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# Biocontrol of Post-harvest Fungal Diseases of Pineapple (*Ananas comosus* L.) Using Bacterial Biopesticides

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**Abstract** Pineapple (*Ananas comosus* L.) is a monocotyledon, herbaceous, of the Bromeliad family. Côte d'Ivoire is the leading supplier of fresh pineapple to the European market. For reasons of sanitary quality and also due to the deterioration under the action of several factors of the marketable quality of the fruits, pineapple suffers a slump in the European market. To deal with the problem linked to the deterioration of fruits under the action of microorganisms, phytosanitary products are used. However, these foods present risks for consumers and may be responsible for public health problems. The objective of the present study is to reduce post-harvest losses of pineapple fruit due to fungal contaminants using bacterial biopesticides such as *Bacillus subtilis* GA1, *Pseudomonas fluorescens* F19 and *Pseudomonas fluorescens* CI. To this end, isolations followed by identification by PCR-ITS-RFLP of the fungal strains carried out on 200 samples composed of healthy and altered pineapple fruits in order to determine the main fungal strains responsible for their alteration have been made. The antifungal activity of biopesticides has been tested *in vitro* and *in vivo* to assess the potential for inhibition against isolated fungal strains. The results indicate that 5 fungal genera namely *Rhizopus*, *Geotrichum*, *Neurospora*, *Candida* and *Aspergillus* were isolated. Antagonist tests using all three biopesticides reduced spoilage. This study contributed to the development of biopesticides in the fight against fruit spoilage fungi in Côte d'Ivoire.

***Keywords:*** *pineapple fruit, fungal spoilage, biopesticides, Côte d'Ivoire*

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## 1. Introduction

Pineapple (*Ananas comosus* (L) M) is a monocotyledon, herbaceous, of the Bromeliad family. It is the eleventh most cultivated fruit, with a global production of 25.8 million tonnes in 2016 [1]. This world production has grown steadily and increased by more than 8 million tonnes between 2000 and 2013. Pineapple cultivation is highly developed in the south-eastern part of the Côte d'Ivoire. It occupies an area of 16,000 ha and contributes 0.6% to the national GDP [2]. With 33,976 tonnes of fruit exported in 2014, Côte d'Ivoire is the leading African exporter, a head of Ghana, which produces 33,175 tonnes [3]. *Ananas comosus*, a species cultivated for its fruit, includes several varieties, eight of which are cultivated in Côte d'Ivoire. The best known are the "Smooth Cayenne", the "Queen" and the "MD2". These species are cultivated by 2,500 small planters with an average farm area of 5 ha. They carry out 80% of the production and are affiliated for the most part to cooperatives for the consolidation, packaging and transport of their production for export. In contrast, there are industrial-type farms owned by large fruit distribution groups around the world. This is the case of BCS (Banana Cultivation Society) belonging to the international group DOLE, practicing intensive production of fruits for export, with farms averaging 500 ha. Côte d'Ivoire, the leading supplier of fresh pineapple to the European Union with a coverage rate of 97 % in the years 1986 [4], has seen a sharp drop in production for several years. Pineapple production has been in constant decline since 2001, dropping from 200,000 tonnes in 2001 to around 35,000 tonnes in 2012 [4]. This regression is due to several problems including non-compliance with the maximum residual limits for etephon (chemical substance used for ripening) set at 2 mg / kg on pineapple [5] and post-harvest losses. This is because the quality of tropical fruits such as pineapple is generally affected by post-harvest diseases such as fruit rot, which are mainly caused by improper handling and storage during transport and marketing [6]. About 20-25% of harvested fruits are

spoiled by microorganisms during post-harvest handling, even in developed countries; this leads to a depreciation of the economic value of the fruits. Post-harvest losses represent about 40-50% of world production annually [3]. In order to have on the one hand, pineapple fruits of good sanitary quality and on the other hand, to preserve their taste quality while prolonging their shelf life, many proposals have been made. These include storage in a modified atmosphere, the use of antioxidants such as ascorbic acid, citric acid and calcium, and the use of firming agents [7]. However, despite these concerns, there is growing demand from consumers and suppliers to demand a reduction in the use of chemical pesticides. Although these chemicals are considered to be the most effective means to combat pests, unfortunately they have harmful consequences [8,9]. On the one hand, at the level of the environment through the accumulation of residues and soil pollution and on the other hand, the appearance and generalization of resistance mechanisms in pathogens and the ecological imbalance, due to the fact that these compounds of synthesis have a wide spectrum of action. These chemicals destroy not only harmful agents but also other populations in the ecosystem. In view of these harmful consequences, it is important to find alternative solutions which will make it possible to continue to fight against phytopathogens while reducing the use of chemicals. These may involve the rationalization of agricultural practices, the use of resistant plant varieties and / or the development of biopesticides [9]. There are plant, animal and bacterial biopesticides. Among the latter, there are microorganisms used in biological control.

# 2. Material and Methods

## 2.1. Material

The study material consists of healthy and spoiled pineapple fruits of the MD2 variety obtained directly from the markets of Yopougon, Abobo, Adjamé and Plateau and three bacterial biopesticides including *Bacillus subtilis* GA1 obtained from the collection of the Wallon Center for Industrial Biology (WCIB-Belgium), *Pseudomonas fluorescens* F19 isolated from tomatoes from Algeria and *Pseudomonas fluorescens* CI isolated from tomatoes from Côte d'Ivoire.

## 2.2. Methods

Two hundred (200) samples of pineapple fruits taken directly from the markets of 4 municipalities in the city of Abidjan (Abobo, Adjamé, Plateau, Yopougon,) at a rate of 50 fruits per site distributed as follows: 10 spoiled pineapples, 10 healthy pineapples used for fungal isolation and the remaining 30 healthy for biocontrol testing. Pineapple fruits collected directly from the markets were collected during the period from June to December 2016, ie 6 months of collection.

