Journal of Experimental Agriculture International

41(1): 1-11, 2019; Article no.JEAI.51453
ISSN: 2457-0591
(Past name: American Journal of Experimental Agriculture, Past ISSN: 2231-0606)

Sugarcane Leaf Scald Disease in Côte d'Ivoire: Pathogenicity and Biocontrol of *Xanthomonas albilineans* Isolates

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Authors' contributions

This work was carried out in collaboration among all authors. Authors NAC and KKD designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors KKD and KKFJ managed the analyses of the study. Author KKFJ managed the literature searches. Authors KKG, KDR, YKJ and KD read and approved the final manuscript.

Article Information

DOI: 10.9734/JEAI/2019/v41i130388
Editor(s):
(1) Dr. Süleyman Korkut, Duzce University, Department of Forest Industrial Engineering, Division of Wood Mechanic and Technology, Turkey.
Reviewers:
(1) R. K. Lal, Central Institute of Medicinal and Aromatic Plants, India.
(2) Clint Magill, Texas A&M University, USA.
(3) Salik Nawaz Khan, University of the Punjab, Pakistan.
Complete Peer review History: https://sdiarticle4.com/review-history/51453

Original Research Article

Received 09 July 2019
Accepted 14 September 2019
Published 01 October 2019

ABSTRACT

Aims: The present investigations describes bactericidal potential of essential oil for management of *Xanthomonas albilineans* (Ashby. 1929) Dowson 1943, pathogen responsible for sugarcane Leaf scald disease in Côte d'Ivoire.
Study Design: the study was conducted at the Ferké 1 sugar mill and the Laboratory of Plant Physiology of Felix Houphouët BOIGNY University.

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Methodology: Diseased leaves specimens showing signs of *X. albilineans* presence were collected from sugarcane industrial plantations in Côte d'Ivoire. Pathogenicity was confirmed by observing Koch's postulates under semi-controlled conditions. Asymptomatic leaves, obtained after 60 days of culture, were inoculated at three points by infiltration with bacterial suspension calibrated at 10^8 bacteria/ml. Apparition of small tan-brown necrotic lesions on the leaf blade, parallel to the veins and prolonged to the ends by a discolored vascular bundle confirmed that disease is caused by *Xanthomonas albilineans*. Three essential oils of aromatic plants whose antibacterial properties are recognized were used at 100; 300; 500; 1000 and 2000 ppm in comparison with Callicuivre (56% copper oxychloride) used as reference product.
Results: The aqueous solutions based on these essential oils induced an inhibition zone of bacterial growth proportional to the dose applied and the incubation period. Thus, on day 5 of incubation, the essential oil solution of *Ocimum gratissimum* L. at 100 ppm induced an average inhibition zone diameter not significantly different from the one induced by the *Cymbopogon citratus* (DC.) Stapf solution at 300 ppm and the one of copper oxychloride at 1000 ppm. At 1000 ppm, *Zingiber officinale* Roscoe*, Cymbopogon citratus* (DC.) Stapf and *Ocimum gratissimum* L essential oils induced equivalent inhibition rates (6 mm) that were higher than that of the control (3.5 mm).
Conclusion: sugarcane varieties cultivated at the Ferké 1 sugar mill complex, despite the selection for resistance are threatened by Leaf Scald Disease, which is significantly expressed on variety R585. *Cymbopogon citratus* (DC.) Stapf and *Ocimum gratissimum* L oils have the strongest antibacterial activity and may be useful to control Leaf Scald Disease

Keywords: Saccharum officinarum; leaf scald disease; Xanthomonas albilineans; essential oil.

1. INTRODUCTION

Leaf scald disease is a bacterial blight which affects sugarcane crops (*Saccharum officinarum* L.), caused by the Gram-negative bacterium *Xanthomonas albilineans* (Ashby. 1929) Dowson 1943. It causes xylem vessel occlusion and prevents chloroplast differentiation [1]. This bacterial disease has three different phases in its progression. A latent phase where no symptoms are apparent on the leaves, then a chronic phase, that appears three months after infection and is characterized by stem necrosis and appearance of white streaks parallel to leaf midveins. Followed by an acute phase, during which immediate death of the seedling is observed [2]. Leaf Scald Disease is one of the most damaging diseases in sugarcane cultivation around the world [3].

