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

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Abstract

Agriphages or bacterial viruses are ubiquitous in the environment. The discovery of virulent phages against phytobacteria improves crop growth and proposes biopesticide uses for plant diseases. In Africa, many phytobacteria

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such as Ralstonia, Clavibacter, and Xanthomonas were reported in several regions. This paper focuses on evaluating the presence of agriphages for the biocontrol of phytobacteria in Côte d'Ivoire. Leaves and soil samples were collected from healthy and diseased plants in three sites located in Anyama, Abidjan, and Bingerville. The pretreatments occurred 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 ΦXanS1 and ΦXanS2 have 93% similarities, while ΦXanS1 and ΦXanS2 are 62% similar to ΦXanF1. This study is the first reported agriphages in West Africa, alongside their potential uses against phytobacteria for biocontrol infection in crops.

Keywords: Agriphages, Vegetable crops, RAPD-PCR, West Africa, Phytobacteria

1. Introduction

Phytobacteria are responsible for several plant diseases worldwide, which results to 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, as well as 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 such as tomatoes, eggplants, and bell pepper is increasing due to population growth. The increase in the production of these foodstuffs causes the growth of phytobacteria in these crops.

The use of chemical phytosanitary products has made it possible to limit the extent of the damage caused by phytobacteria. However, phytosanitary products of chemical origin have proven to be ineffective over time in eliminating bacteria. They have adverse effects on human and environmental health (FAO, 2015) for consumers and also promote multi-resistance (Jovana et al., 2014). Many phytosanitary products such as copper

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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. Subsequently, using phytosanitary products of biological origin such as agricultural production, resistant plant varieties, or biopesticides makes it possible to defeat pests while respecting nature. Microbial biopesticides are ecologically more compatible than chemical products and have increased specificity towards the pathogens against which they are directed. Among the biopesticides are agriphages.

Agriphages are ubiquitous in the environment. As a result, 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 increase 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 in Côte d'Ivoire to control some phytobacteria.

1. Material and Methods

2.1 Sites of Study

This study is an experimental with tree based agro-systems in the field. The sampling sites were all biological fields and no chemical products were used. Site 1 was located in a forest area in the city of Anyama. Soils, eggplant leaves, and chili samples were collected in this site. Site 2 was located in an urban area (Abidjan City) for the sampling of soil and tomato leaves. 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*).

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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)

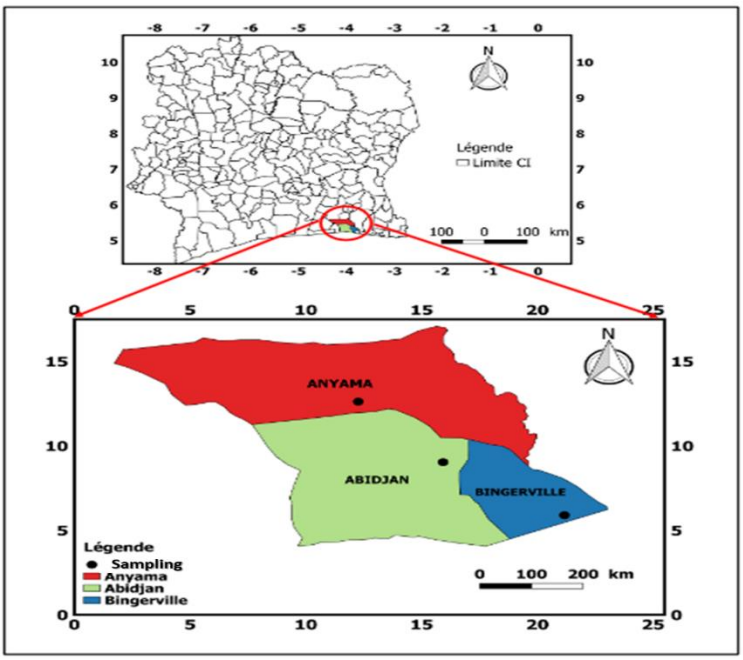

Figure 2. Geographical location of sampling sites (Source: CNTIG, 2022 QGIS 3.10)