### 2.2.1. Identification of Fungal Microflora

**-Isolation of fungal flora**

The microorganisms were isolated by direct contact on Potatoe Dextrose Agar (PDA) (Merck, Germany) as described by [10]. Thus, the healthy and spoiled fruits were washed with tap water, rinsed three times with sterile distilled water, and disinfected using household paper soaked in 70% ethanol. A flamed blade was used to cut the "eyes" of the pineapple. The “eyes” taken were inoculated in Petri dishes containing Potatoe Dextrose Agar (PDA) with Chloramphenicol (CHL) medium. The dishes were then incubated at 28°C for five days. To obtain a pure strain, several subcultures on Malt Extract Agar (MEA) and Yeast Extract Peptone Glucose (YPG) (Merck, Germany) media were carried out from each colony observed on the PDA agar.

**-Identification by classical method**

First, an identification of the fungal strains isolated was carried out on the morphological (appearance, shape, relief, color) and microscopic criteria (thallus, spores, etc.) in order to carry out a first screening allowing the different strains isolated to be classified according to their kind.

- **Macroscopic identification of fungal strains**

The selected strains were subjected to macroscopic identification by examination of the culture on MEA and PDA with Chloramphenicol [11]. The cultural characteristics determined were the appearance, form, relief and color of the crop.

- **Microscopic identification of fungal strains**

Fungal isolates were identified on the basis of their morphological characteristics. Microscopic examination was performed using cotton blue solution. A drop of the solution was placed on a clean slide and a fragment of the isolate was placed on the drop. The isolate was evenly distributed on the slide using a sterile platinum loop. A coverslip was gently placed on the slide to remove air bubbles. The slide was subsequently mounted and examined under a microscope at 100x magnification. Identification was made as described by [12] focusing on thallus and spores.

**- Identification by molecular method of fungal strains**

- **DNA extraction**

On a 2 to 3 day culture of yeast in solid YPG medium, 5 mL of sterile distilled water containing 0.05% (v / v) Tween 80 was added and the surface of the medium was gently scraped using disposable sterile inoculator. The cells were then collected in a 2 ml tube then centrifuged at 10,000 xg for 4 min and stripped of the supernatant before DNA extraction. The mycelium was then recovered and then centrifuged at 10,000 g for 4 min to obtain a mycelium pellet of between 150 mg and 200 mg by fresh weight. The pellet was stored in 2 ml microcentrifuge tubes. For the extraction of DNA from fungi in an amount of 100 mg of mycelium or cells previously centrifuged at 10,000 xg for 4 min contained in a 2 mL microcentrifuge tube using the Miniprep EZNA® Mushroom DNA Extraction Kit (Omega Bio-tek, Doraville, USA) was added to 500 µl of CSPL buffer and 10 µl of 2-mercaptoethanol. The mixture was vortexed to disperse all of the clumps and was incubated at 65 ° C for 15 min. Then 800 µl of chloroform was added and the mixture was vortexed. After centrifugation at 10,000 xg for 5 min, the supernatant containing the fungal DNA was transferred to another 2 ml microcentrifuge tube to which are successively added 150 µl of CXD buffer and 300 µl of absolute ethanol (100%). For the next step, DNA is eluted with 100 µl H2O using Hi-bond®spin columns.

• **DNA assay**

The assay and quality control of the extracted DNAs were performed before each use by spectrophotometry using a nanodrop (Thermo Scientific, USA). The OD ratios (260nm / 280nm and 260nm / 230nm) are determined in order to assess the quality of the samples. The quality of the extracted DNAs is also checked by electrophoresis on 2% agarose gel (30 minutes migration at 75-80 volts). The DNAs are then stored at -20 ° C before analysis.

• **PCR ITS-RFLP**

The ITS region (Internal Transcribed Spacers) widely described by various studies [13,14,15,16] was chosen as the target region for a identification of fungal species. More precisely, the sequence ITS1-5.8S-ITS2 exhibits a region conserved in the majority of fungal species and variable regions which allow its use for population studies. The primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3'), ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5 ′GGAAGTAAAAGTCGTAACAAGG-3') described as universal primers by White et al. (1990) were used for this study. The PCR conditions for ITS1 and ITS4 used as well as the preparation of the reaction mixture are given below (Table 1).

**Table 1. Composition of the reaction mixture (final volume 50 µl)**

| | C. initial stock solution | V. sampled (µl) | C. final in the reaction mixture |
|---|---|---|---|
| Water MilliQ | | 28,75 | |
| 10X DreamTaq Green Buffer avec 20 mM $MgCl_2$ inclued | | 5 | |
| dNTP 10mM | 10 mM | 1 | 0,2 mM |
| ITS1 | 10 µM | 2,5 | 0,5 µM |
| ITS4 | 10 µM | 2,5 | 0,5 µM |
| ITS5 | 10 µM | 2,5 | 0,5 µM |
| DreamTaq DNApolymerase (Promega) | 5 U x $\mu l^{-1}$ | 0,25 | 0,025 U x $\mu l^{-1}$ |
| ADN | 100-150 ng x $\mu l^{-1}$ | 10 | ng x $\mu l^{-1}$ |

Amplification was performed in the thermal cycler (MultiGene, USA) according to the program described by [17]:

*First step* : *Initial denaturation at* 94°*C for* 3 *minutes*
*Second step* : *Denaturation at* 94°*C for* 1.5 *minutes*;
– *Hybridization at* 55°*C for* 1.5 *minutes*;
– *Elongation at* 72°*C for* 2 *minutes*;
} × 34
*Third step* : *Final elongation at* 72°*C for* 15 *minutes*
*Final step* : 1*X* ∞ *at* 4°*C*

The PCR products are visualized by electrophoresis (migration for 45 minutes at 90 volts) in 2% agarose gel + 5 µl of ethidium bromide at 10 mg.mL-1 (BET, Promega) in a buffer of 1X TBE electrophoresis (Tris-Borate-EDTA).