In Côte d'Ivoire, sugarcane is cultivated in two industrial complexes (SUCAF-CI and SUCRIVOIRE) which exploit a surface area of 25 400 hectares of cane crop [4]. The country ranks 53rd worldwide and 16th at the African level in terms of sugar production [5]. This plant is mainly grown in the North and Center of the country where it is prone to leaf scald disease which is expressed on a certain number of varieties. For sensitive varieties (R585, N21, NCo376), the pathogen can cause total destruction of the plantation. The pathogen (*Xanthomonas albilineans* (Ashby. 1929) Dowson 1943) is spread through the use of contaminated cuttings and cutting equipment soiled by the bacteria [6]. Various control methods are popularized among producers, including the use of resistant varieties [1], the disinfection of cuttings by thermotherapy and the chemical disinfection of maintenance and harvesting tools [7]. Chemical control is predominantly used but is restrictive and harmful to the health of the applicator as well as to the environment.

The use of essential oils extracted from aromatic plants could serve as an alternative to synthetic bactericides in the control of Leaf scald disease. These essential oils showed their toxicity on Enterobacteria and Staphylococci [8], also on *Bacilus cereus, Bacillus subtilis, Staphylococcus aureus, Enterococcus faecalis* and *Escherichia coli* [9].

This study was conducted in order to assess the pathogenicity of *Xanthomonas albilineans* (Ashby. 1929) Dowson 1943 strains and the sensitivity of the main varieties grown in Ferké 1 industrial sugarcane field. It also helped assess the bactericidal effect of 3 essential oils of aromatic plants so as to propose them in the strategies of sugarcane Leaf scald disease management.

2. MATERIALS AND METHODS

2.1 Study Area

This study was conducted at the Ferké 1 sugar mill in northern Côte d'Ivoire. It is located between 09° 14'-09° 35' north latitude and 005° 15'-005° 24' west longitude, at a distance of 610 km from Abidjan, 35 km from Ferkessédougou and 45 km from Korhogo [4]. The Sudanese "sub humid tropical" climate marked by a dry, hot season, which runs from November to March, and a unimodal rainy season from April to October. The average annual rainfall is between 1000 and 1600 mm [10]. The maximum and minimum temperatures are 32.5ºC and 21ºC. The vegetation is a savannah (Guinean or sub-Sudanese) wooded, with varying levels, containing small detached fragments of forest. The soils of the area are mainly ferralitic with shallow topsoil (40 to 60 cm) limited by indurations. The fertility of these soils is moderate, with relatively low pH, low cation exchange capacity, and rather poor base saturation. This situation is characteristic of areas of the savannah region in West Africa. However, the conditions in the area of Ferkessédougou are suitable for sugarcane cultivation [4].

The study area is favorable for the incidence of Leaf scald disease caused by the Gram-negative bacterium *Xanthomonas albilineans*, whose optimal growth occurs at temperatures between 25 and 30ºC.

2.2 Plant Material

Three (3) sugarcane varieties R585, NCo376, CP921167 were used to assess the pathogenicity of *Xanthomonas albilineans* (Ashby. 1929) Dowson 1943 strains (Table 1). Fresh leaves of *Cymbopogon citratus* (DC.) Stapf*, Ocimum gratissimum* L and rhizomes of *Zingiber officinale* Roscoe were used to produce essential oils by hydro distillation.

Table 1. Sugarcane varieties assessed under leaf scald disease

Varieties	Origin
R585	Reunion (Reunion Island)
NCo376	Natal and Coimbatore (South Africa and India)
CP921167	Canal Point (Florida)

2.3 Bacterial Strain

Xanthomonas albilineans (Ashby. 1929) Dowson 1943 strains were isolated from stem and leaf fragments of sugarcane variety R585 collected from the Ferké 1 sugar mill complex showing Leaf scald disease symptoms.

2.4 Bactericidal Product Used

Callicuivre (56% copper oxychloride), copper-based bactericide is the reference product used to compare the antibacterial power of extracted essential oils.

2.5 Methods

2.5.1 Collection of organs infected by leaf scald disease and production of *Xanthomonas albilineans* isolates

The isolation of the different strains of *X. albilineans* was carried out from fragments of stems and leaves collected on the Ferké 1 industrial complex and showing the characteristic symptoms of sugarcane Leaf Scald Disease (white streaks parallel to the midvein). Samples were collected from five (5) industrial plots of 20 ha each and included symptomatic seedlings randomly selected from each plantation row. A total of 20 seedlings were collected at a rate of four (4) samples per industrial plot, packaged in referenced envelopes (date, place, cultivar) and then transferred to the laboratory for storage in the freezer at -4ºC.