2.2 Collection of Samples

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

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2.3 Bacterial Strains and Culture Conditions

Although the bacteria strains used in this study were obtained from the Pasteur Institute Côte d'Ivoire, 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 solanacerum, 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. 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 (Tryptic Soy Broth, 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 (Saline Medium) buffer (10 mM Tris-HCl, pH 7.5; 100 mM NaCl; 10 mM $MgSO_4$). This was 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 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 was inoculated. Five microliters of phage filtrate were spotted on LB agar (Twest & Kropinski, 2009).

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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) were added and incubated at 56°C for 90 minutes. After lysis, 200 µl of the solution was 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%) was added and mixed by vortexing and centrifugation (10 000 rpm, 1 minute). Thereafter, the column was placed in a new collecting tube and 500 µl of buffer AW1 was 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; Jakočiūnė & 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 µL 5X Reaction Buffer, 3.0 µL $MgCl_2$ (25 mM), 1.0 µL dNTPs (25 mM) (Promega, Wisconsin, USA), 1.0 µL primer (10 µM), and 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 minutes), followed by 32 cycles of denaturation (95°C for 30 seconds), hybridization (54°C for 30 seconds), and elongation (72°C for 1 minute). Accordingly, 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, Wisconsin, USA).

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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).

2. Results

2.1 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 did not detect phages. The bacteriophages were called ΦXanS1, ΦXanS2, ΦXanS3, ΦXanF1, and ΦXanF4. Two agriphages, ΦXanF1 and ΦXanF4, were isolated from the leaves and three agriphages, ΦXanS1, ΦXanS2, ΦxanS3, were isolated from soils (*Figures 4 & 5*). All agriphages were isolated from a single bacterium host *Xanthomonas campestris*.

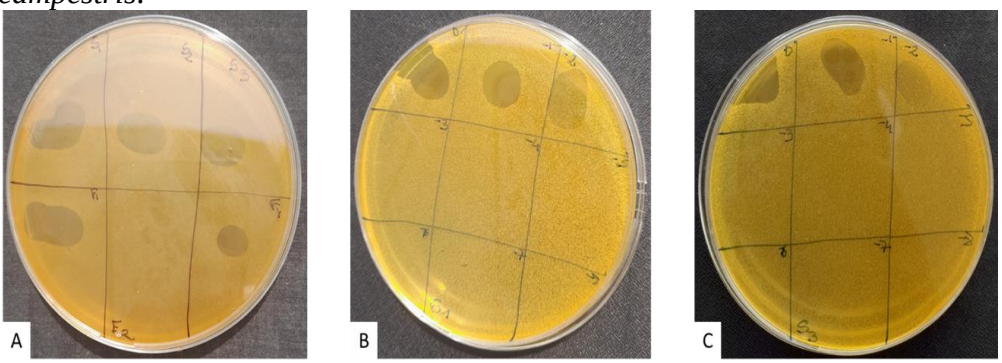

Figure 3. Lysis plaque on nutrient agar
A: Lysis plaque on five isolated agriphages against *Xanthomonas campestris;* B: Lysis plaque of phage ΦXanS1; C: Lysis plaque of phage ΦXanS3