• **Enzymatic digestion**

In order to identify the different species of molds and to be as discriminating as possible, ITS1-5.8SrDNA-ITS2 sequences of molds from the EMBL database (http://srs.ebi.ac.uk) were used in a theoretical restriction program (http://biotools.umassmed.edu/tacg4/). Six restriction enzymes *Hae*III, *Hinf*I, *Hha*I, *Nla*III, *Rsa*I and *Sdu*I were thus retained, as being the most discriminative for our study.

Site of cleavage of the enzymes selected:

**-*Hae*III:**
5'…GG^CC…3'
3'...CC^GG....5'

**-*Hinf*I:**
3'…CTNA^G...5'
5'...G^ANTC...3'

**-*Sdu*I:**
5'...GdGCh^C...3'
3'...C^hCGdG...5'

**-*Hha*I:**
5'...GCG^C...3'
3'...C^GCG...5'

**-*Nla*III:**
5'...CATG^...3'
3'...^GTAC...5'

**-*Rsa*I:**
5'...GT^AC...3'
3'...CA^TG...5'

The reaction mixture was carried out as follows:
10 µL PCR product
MilliQ sterile water 7 µL
Buffer 2 µL
Enzyme 10 U. µL-1 1 µL
The final volume of the reaction mixture is 20 µL.
The reaction mixture is placed in a water bath for 4 h at an optimum temperature of 37 ° C.

• **Obtaining ITS-RFLP PCR Profiles**

After restriction, the restriction profiles are obtained by electrophoresis (migration for 60 minutes at 90 volts) in 2% (m / v) agarose gel (5 µl of BET at 10 mg.mL-1 and TAE 1X). The size of the DNA fragments is estimated by comparison with a size marker (GeneRuler 100bp DNA Ladder, Fermentas, France) and the profiles were analyzed using the Quantity One 4.6.5 BioRad program.

• **Sequencing**

In order to finalize the identification, a sequencing of the DNA fragments obtained was carried out. The amplified DNA fragments are extracted from the agarose gel and purified using the Qiagen "QIAquick Gel Extration Kit" (according to the manufacturer's recommendations). The fragments thus extracted and purified were then cloned using the TOPO TA Cloning kit (Invitrogen). The sequencing of the fragments of interest is performed by the platform Beckman Coulter Genomics, UK. The sequences obtained were then analyzed by comparison with sequences existing in the databases (GenBank) using the Blast program of the NCBI server (http://blast.ncbi.nlm.nih.gov). The alignments of the sequences were carried out with the software http://www.ebi.ac.uk/Tools/clustalw2/index.html. Only sequences with an identity percentage greater than 98% were retained

### 2.2.2. Analysis of Biocontrol Activities

**- Determination of the *in vitro* antifungal activity of bacterial biopesticides**

The test was performed on PDA agar to verify the existence of a possible inhibitory action of bacterial biopesticides against isolated fungi. Using a sterile

platinum loop, the biopesticide is inoculated as a straight streak that divides the Petri dish into two equal parts. Two disks, each having the diameter of the tip of a Pasteur pipette, are obtained by punching an isolated mushroom culture. The discs were placed on either side of the cutmark 3 cm from the cutmark. The control dishes were not inoculated with the biopesticide. When the fungus in the control dish has covered the culture medium, the percentage of growth of the fungus in the other dishes is determined using the method of [18], then the inhibition rate is deduced according to the formula:

$$Inhibition\ Rate\ (\%) = \left(\frac{R-r}{R}\right) \times 100$$

R is the radial growth of the microorganism without antagonist confrontation

r is the radial growth of the microorganism with antagonist

**- Determination of the *in vivo* antifungal activity of Bacterial biopesticides**

The efficacy of the biopesticide strains against the isolated fungi was evaluated on the wounds of healthy pineapples as described by [19], with some modifications. The fruits were disinfected and injured with a scapel (three wounds per pineapple). Dilution of biopesticides and fungi was performed in a buffered peptone water solution. Thus, suspensions of 50 µL of the biopesticides were applied to the wounds at concentrations of $10^5$, $10^6$ and $10^7$ CFU / ml. After 24 hours of application, a 50 µl suspension of the fungi was inoculated into the wounds. The pineapples were incubated, in closed plastic boxes containing moistened filter paper, at 25°C in the dark for five days. Lesion diameters occurring on the fruits thus preserved were observed.

### 2.2.3. Statistical Analyzes

Statistical analyzes were performed with the STATISTICA version 7.1 software (StatSoft). An ANOVA (analysis of variance) study associated with the Newman-Keuls test made it possible to analyze the differences observed in the antagonist studies. Probability values $p < 0.05$ were considered to be statistically different.

## 3. Results and Discussion

### 3.1. Results

#### 3.1.1. Pineapple Fruit Fungal Profile

- **Phenotypic characteristics of fungal genera**

Table 2 and Figure 1 show the macroscopic and microscopic characteristics of the fungal genera isolated from pineapple fruits sold in the markets. On the basis of the identification keys taking into account the macroscopic and microscopic characters, 5 fungal genera were isolated. These are *Aspergillus*, *Rhizopus*, *Geotrichum*, *Neurospora* and *Candida*.

#### 3.1.2. Molecular Characteristics of Fungal Genera

The molecular characterization of fungal strains isolated from pineapple fruits has made it possible, through enzymatic digestion, to establish the diversity of the strains involved in the spoilage of pineapple fruits. The electrophoretic profile of the restriction fragments of ribosomal DNA for the *Aspergillus* strains after digestion with the endonucleases *Rsa*I and *Hha*I was established (Figure 2). For strains 1-3 and 5-7, when digestion is carried out with RsaI, two fragments are obtained (79 + 497) on the other hand with HhaI, four fragments are generated (75 + 138 + 178 + 185). Regarding strains 4 and 8, *Rsa*I gives two fragments (77 + 522) and *Hha*I four fragments (89 +125 +178+ 207). The size of the fragments is expressed in base pairs (bp). The different composite profiles obtained from the isolated *Aspergillus* have shown that they are two different species.