The isolation of bacterial strains was conducted according to the modified method of Persley (1972) [11]. It consisted in sterilizing leaf or stem fragments of each sample collected, with a very fine white streak, in 10% bleach for one minute, then rinsed twice in sterilized distilled water. The sterilized samples were individually aseptically dissected in a few drops of sterile water and allowed to macerate for 4 h at 28ºC. Then, 0.5 µl of the suspension obtained was spread per parallel streaks on a petri dish containing YPGA culture medium (2% agar, 1% dextrose, 0.2% yeast, 0.1% peptone). The Petri dishes were then incubated underside up at a temperature of 28ºC.

2.5.2 Pathogenicity of *Xanthomonas albilineans* isolates

Respective cuttings of R585; NCo376 and CP921167, three sugarcane varieties exploited

on the Ferké 1 sugar mill complex were cultivated under shelter in pots containing steam-sterilized soil. The asymptomatic leaves, obtained after 60 days of culture, were inoculated by infiltration as described by Meyer et al. (2005) [12]. For this mechanical transmission of isolated strains, a bacterial culture fragment was retrieved using the inoculation loop and then diluted in 1 ml of sterile water and vortexed for 1 min.

From a syringe containing a bacterial suspension calibrated at an optical density (OD) of 0.2 at a wavelength of 600 nm, corresponding to 10^8 bacteria/ml, three inoculation points were made on each side of the central vein (Fig. 1).

After inoculation, the seedlings were subjected to illumination conditions of a 12-h photoperiod. Regular watering every three days was carried out to maintain saturating moisture conditions, favorable to disease expression. Sterile distilled water served as a negative control and this treatment involved 10 seedlings per sugarcane variety. The inoculation tests were repeated three times. The search for characteristic symptoms of sugarcane Leaf Scald Disease (small tan-brown necrotic lesions on the leaf blade, parallel to the veins and prolonged to the ends by a discolored vascular bundle) was carried out during seedlings follow-up period under semi-controlled conditions.

Fig. 1. Pathogenicity test by injection of *X. albilineans* strains

2.5.3 Production of essential oils

The essential oils of the aromatic plants used in this study were obtained from fresh organs by distillation with saturated steam, carried out in a Clevenger-type apparatus for 2 h. This method consists of a conventional distillation in which the organ is not in direct contact with water. Indeed, in the distillation with saturated steam, the plant material is placed on a grid and penetrated by steam. During the passage of steam, through the plant material, the cells burst out and release the essential oils which are led to the condenser and then the essence jar. The oil is collected by decantation.

2.5.4 Comparison of callicuivre *in vitro* bactericidal activity with that of essential oils

The technique used is the modified Fauchère and Avril (2002) method [13]. It consists in depositing a sterile disc of filter paper soaked in essential oils or callicuivre, on a microbial mat and then measuring the area where the bacteria could not develop. The inhibition diameter, which reflects the antibacterial activity of the essential oil or callicuivre, is thus determined as a translucent halo at the location of the disc. To this end, different solutions of each of the essential oils tested were prepared at doses of 100; 300; 500; 1000 and 2000 µl/l by adding 0.1% Tween 20. In addition, the same doses of callicuivre were prepared from 100 ml of a stock solution of this bactericide obtained by diluting the commercial product in sterile distilled water.

A suspension of one of the isolated *Xanthomonas albilineans* (Ashby. 1929) Dowson 1943 strains with proven pathogenicity was prepared at the density equivalent to Mac Farland Standard 0.5 (10^8 CFU.ml^{-1}) by suspending some bacterial colonies in saline solution (0.9% NaCl). The Petri dishes containing the YPGA culture medium were inoculated in layer with the inoculum. On the surface of each dish, a disc of sterile 6 mm-diameter filter paper soaked in 20 µl of essential oil solution at different concentrations and callicuivre was deposited. A disk soaked in 20 µl of an aqueous solution of 0.1% Tween 20 was used as a positive control. The dishes were left for one hour at room temperature to allow the diffusion of the essential oil or callicuivre, then incubated at 28ºC for 120 h. After incubation, the antibacterial activity was expressed by inhibition zones around the filter paper which were proportional to the intensity of the antibacterial activity. The reading was carried out daily during the 5 days of incubation by measuring inhibition diameters around the filter papers for each dose of products (solution of essential oils or callicuivre).