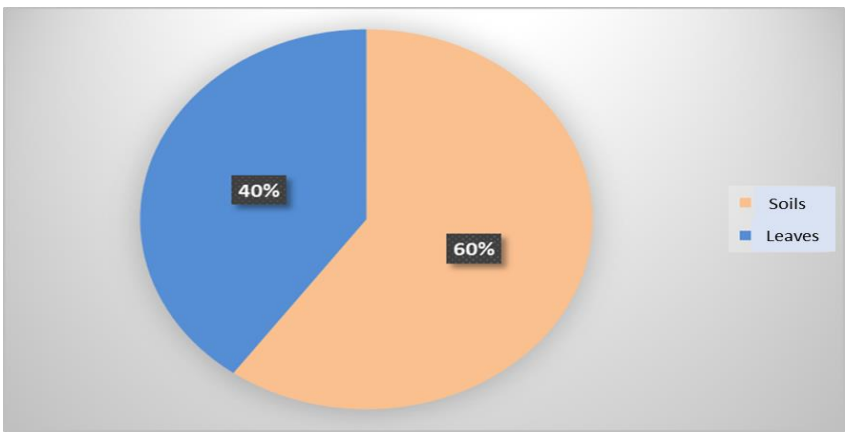

Figure 4. Distribution of agriphages according to the type of samples

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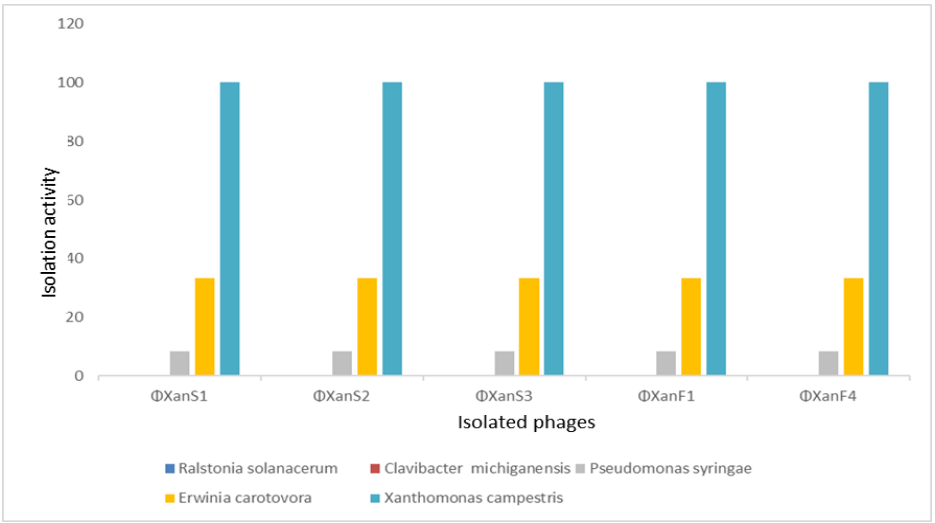

Figure 5. Distribution of the activity of the isolated phages according to the bacteria *Ralstonia solanacerum, Clavibacter michiganensis, Pseudomonas syringea, Erwinia carotovora,* and *Xanthomonas campestris*

3.2 Lytic Activity of Agriphages

Five bacterial strains were tested with agriphages to determine sensibility or resistance effects. 3 bacterial strains (*Xanthomonas*, *Erwinia*, and *Pseudomonas*) 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
	Xanthomonas campestris	***Erwinia carotovora***	***Pseudomonas syringae***	***Ralstonia solanacerum***	***Clavibacter michiganensis***	
ΦXanS1	+	+	+	-	-	**60%**
ΦXanS2	+	+	+	-	-	**60%**
ΦXanS3	+	+	+	-	-	**60%**
ΦXanF1	+	+	+	-	-	**60%**
ΦXanF4	+	+	+	-	-	**60%**
Total	**5/5**	**5/5**	**5/5**	**0/5**	**0/5**	

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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. According to the P1 primer amplification, the profiles showed that phages ΦXanS1, ΦXanS2, and ΦXanF1 are DNA phages with some similar profiles (*Figure 6A*).