**Table 2. Macroscopic and microscopic characteristics of mold genera isolated from pineapple fruit samples**

| | | | | | | |
|---|---|---|---|---|---|---|
| **Macroscopic characteristics** | **Appearance of colonies** | Cottony, Filamentous | Powdery | Fluffy | Cottony | creamy |
| | **colonies Forms** | Intrusive, rapid development | No intrusive | No intrusive | Intrusive | No intrusive |
| | **Colonies color** | Beige turning greyish black with age | Black, brunette | White | Pinkish white | White |
| | **Reverse** | Does not diffuse into agar | Diffuse into agar | Does not diffuse into agar | Diffuse into agar | Does not diffuse into agar |
| | **Reverse color** | Black | Colorless to pale yellow | Colorless | Slightly cream | Colorless |
| **Microscopic characteristics** | **Hypha** | Not partitioned | Partitioned | Partitioned | Partitioned | Partitioned |
| | **Conidial head** | Rounded columella | Spherical or in a club | Absent | Absent | Absent |
| | **Spores and conidia forms** | Small, round | Rounded | Rectangular | Fusiform, curved | Rounded |
| | **Conidiophore** | Unbranched | Unbranched | Branched | Branched | Branched |
| **Type of molds** | | ***Rhizopus*** | ***Aspergillus*** | ***Geotrichum*** | ***Neurospora*** | ***Candida*** |

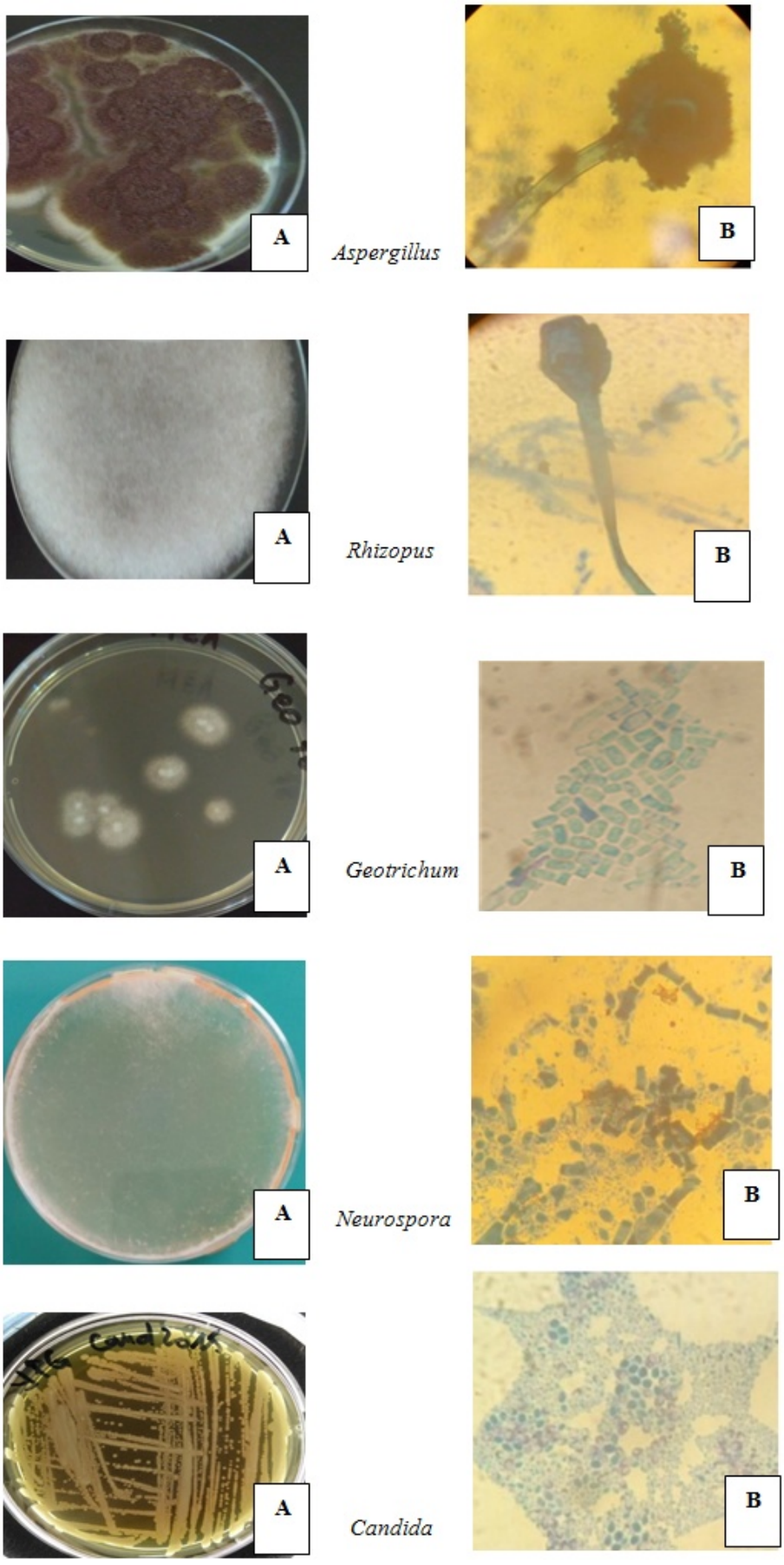


**Figure 1.** Macroscopic (A) and microscopic (B) aspects of the mold genera isolated from pineapple fruit samples

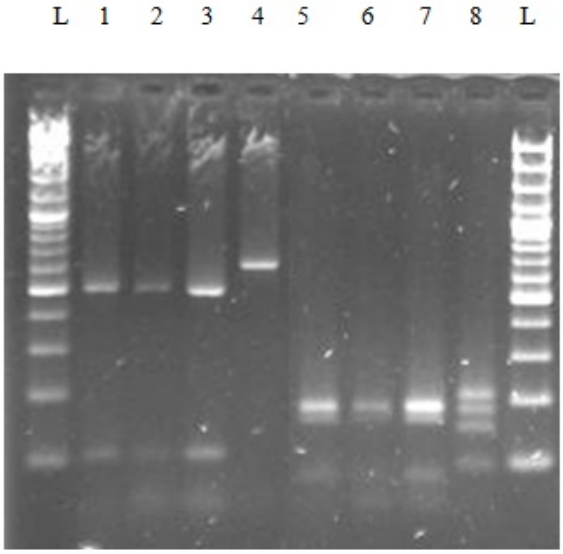


**Figure 2.** Electrophoretic profile of ribosomal DNA restriction fragments for Aspergillus strains after digestion with *RsaI* and *Hha*I endonucleases. (1-4: *Aspergillus* digested with *Rsa*I; 5-8*: Aspergillus* digested with *Hha*I; L = 100 bp)