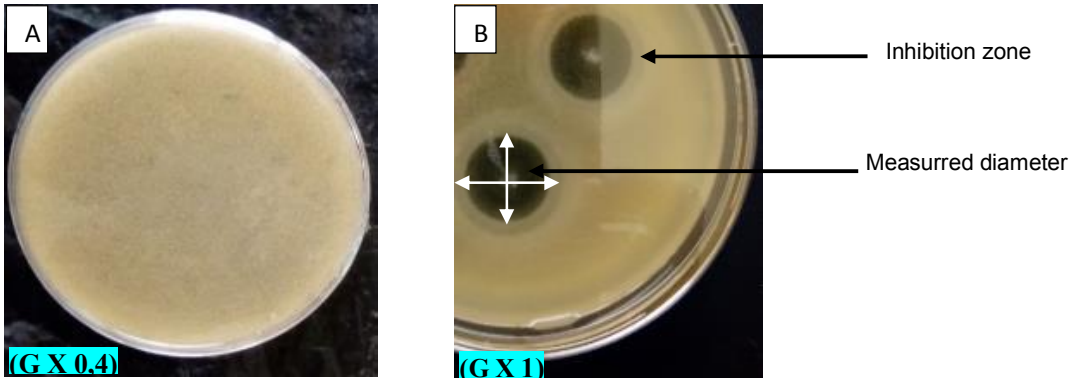


Fig. 2. Proliferation of *Xanthomonas albilineans* (Ashby. 1929) Dowson 1943 colonies on YPDA medium at 28°C for 5 days of incubation

A: Proliferation on Control medium; B: Proliferation on amended medium

2.6 Statistical Analyses

A complete randomization with three repetitions was used for the collection of essential oil inhibition data. The results obtained were analyzed using STATISTICA 7.1 software. The multiple factor analysis of variance (ANOVA) test was used to assess the effect of each essential oil at different concentrations on *Xanthomonas albilineans* (Ashby. 1929) Dowson 1943 proliferation. In the event of significant difference between the averages, the Newman-Keuls multiple comparison test at 5% threshold was performed to classify them into homogeneous groups.

3. RESULTS

3.1 Identification of Isolated Bacterial Strains

After 48-h incubation at 28ºC, the macerated leaves or stems streaked on the YPDA culture medium revealed bacterial colonies. They were honey-yellow in color, circular in shape, translucent, convex and shiny in appearance (Fig. 3). Three isolates were identified, Xam_1 and Xam_3 isolated from stem fragments and Xam_2 obtained from leaf fragments.

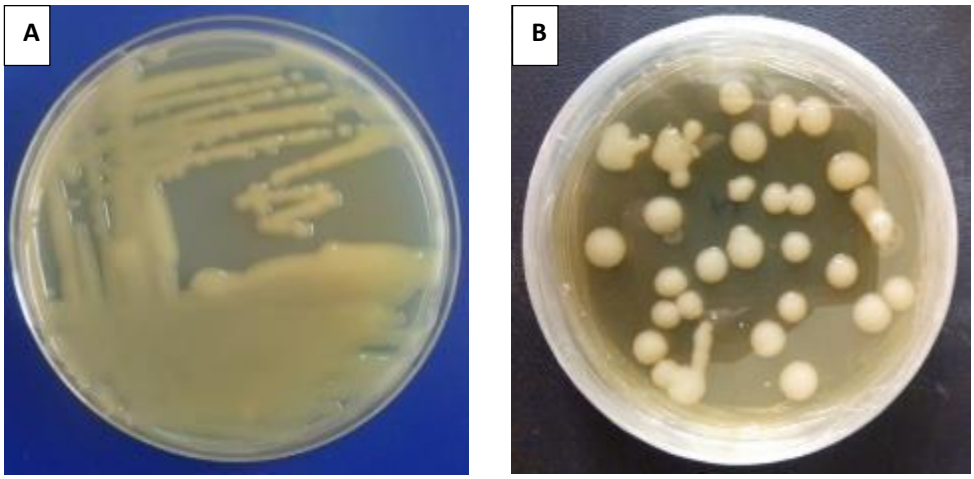


Fig. 3. Macroscopic appearance of two *Xanthomonas albilineans* (Ashby. 1929) Dowson 1943 isolates