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

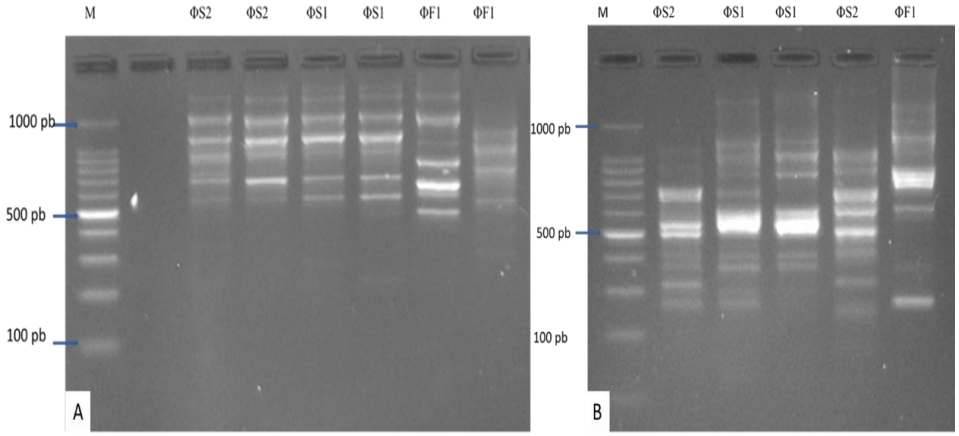

Figure 6. RAPD-PCR Genomic profile of agriphages

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

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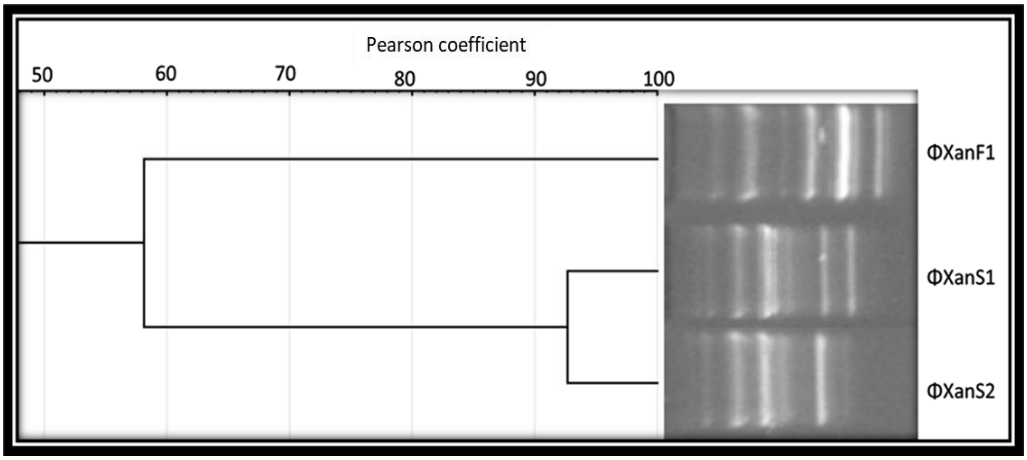

Figure 7. Dendogram after RAPD-PCR analyzing of tree isolated phages

3. Discussion

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

This study further 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. According to a previous study, the plot was formerly 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. In addition, 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 because 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).

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All the isolated phages are from the same bacterial species *Xanthomonas campestris*. This suggests the predominance of *Xanthomonas* in these sites. Adioumani *et al*. (2022) have shown that almost 70% of *Xanthomonas sp* strains were in the site. According to the phage infection mechanism, the agriphages are specific to the most abundant bacteria in a biotope. This has resulted to the specificity of the agriphages for this strain (Flores et al., 2020). The phages found on this site reflect the abundance of *Xanthomonas sp*. 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 such as 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.

Therefore, with link clustering, which allows 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).

Subsequently, these phages will be explored in the field with different challenges, such as 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 phytobacterial infection instead of antibiotic use in West Africa.

Conclusion

The 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 from *in vitro* and *in vivo* efficacy tests will allow the selection of the best candidates for field applications.

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Data Availability: All of the data are included in the content of the paper.

Conflict of Interest: The authors reported no conflict of interest.

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