

ANTAGONISTIC ACTIVITIES OF HUMAN ORAL FLORA AGAINST FUNGAL PLANT PATHOGENS

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Abstract: This study was carried out to investigate the antagonistic activities of tongue isolates; *Micrococcus luteus*, *Streptococcus mutatis*, and *Corynebacterium* sp. and *Aspergillus* sp. against five test pathogenic fungi; *Colletotrichum lindemuthianum*, *Colletotrichum capsici*, *Colletotrichum pisi*, *Sclerotium rolsii*, and *Fusarium oxysporum*, monitored over a period of five days incubation. All isolates were subjected to screening against the plant pathogens and most of the isolates showed antagonistic activity. *Streptococcus mutatis* (13.8 ± 0.1 ; $P=0.05$) showed the highest antagonistic activity after 24h; and least, *Micrococcus luteus* (0.1 ± 0.0) against *Colletotrichum capsici*. The experimental results demonstrated the fungicidal effect of tongue microflora and revealed the possibility of the metabolites produced by these microorganisms to be used as potential biocontrol agents against these fungal species. This research paper further discussed the use of oral microflora as possible source(s) of biocontrol agents in control of plant disease, especially those caused by the selected test fungal pathogens. It also highlights factors that contribute to the limited use of biocontrol agents (BCAs).

Keywords: Antagonism, pathogenic, fungi, tongue isolates, biocontrol

INTRODUCTION

The antimicrobial properties of microbes have led to the characterization of various antimicrobial substances as organics acids (lactic acid and formic acids), diacetyl, and hydrogen peroxide alone or in combination. Other antimicrobial substances with antagonistic properties include biocides, sterilants and probiotics, the production of specific protein or protein complexes; bacteriocin that inhibits some Gram positive bacteria, mainly homologous species and some sphaeroplast of Gram negative bacteria (Marsh, 2009). Antagonistic relationship is an interaction between microorganisms in which the growth of one inhibits the growth of another microorganism.

This is usually evident with microorganisms that are capable of producing antibiotics which inhibit the growth or the activity of another microorganism (Marsh, 2009).

The oral microflora (Marsh *et al.*, 1992; Percival *et al.*, 1991) is known to undergo dramatic change, both quantitatively and qualitatively, with age, especially in edentulous individuals (people who have partial or complete loss of teeth). In an edentulous oral cavity, bacteria tend to colonize mainly the tongue, oral mucous membranes and denture surfaces, and are transferred to other sites by the saliva. The oral cavity is comprised of many surfaces, each coated with a plethora of bacteria, the proverbial bacterial biofilms. Some of these bacteria have

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been implicated in oral diseases such as caries and periodontitis, which are among the most common bacterial infections in humans (Albandar *et al.*, 1999). In addition, specific oral bacterial species have been implicated in several systemic diseases, such as bacterial endocarditis (Becker *et al.*, 2002) aspiration pneumonia (Scannapieco, 1999), osteomyelitis in children (Dodman *et al.*, 2000), preterm low birth weight (Buduneli *et al.*, 2005) and cardiovascular disease (Wu *et al.*, 2000). Normal flora is a dynamic and complex mixture of microbes that have diverse functions including digestion of essential nutrients, maturation of intestinal physiology, stimulation of immune system, systemic effects on blood lipids and the inhibition of harmful bacteria. Far from having a passive relationship with the host, recent research has confirmed earlier (and largely forgotten) studies that demonstrated that the resident microflora of animals and humans play a positive role in the normal development of the host. These resident microflora also play active roles in the maintenance of the healthy state by contributing to the host defenses and preventing colonization by exogenous microorganisms (Marsh, 2000).

Antagonism of microorganisms which is also known as antibiosis is the suppression of some species of microorganisms by others (Marsh, 2009). The mechanism of antagonism is varied and often obscure. Antagonists more often than not act on their competitors with metabolic products (allelopathy), including antibiotics, or displace the competitors by means of more intensive reproduction or primary utilization of food. Repeated attempts

were made as early as the 19th century to treat diseases caused by bacteria. However, these attempts were unsuccessful because of the use of unpurified preparations. Microbial antagonists are extensively used in the production of antibiotics and greatly influence soil fertility. Antagonists can also be used in many branches of the food industry (Marsh, 2009). The microorganisms that produce antibiotics useful in preventing or treating disease include the bacteria *Bacillus* and *Streptomyces*; and the fungi *Penicillium*, *Cephalosporium*, and *Micromonospora*.