A: Xam_1, yellow bacterial colonies mucous isolated from stems; B: Xam_2, rounded bacterial colonies isolated from leaves

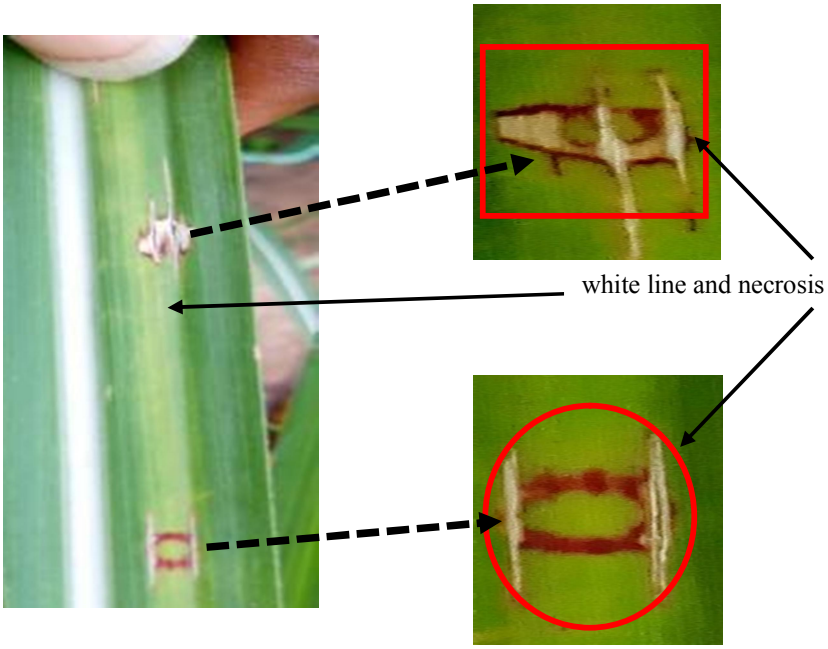


Fig. 4. Whitish streaks on leaves after inoculation of Cv R585 with Xam_1 strain

Fig. 5. Absence of leaf scald disease symptoms on Cv R585 leaves after inoculation with water

At the end of the mechanical transmission of Xam_1 strain by injection, the first symptoms on the cultivar (Cv R585) appeared five (5) days after inoculation in the form of a fine white streak, on both sides of the injection, on the inoculated leaf (Fig. 4). Subsequently on the newly formed leaves, we noticed fine continuous white lines, characteristic of *Xanthomonas albilineans* (Ashby. 1929) Dowson 1943. The seedlings inoculated with Xam_3 strain showed identical symptoms. The bacteria were then isolated from the symptomatic seedlings. Control seedlings inoculated with water showed none of these symptoms (Fig. 5).

On sugar cane seedlings Cv R585; NCo376 and CP921167 no symptoms of Leaf Scald Disease were noticed after inoculation with Xam_2 strain.

3.2 Comparative Antibacterial Activities of Essential Oils and Callicuivre (56% copper oxychloride) under *in vitro* Culture Conditions

Inhibition zones were observed in all the Petri dishes on the YPGA media amended with the solutions of the essential oils and the reference product (Callicuivre 56%). A proportionality between the diameter of the translucent halos representing the inhibition zone, the doses in products brought and the duration of the incubation period was revealed (Fig. 6).

At a dose of 100 ppm, the solutions of *Ocimum gratissimum* L and *Cymbopogon citratus* (DC.) Stapf essential oils induced statistically identical translucent halo diameters with 3.28 mm and 2.44 mm, respectively. The one of *Zingiber officinale* Roscoe had the smallest

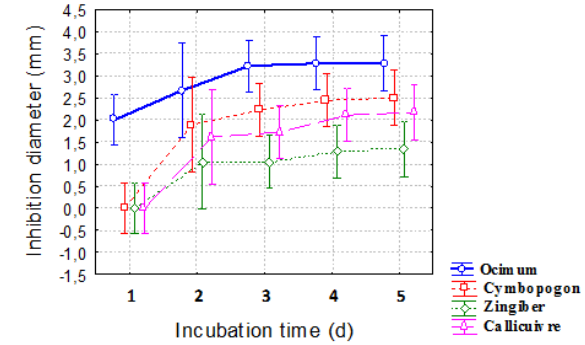
inhibition diameter, that is, 1.33 mm. The Callicuivre solution induced a translucent zone with a diameter of 2.16 mm, less than the one assessed in Petri dishes treated with *Ocimum gratissimum* L and *Cymbopogon citratus* (DC.) Stapf essential oil-based solutions (Fig. 6a).