The electrophoretic profile of the restriction fragments of ribosomal DNA for the strains of *Neurospora*, *Rhizopus* and *Geotrichum* after digestion with the endonucleases *Sdu*I, *Hinf*I and *Hae*III is shown in Figure 3. Restriction with SduI from *Neurospora* generated two fragments (139 + 448), in contrast *Hinf*I generated four fragments (8 + 62 + 246 + 271) and *Hae*III two (106 + 481). Enzymatic digestion at the level of the *Rhizopus* genus showed three fragments (176 + 181 + 272) for *Sdu*I, four (8 + 111 + 200 + 310) for *Hinf*I and one fragment (629) for *Hae*III. The *Geotrichum* restriction profile is composed of one fragment (372) for *Sdu*I, three fragments (86 + 105 +181) for *Hinf*I and one fragment (372) for *HaeI*II. The different restriction profiles obtained indicate the presence of three different species.

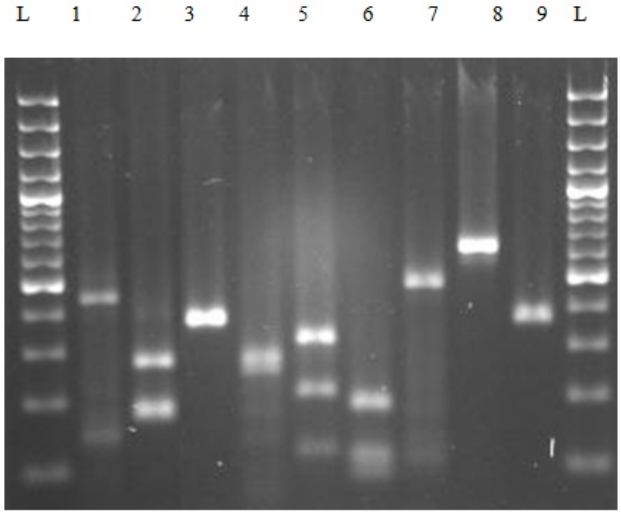


**Figure 3.** Electrophoretic profile of restriction fragments of ribosomal DNA for other mold strains after digestion with *Sdu*I *Hinf*I and *HaeI*II endonucleases (1–3: *Neurospora* digested with *Sdu*I .; 4–6: *Rhizopus* digested with *Hinf*I; 7-9: *Geotrichum* digested with *Hae*III; L = 100 bp)

The electrophoretic profile of the restriction fragments of ribosomal DNA for the strains of *Candida* after digestion with the endonucleases *Hha*I, *Hinf*I and *Hae*III, shows three strains of *Candida* of which two are identical (2-3, 5-6, 8-9) (Figure 4). Strains 1, 4 and 7 present two fragments (290 + 300) derived from *Hha*I, two fragments (300 + 290) generated by *Hinf*I and three fragments (400 + 100 + 90) for *Hae*III. The strains (2-3, 5-6, 8-9) present two fragments (210 + 200) for *Hha*I, two fragments (200 + 160) for *Hinf*I and two fragments (400 + 100) for *HaeI*II. The different restriction fragments obtained distinguish two species of the genus *Candida*.

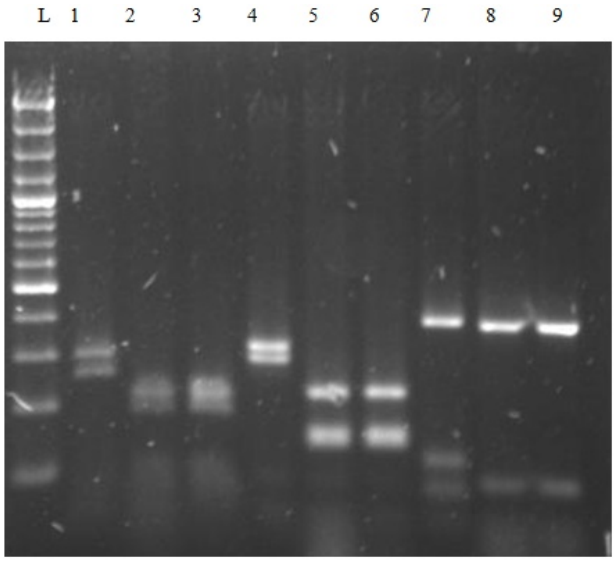


**Figure 4.** Electrophoretic profile of ribosomal DNA restriction fragments for *Candida* strains after digestion with endonucleases *Hha*I, *Hinf*I and *Hae*III (1–3: *Candida* digested with *Hha*I; 4–6: *Candida* digested with *Hinf*I; 7-9: *Candida* digested with *Hae*III; L = 100 bp)

### 3.1.3. Fungal Species Isolated from Pineapple Fruit

The different restriction profiles obtained did not make it possible to identify the different fungal strains at the species level. One representative from each group was assigned to sequencing. The sequences obtained (Figure 5) were then analyzed by comparison with existing sequences in the databases (GenBank) using the Blast program of the NCBI server. Only sequences with a percentage identity or homology greater than 98% were retained (Table 3).

