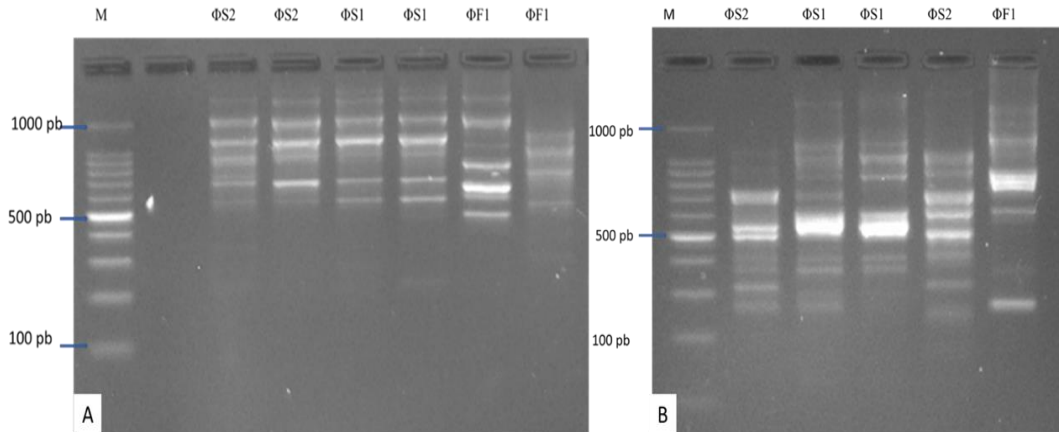


Figure 6. RAPD-PCR Genomic profile of agriphages.

A : RAPD-PCR Genomic profile of agriphages using primer P1; B: RAPD-PCR Genomic profile of agriphages using primer P2.

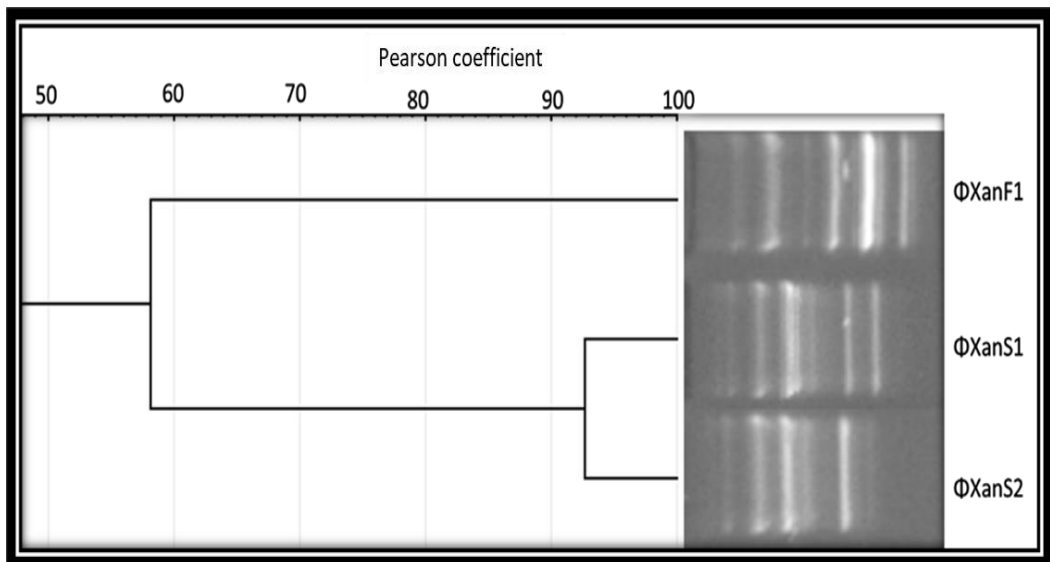


Figure 7. Dendrogram after RAP-PCR analyzing.

Discussion

This study presents the first isolation and characterization of agriphages for the control of phyto-bacteria infection in West Africa. The isolated agriphages were virulent by lysing of three bacterial strains and exhibited great potential to control infections in vitro of *Xanthomonas*, *Erwinia*, and *Pseudomonas*.

This study proposed a collection of five agriphages, and three were DNA phages. These agriphages were isolated in site 2. The percentage of bacteria isolated from this site is higher than the other two sites. Indeed,

according to a previous study, the plot was previously used as a household waste dump. This waste was the growth biotope of several bacteria, viruses, and parasites (Adioumani et al., 2022). Several studies have shown that a contaminated biotope represents a high risk for cultivated plants. It has also been shown that agriphages are present in environments, where the bacterial population is also abundant (Son et al., 2012).

The agriphages need different degrees of tolerance depending on the experimental conditions where they are living. Thus, many conditions in crop plots are beneficial to their proliferation. Low exposure to UV rays, temperature, soil pH, and humidity are essential for the good growth of agriphages (Flores et al., 2020).

60% of agriphages were isolated from the soil and 40% from leaves. The conservation of the agriphages in the phyllosphere is quite complex in the sense that they are constantly exposed to temperature variations, the rays of the sun, and the rains. During the day, the agriphages present on the surface of the leaves are exposed to the sun which is the greatest limiting factor to their proliferation (Jones et al., 2012).

All the isolated phages are from the same bacterial species *Xanthomonas campestris*. This suggests the predominance of *Xanthomonas* in these sites. Indeed, Adioumani et al., 2022 have shown that almost 70 % of *Xanthomonas* sp strains are in the site. And according to the phage infection mechanism, the agriphages are specific to the most abundant bacteria in a biotope. Hence the specificity of the agriphages for this strain (Flores et al., 2020). The phages found on this site reflect the abundance of *Xanthomonas* sp. Because according to the theory of Kill-the-Winner, the phages preferably infect the most abundant species in the environment (Koskella & Meaden, 2013).

In order to reduce the high concentration of *Xanthomonas* sp on this site, the agriphages have developed an increased specificity for their bacteria host. Despite this specificity, isolated agriphages are virulent on other species like *Erwinia* sp and *Pseudomonas* sp. The broad range of agriphages to other bacteria strains is particular for phages in tropical countries. Previous studies have shown the circulation of polyvalent phages in West Africa (Addablah et al 2021; Kakou-Ngazoa et al 2020).

Using RADP-PCR methods, the genomic profile of the agriphages allowed the confirmation of 60% of isolated phages like DNA phages. This is an excellent approach for typing phages infecting bacteria of the same or different species (Gutierrez et al., 2011; Addablah et al., 2021). The genomic profile showed the genetic diversity of the isolated phages.

And with link clustering, for one group to assimilate to another, all objects must be linked to the given similarity (Gutiérrez et al., 2011). The molecular typing dendrogram was used to assess the diversity of the isolated phages.

These phages isolated from the same bacterium have been grouped like a cluster (Hatfull, 2008).

These phages will be explored in the field with different challenges, such as the tolerance to environmental conditions, including the soil pH, UV radiation, and temperature. Molecular investigation using Next Generation Sequencing (NGS) can provide more identities or similarities of these agriphages. This study promotes the use of viral bacteria (phages) to control phyto-bacterial infection instead of antibiotic use in West Africa.

Conclusion

This study allowed a collection of five agriphages, and the first isolation of agriphages in West Africa. These agriphages have lytic activity on three bacterial strains; *Xanthomonas campestris*; *Erwinia carotovora* and *Pseudomonas syringea*. The phagogram shows a broad spectrum of action of agriphages with a virulence rate of 60%. The characterization of agriphages in vitro and in vivo efficacy tests will allow the selection of the best candidates for field future applications.

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