The solution at the 300 ppm dose of *Ocimum gratissimum* L induced the highest diameter with 4.55 mm. The one of *Zingiber officinale* Roscoe at the same dose induced a translucent zone with a diameter of 1.33 mm which was not significantly different from the one assessed in the Petri dishes treated with Callicuivre solution (Fig. 6b).

For the 500 ppm dose, the *Ocimum gratissimum* L and *Cymbopogon citratus* (DC.) Stapf solutions had the highest inhibition zone diameters of 4.77 mm and 4.28 mm, respectively. *Zingiber officinale* Roscoe and Callicuivre induced diameters of 2.5 mm and 3 mm (Fig. 6c).

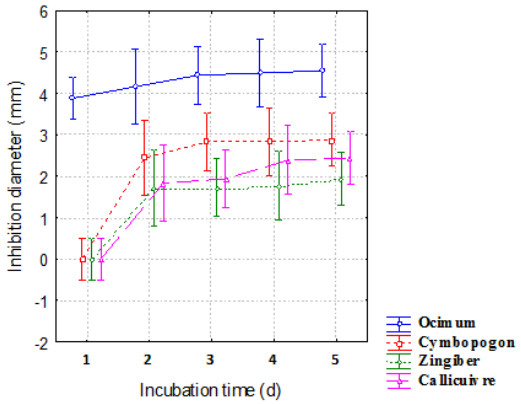
At a dose of 1000 ppm, the essential oils of *Zingiber officinale* Roscoe, *Cymbopogon citratus* (DC.) Stapf and *Ocimum gratissimum* L brought about translucent halo diameters that were not statistically different (Fig. 6d). They varied from 5.83 mm for *Zingiber officinale* Roscoe essential oil to 6.00 mm for *Cymbopogon citratus* (DC.) Stapf one. The imbibition of the filter paper with the solution of *Ocimum gratissimum* L essential oil induced a translucent zone of 5.94 mm. As for the imbibition with callicuivre solution, it induced the smallest inhibition zone diameter with an average value of 3.55 mm (Fig. 6e).

The inhibition results obtained by the essential oils and the synthetic bactericide on Xam_1 strain were significantly different for the same dose used on the 5th day of incubation (Table 2).



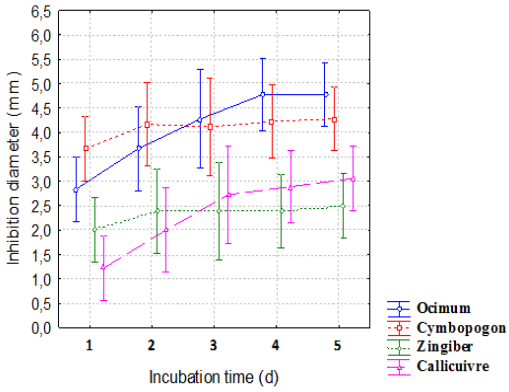
100 ppm

(a)



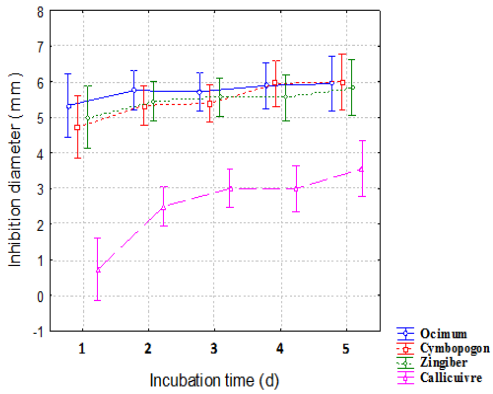
300 ppm

(b)