**Table 3. Fungal strains identified on the pineapple fruits studied**

| Group | Nc | %H | Frequency (%) | Species |
|---|---|---|---|---|
| I | 536 | 100 | 34,31 | *Aspergillus aculeatus* |
| II | 570 | 100 | 14,71 | *Aspergillus niger* |
| III | 530 | 100 | 12,75 | *Candida carpophila* |
| IV | 420 | - | 6,86 | *Candida* sp. |
| V | 577 | 100 | 11,77 | *Rhizopus oryzae* |
| VII | 551 | 100 | 8 ,82 | *Neurospora tetrasperma*. |
| VIII | 540 | 100 | 10,78 | *Geotrichum candidum* |

Nc : Number of nucleotides compared. %H : percentage of sequence homology.

## 3.2. *In vitro* Activities

The antifungal activities observed by comparison of the main isolated fungal strains and bacterial biopesticides, namely *Bacillus subtilis* GA1, *Pseudomonas fluorescens* F19 and *Pseudomonas fluorescens* CI are shown in Table 4. These figures show that in the presence of biopesticides, the All of the fungal strains could not

achieve total growth in the Petri dish containing the culture medium. There are spaces of inhibition or demarcation between the fungal growth and the biopesticides used. These results therefore indicate the capacity of these bacterial biopesticides to inhibit the growth of the main fungal strains.

*Bacillus subtilis* GA1 showed inhibition rates in the range of 57.14% to 81.82%. *Pseudomonas fluorescens* F19 gave levels ranging from 53.33% to 90%. *Pseudomonas fluorescens* CI showed inhibition rates ranging from 50% to 86.67% (Table 4).

**Table 4. Rate of inhibition of bacterial biopesticides against the main fungal strains isolated**

| Molds | Inhibition rate of B. *subtilis* GA1(%) | Inhibition rate of P. *fluorescens* F19 (%) | Inhibition rate of P. CI (%) |
|---|---|---|---|
| ***A. aculeatus*** | 81,42±0,21[a] | 60,33±0,41[a] | 63,17± 0,24[a] |
| ***Rhizopus oryzae*** | 64,00±0,18[b] | 53,33±0,43[b] | 50,00±0,48[b] |
| ***Neurospora tertasperma*** | 57,14±0,45[c] | 60,16±0,45[a] | 62,34±0,35[a] |
| ***G. candidum*** | 66,67±0,34[b] | 66,67±0,24[c] | 66,67±0,37[c] |
| ***C. carpophila*** | 60,00±0,20[b] | 90,00±0,34[d] | 86,67±0,38[d] |

Values bearing the same letter in the same column do not show a significant difference at the 5% level according to the Newman-Keuls test.

## 3.3. *In vivo* Activities

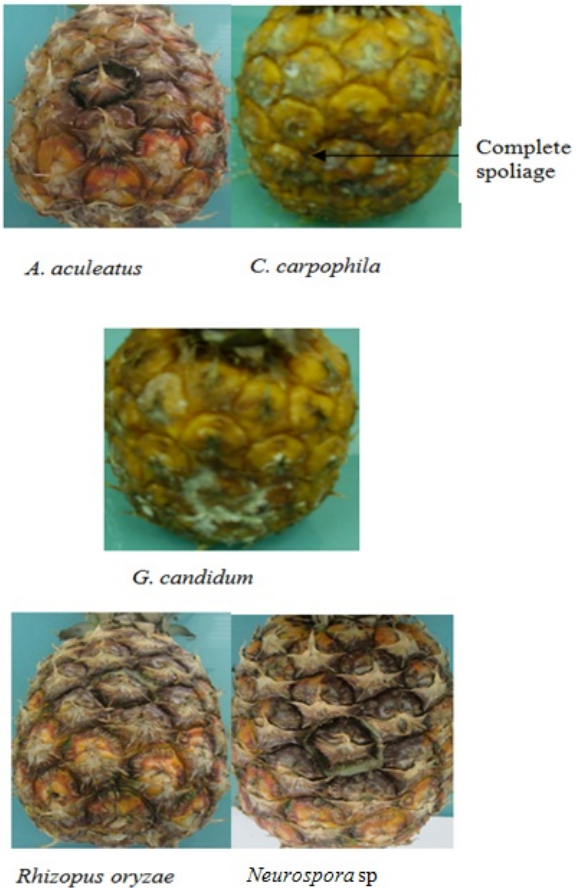


**Figure 5**. Aspects of pineapple fruit without biopesticides (control)

The biopesticides as a whole showed a considerable reduction in spoilage of fruit which resulted in a reduction in spoilage diameter (Figure 5, Figure 6, Figure 7 and Figure 8). The fruits not inoculated with the biopesticides all showed deterioration (Figure 5). Also *Bacillus subtilis* GA1 and *Pseudomonas fluorescens* CI showed much greater efficacy unlike *Pseudomonas fluorescens* F19.