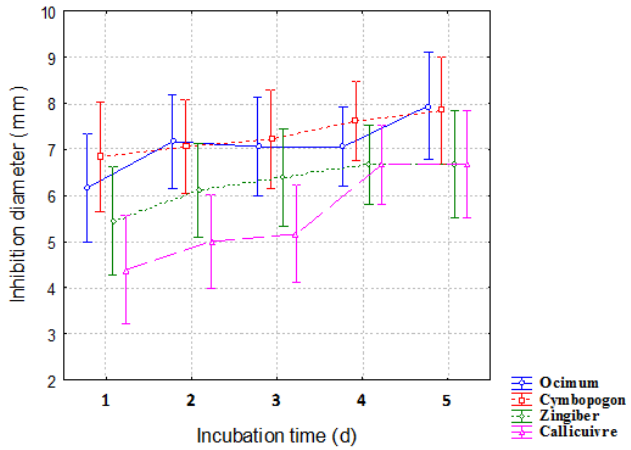
500 ppm

(c)



1000 ppm

(d)



(e)

Fig. 6. Diameters of the bacterial inhibition zone at different doses of essential oils and callicuivre (56%)

Table 2. Diameters of the inhibition zones at different doses of the products assessed at the 5th day of incubation

Doses / Products	Diameter of inhibition zones (mm)				
	100 ppm	300 ppm	500 ppm	1000 ppm	2000 ppm
Ocimum gratissimum L.	3.28 ± 0.3 ef	4.55 ± 0.36 cd	4.78 ± 0.33 bc	5.94 ± 0.38 b	7.95 ± 0.86 a
Cymbopogon citratus (DC.) Stapf	2.44 ± 0.9 fg	2 .89 ± 0.33 ef	4.28 ± 0.2 cde	6.0 ± 0.25 b	7.84 ± 0.33 a
Zingiber officinale Roscoe	1.33 ± 0.25 g	1.94 ± 0.24 fg	2.5 ± 0.2 fg	5.83±0.2 b	6.67 ± 0.19 ab
Callicuivre (56%)	2.17 ± 0.25 fg	2.72 ± 0.22 fg	3.06 ± 0.29 ef	3.55±0.2 ef	6.67 ± 0.34 ab

The *Ocimum gratissimum* L essential oil solution at 100 ppm induced an average inhibition zone diameter which was not significantly different from the one induced by the *Cymbopogon citratus* (DC.) Stapf solution at 300 ppm and that of Callicuivre (56%) at 1000 ppm (Table 2).

At 2000 ppm, the maximum dose assessed during this work, the solutions of *Ocimum gratissimum* L and *Cymbopogon citratus* (DC.) Stapf essential oils brought about the highest inhibition diameters. They prevented the bacterial strain from reaching an area of 7.94 mm and 7.83 mm in diameter for *Ocimum gratissimum* L and *Cymbopogon citratus* (DC.) Stapf oil, respectively. *Zingiber officinale* Roscoe essential oil and Callicuivre brought about a translucent halo diameter of 6.66 mm free from bacterial strain Xam_1 colonies.

4. DISCUSSION

The cultural, morphological characters, the symptoms caused by the inoculation of isolated strains (Xam_1 et Xam_3) of sugarcane, var. R 585 from Ferke 1 industrial plots indicate that they can be identified with *Xanthomonas albilineans* (ASHBY) DOWSON. The isolates obtained showed colonies that were circular, convex in shape, smooth, shiny, and translucent, with yellow-honey color [14]. After inoculation, the isolates showed symptoms characteristic of Leaf Scald Disease. They appeared in the form of small tan-brown necrotic lesions on the leaf blade consistent with those described by Daugrois et al. (2003) [15]. White lines subsequently appeared and developed into the yellowing and death of the seedlings. CIRAD researchers have confirmed that necrotic lesions appear 3 to 5 weeks after the detection of

bacteria: the internal contamination of leaf tissue is therefore posterior to leaf contamination. Leaf contamination is therefore a crucial step in the infectious cycle and the spread of the disease in the humid tropics such as Guadeloupe.

Control methods showed significant effects on the bacterial activity of the isolates under *in vitro* culture conditions. Inhibition tests have shown the effectiveness of *Cymbopogon citratus* (DC.) Stapf*, Ocimum gratissimum* L*, Zingiber officinale* Roscoe essential oils and Callicuivre on the growth of *Xanthomonas albilineans* . The results obtained show that the three oils tested have antibacterial effects. Similar results were obtained by Affery et al. (2017) [16] who showed the effectiveness of NECO, *Ocimum gratissimum* L essential oil-based biofungicide on *Xanthomonas axonopodis* pv. *manihotis*, the pathogen of Cassava Bacterial Blight. These studies revealed that NECO showed antibacterial activity with inhibition zone diameters ranging from 0.34 to 0.46 cm. They also showed that *Ocimum gratissimum* L essential oil induced, *in vitro*, inhibition diameters greater than those of 56% copper oxychloride. This study also shows that the inhibition diameters obtained are greater than those induced by copper oxychloride. This indicates that a biological control by the use of its vegetable substances can be considered. They can be used as an alternative to the synthetic product in Cassava Bacterial Blight control. Equally, these oils can be applied on-farm and also for the treatment of cuttings [17]. At 1000 ppm, the three oils effectively inhibit the growth of pathogenic bacterial strains. Treating the cuttings with these products at a rate of 3000 to 10000 ppm before planting could significantly reduce the bacterial population and then an aerial spraying could eliminate the epiphytic population (on the leaves) and in the air.

The activity of these oils might be due to the antibacterial properties that gives them their natural organic characteristic. The strong antibacterial activity of these three plant species might be due to their chemical composition. Indeed, the work of Kanko (2010) [18] showed that the antimicrobial activity of *Ocimum gratissimum* L essential oil might be due to the major compounds such as thymol, γ-terpinene and p-cymene. For *Cymbopogon citratus* (DC.) Stapf oil of, it is due to geranial, mineral and myrcene which are the majority compounds of this oil.

The antibacterial activity of certain low molecular weight phenolic compounds such as thymol and carvacrol as been confirmed [19]. The latter have the ability to adhere to bacteria by binding to proteins and parietal lipopolysaccharides through their functional groups and thus reach the more vulnerable inner membrane.

The essential oils studied showed a relatively strong antibacterial activity compared to that of Callicuivre. The work of Bolou et al. (2015) [20] on tomato dry rot also showed strong fungicidal activity of the essential oils of *Xylopia aethiopica* (Dunal) A. Rich. fruits and leaves on *Sclerotium rolfsii* causative agent of tomato crown rot compared with Mancozeb and Banko-plus. The inhibitory action of *X. aethiopica* (Dunal) A. Rich essential oils might be due to the presence of two compounds which are β-pinene and terpinene 4-ol. These compounds are oxidizable into quinones, inhibitors of the hydrolytic enzymes of fungi [21].

5. CONCLUSION

The purpose of this study was to propose an effective and sustainable control method against *Xanthomonas albilineans* (Ashby. 1929) Dowson 1943, causative agent of sugarcane Leaf Scald Disease in Côte d'Ivoire. It must be remembered that sugarcane varieties cultivated at the Ferké 1 sugar mill complex, despite the selection process used and the phytosanitary watch, are threatened by numerous diseases, including Leaf Scald Disease, which is significantly expressed on variety R585. Surveys have shown that other varieties are susceptible (M1176/77; N21) and that if nothing is done, some resistant varieties could be affected with the amplification of parasite pressure. The isolates of *X. albilineans* obtained proved to be aggressive in the pathogenicity test, with pronounced symptoms in less than a month.

We obtained very interesting results during *in vitro* inhibition tests with natural substances that give better effects than the synthetic product. The essential oils used proved effective in stopping colony growth and in their antibacterial properties. These oils also act in low doses. *Cymbopogon citratus* (DC.) Stapf and *Ocimum gratissimum* L oils have the strongest antibacterial activity compared to Callicuivre. *Zingiber officinale* Roscoe oil is just as effective and is a promising production activity in the Ivorian agricultural sector.

These organic products have the potential to provide effective control of Leaf Scald Disease and other diseases such as smut, sugarcane rust and some insects, as other similar studies have shown, and will also to add value to agriculture in Côte d'Ivoire in the sense that the cultivation of its plant species will produce an income for farmers since it does not require the mobilization of substantial resources for their establishment. As the environmental component can not remain on the margins of this study, it should be emphasized that the use of these products has no negative effect on the health of users (humans) and the environment; they are easier to handle than products of synthetic or chemical origin.

ACKNOWLEDGEMENTS

The authors thank managers of Ferké 1 sugar complex for allowing access to the plots and obtaining various isolates from the samples taken.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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