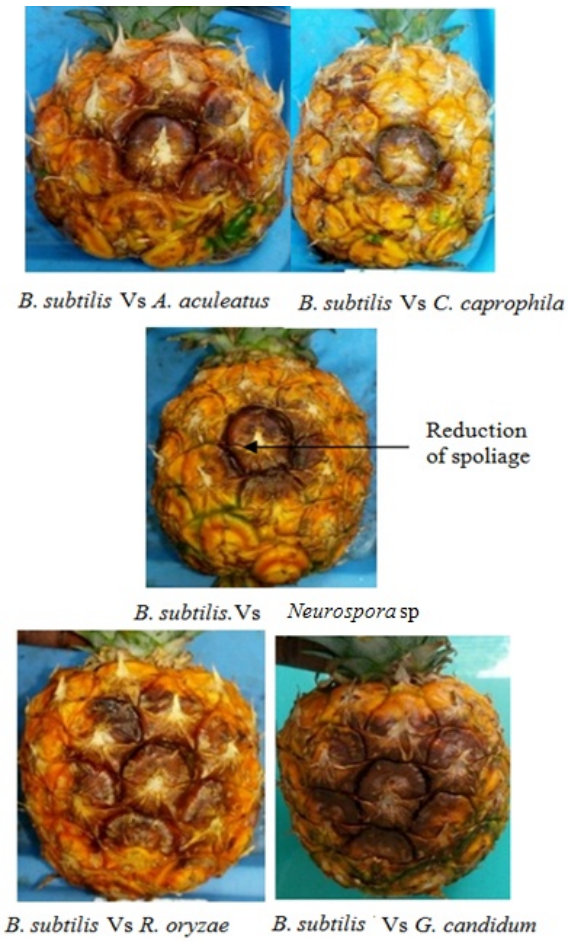


**Figure 6**. *In vivo* antifungal activities of *B. subtilis* GA1

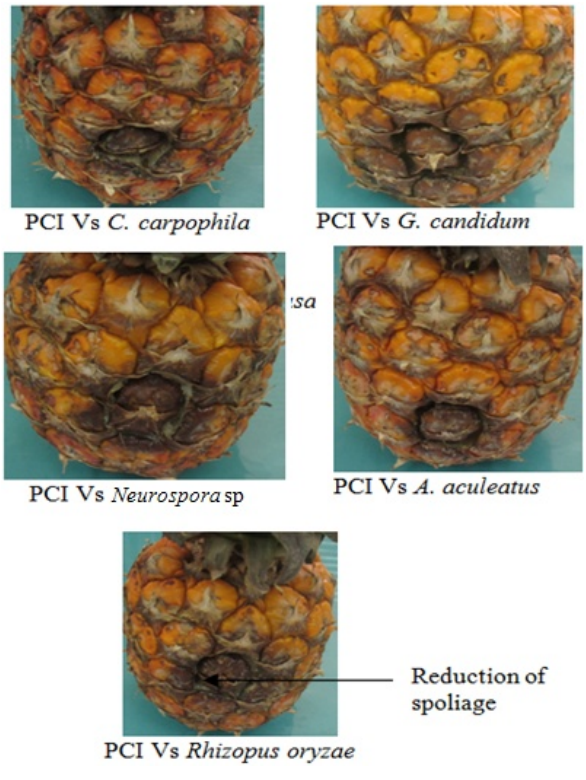


**Figure 7**. *In vivo* antifungal activities of *Pseudomonas fluorescens* CI

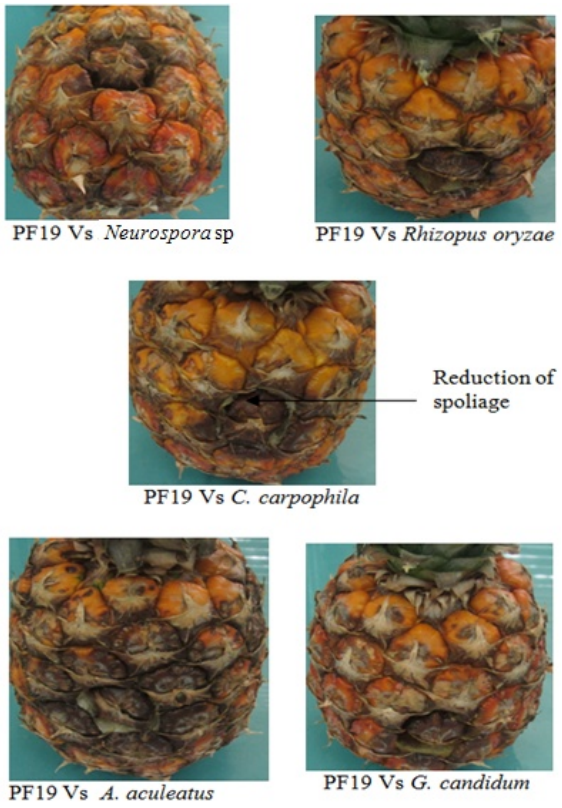


**Figure 8**. *In vivo* antifungal activities of *Pseudomons fluorescens* F19

## 4. Discussion

The phenotypic identification was carried out according to the classic criteria of macro and microscopic observation. This identification, although known not to be 100% reliable in terms of identification, made it possible to carry out a screening of strains at the level of the genus. The PCR-ITS-RFLP technique developed in this work made it possible to carry out an exhaustive study of the fungal strains present on fruits at the species level. The combination of two endonucleases or three endonucleases followed by sequencing allowed the complete identification of these fungal strains at the species level. This result is similar to that of [20] who reported that the PCR-ITS-RFLP method using a combination of four restriction enzymes was necessary to discriminate between fungal genera at the species level. This result agrees with those of [21] who indicated that three endonucleases were sufficient to differentiate *A. niger*, *A. tubingensis*, *A. carbonarius* and *A. aculeatus*. In addition, [22] also reported that three endonucleases were necessary to differentiate yeasts.

The main fungal strains isolated and phenotypically identified were confirmed by molecular identifications. This result confirms the work of [23] According to this author, the basis for the identification of fungal strains is based on macroscopic and microscopic observations. Indeed, the identification of the genus gives an orientation on the choice of endonucleases for the PCR-ITS-RFLP technique.

Among the strains isolated, *Aspergillus* species were dominant. These results corroborate those of [24] and [25] who reported that *Aspergillus* species are commonly associated with tropical and warm regions. The development of these fungi in the samples analyzed is believed to be due to the hot and humid environmental conditions of the Côte d'Ivoire. Indeed, according to [26] and [27], the hot and humid environmental conditions of tropical regions of Africa are ideal for the growth of molds contaminating foodstuffs. Contamination of pineapple fruit is also believed to be due to storage conditions. According to [23], fungal contamination of foodstuffs can take place either during the manufacturing process, or after processing or even during storage. [28,29] and [11] demonstrated the predominance of species of the genus *Aspergillus* in the contamination of pineapple fruits in Thailand and Nigeria, in particular *A. aculeatus* and *A. niger*. The presence of *Aspergillus* involves a risk of mycotoxin production which poses a risk to the health of consumers [30].

Two fungal species of the genus *Aspergillus*, notably A. niger, and A. aculeatus were identified in this work. This result thus demonstrates that pineapple fruits are vulnerable to fungal contamination, in particular that of *Aspergillus*. These results confirm those of the work of [29] and [11] who reported that these species are the major contaminants of pineapple fruits. This result could be explained by the fact that the pineapple fruit provides a favorable environment for fungal growth given its biochemical composition. This is because these products contain proteins, oils, carbohydrates and minerals which provide a nutrient-rich medium for fungal growth. The presence of Aspergillus in these samples is believed to be due to the fact that this species is generally present in most foods produced in tropical countries, with a special affinity for pineapple fruits [31].

For [32], *Aspergillus* is found to be widespread in residential, urban and air environments. Contamination of pineapple fruit samples is believed to be due to their exposure to air during sale. Isolated fungal species in particular can pose health problems. Indeed, these species are recognized for their ability to produce mycotoxins such as ochratoxin A, a nephrotoxic, carcinogenic and immunosuppressive substance, both in humans and in animals [33,34]. The genus *Neurospora* in pineapple fruit is also a danger to consumers. These species are frequently involved in human infections [35,36]. Thus, several strains of *Neurospora* are active producers of toxins when all the favorable growth conditions are met. According to [37], these metabolites can cause mycotoxicosis following repeated ingestion of food contaminated with these fungi in animals and humans. Toxic effects attributable to the ingestion of *Neurospora* include cytotoxic, nephrotoxic and tremorgene effects (neurological effects of prolonged tremors), as well as immunosuppressive and carcinogenic effects. Beyond mycotoxicosis, the fungi of the *Fusarium* genus are causative agents of keratitis, skin infections, infections in severe burns, sinusitis and lung diseases [38].

The presence of the genus *Rhizopus* in this study confirms the work of [39]. Indeed, the latter have found that the fungi of the genus *Rhizopus* are the second cause of fungal contamination of seeds and fruits sold in the markets of Kenya. Exposure to the genus *Rhizopus* is believed to be dangerous for many people. According to [40], the genus *Rhizopus* is a formidable opportunistic

mold for immunocompromised and diabetic people. It is the cause of serious, invasive and often fatal injuries. The presence of the genus *Candida* on pineapple fruits testifies to human contamination linked to poor hygiene conditions during their handling. Indeed, according to [41], the genus *Candida* generally has mucous membranes, skin and integuments as its habitat. It could infect pineapples during harvest or during handling by traders and customers.

The presence of *Geotrichum* on pineapple fruits would be an accidental presence. Indeed, *Geotrichum* is implicated in the spoilage of citrus fruits. Its isolation is probably the result of cross contamination. Investigation results indicated that some traders store pineapple fruit with other fruits such as citrus. According to [42] the genus *Geotrichum*, is a microorganism commonly associated with the spoilage of several fruits and vegetables such as tomatoes, cucumbers, carrots and many other fruits and vegetables

The results of the pathogenicity tests carried out on pineapples show that all isolated fungi were responsible for spoiling the pineapple fruit. Tests have also shown the link between fruit spoilage and the presence opening in pineapple. This is because the fungi cause the fruits to spoil when they enter pineapple through mechanical injuries, such as bruises and sores. They can also enter fruit through lesions caused by pests of all kinds. This damage is to be feared for the fruits to be stored [43]. The alterations could be explained by the ability of these molds to metabolize the sugars and nutrients contained in pineapple and also, to grow under conditions of very low pH with high water content of pineapple.

The very advanced rot induced by all fungi could be due to a mechanism specific to pathogens. Indeed, pathogens have learned to mask their presence by interfering with the fruit's defence pathways. This deactivation occurs through the secretion by pathogens of effectors called avirulence proteins. They are directly injected into the host cell [44]. Pathogens, like *A. aculeatus*, are also capable of producing pectinolytic enzymes, to break down the protective lining of pineapples. Their speed and the damage caused to pineapples could confirm the short shelf life of the fruits.

The use of bacterial biopesticides in this study demonstrated the ability of these agents to inhibit the main fungal strains responsible for spoilage in pineapple fruit. These results are similar to those obtained by [45] who showed that the inhibition rates of *Bacillus subtilis* GA1 against spoilage germs were between 59.37% and 84.78% in the conservation of mangoes in Côte d'Ivoire. Also in a similar study of the control of *Geotrichum* sp. on tomato, [46] showed that *Pseudomonas fluorescens* F19 had the ability to inhibit the growth of *Geotrichum* sp. at 77.5%. This reduction in the incidence of microorganisms in the spoilage of pineapple fruit could be explained on the one hand by the rapid and extensive colonization of wound sites by biological control agents. This presence would interfere with the establishment of the pathogenic fungus at injury sites by reducing space and available nutrients. [47] reported that application of biopesticides to injury sites prior to infection of the pathogen is necessary to ensure better colonization and maximum rate of inhibition. And on the other hand by the production of molecules of lipopeptide nature by *B. subtilis* GA1, in particular fengycin, surfactins and iturins which are manifested by the bursting of the cell wall of fungi [48]. These can either activate plant defences or have a direct antibacterial or antifungal effect [49]. Also *Pseudomonas fluorescens* F19 produces metabolites such as Phenazines and Pyrrolnitrine and also siderophores which are iron chelating agents preventing its use by pathogens [50]. *Pseudomonas fluorescens* would have acted either by mycoparasitism, which is a trophic relationship established by a microorganism to the detriment of a fungus [51] by the production of lytic enzymes such as glucanase and chitinase, or by induction of resistance or again by antibiosis.

## 5. Conclusion

This study has shown, through macroscopic and microscopic observations and the PCR-ITS-RFLP technique, a diversity of fungal contaminants in pineapple fruits sold in the markets. *Aspergillus* and *Candida* were the predominant fungal genera isolated in this work. It emerges from this chapter that a real risk of spoilage of pineapple fruits exists in Côte d'Ivoire given the diversity of fungal strains isolated and identified. The various microbial biopesticides isolated and acquired have shown their ability to inhibit the main spoilage germs in pineapple fruits. Also, the biocontrol agents *Bacillus subtilis* GA1, *Pseudomonas fluorescens* CI and *Pseudomonas fluorescens* F19 showed significant inhibition rates.

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