Plant diseases are caused mainly by fungi, bacteria, viruses and nematodes. Biocontrol of plant disease involves the use of an organism or organisms to reduce disease (Pal and McSpadden Gardner, 2006). The most common approach to biological control consists of selecting antagonistic microorganisms, studying their modes of action and developing a biological control product. The most common mechanisms for microbial antagonism of plant pathogens are parasitism, predation, competition, induced resistance and the production of antimicrobial substances. Often, several mechanisms act together. Besides the production of antibiotics, several other microbial by-products also contribute to pathogen suppression/control. Hydrogen cyanide (HCN) effectively blocks the cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations. The production of HCN by certain fluorescent pseudomonads is believed to be involved in the suppression of root pathogens. *P. fluorescens* CHA0 produces antibiotics, siderophores and HCN, but suppression

of black rot of tobacco caused by *Thielaviopsis basicola* appeared to be due primarily to HCN production. Volatile compounds such as ammonia produced by *Enterobacter cloacae* were involved in the suppression of *Pythium ultimum*-induced damping-off of cotton. While it is clear that biocontrol microbes can release many different compounds into their surrounding environment, the types and amounts produced in natural systems in the presence and absence of plant disease have not been well documented and this remains a frontier for discovery (Pal and McSpadden Gardner, 2006).

Plant diseases threaten food security. *Colletotrichum sp.* is one of the most important plant pathogens worldwide causing the economically important disease anthracnose (Pakdeevanaporn et al., 2005). Chilli (*Capsicum spp.*), an important economic crop worldwide, is severely affected by anthracnose which may cause yield losses of up to 50%. *Fusarium oxysporum* is a fungal disease of plants that has initial symptoms of vascular wilt including vein clearing and leaf epinasty, followed by stunting, yellowing of the lower leaves, progressive wilting, defoliation and, finally, death of the plant. They cause severe losses in many vegetables and flowers, field crops, such as cotton, and plantation crops, such as banana, date palm and oil palm. *Fusarium oxysporum* plays the role of a silent assassin. The pathogenic strains of this fungus can be dormant for 30 years before resuming virulence and infecting a plant. *Fusarium oxysporum* is infamous for causing a condition called *Fusarium wilt*, which is lethal to plants and swift (by the time a plant shows any outward sign of infection, it is already too late, and the plant dies (Mullen, 2007).

Southern blight, caused by the soil borne fungus *Sclerotium rolfsii* is a serious disease of a wide variety of plants, including field, vegetable, fruit, ornamental crops and also turf. *S. rolfsii* usually infects the lower stem near the soil surface. In some plants, the roots may become infected. The disease occurs worldwide, but predominantly in warm climates. In recent years, southern blight has been especially damaging on cotton in Arizona, peanut and tomato in the Southeast, and sugar beet in California. Despite considerable research on this disease, control of southern blight continues to be a problem. **More than 500 hosts plant species, many woody ornamentals, herbaceous annuals and perennials are continually affected (Mullen, 2007).**

The search for microorganisms that could serve as source(s) of biocontrol agents in the control of plant diseases cannot be overemphasized. Studies have shown that some oral microflora exert their health effect within the mouth by producing substances which suppress the growth of other oral flora. Hence, this study aimed at the isolation of microorganisms from the human tongue, and study of possible antagonistic effect of isolated oral flora on some fungal plant pathogens. Research into the search for antifungal agents with possible novel application in battling plant diseases cannot be overemphasized.

MATERIALS AND METHODS

Collection of samples

Sterile swab stick was used to collect sample from the tongue by using the swab stick to rub the tongue before eating and after eating, under aseptic conditions. Two samples were collected

twice a day for two weeks. Apparently healthy subject gave consent before sample collection.

Media Preparation and Sample Analysis

Media were prepared according to manufacturer's specification (2.8g of Nutrient agar into 100ml of water, 4.8g of MacConkey media into 100ml of water, 5g of Malt extract agar into 100ml of water, 3.6g of Potato Dextrose agar into 100ml of water and 3.8g of Mueller Hinton into 100ml of water). Streak plate method was used for the isolation of the microorganisms. In the streak method, labelled swab stick samples were properly streaked respectively prepared solidified agar plates, incubated for 24h at 37°C (Nutrient Agar for bacteria) and 48-72h at 25°C (Potato Dextrose Agar for fungi) (Cheesbrough, 2006; Fawole and Oso, 2001).

Purification and identification of bacterial and fungal isolates

Distinct colonies of bacteria were purified by repeated subculture on the respective isolation media [Nutrient agar - NA (Oxoid)], and preserved on slants at 4° C according to Olutiola *et al.* (1991). Morphological and biochemical tests to identify isolates were carried out using the methods of Fawole and Oso (2001) and Bergey's Manual of Systematic Bacteriology (Claus and Berkley, 1986). Biochemical tests carried out in the conventional method include fermentation of carbohydrate, catalase, motility, and coagulase tests. Fungal isolates were subcultured using the same isolation media [Potato Dextrose agar - PDA (LabM)] and their identification made possible using

macroscopic and microscopic (stereomicroscope) fungal features. Fungi were identified using the cotton-blue in lactophenol method (Olutiola *et al.*, 2001).

The tongue isolates used in this investigation were maintained by routine culture on agar slants, stored at 4°C between transfers. Additional subcultures (24 hrs, 25°C) were made in fresh medium before use in the experiment.

Collection of test pathogenic fungi

Pure cultures of fungi namely; *Colletotrichum pisi*, *Colletotrichum capsici*, *Collectotrichum lindemuthianum*, *Sclerotium rolfsii* and *Fusarium oxysporum* (plant pathogens) were obtained at the Department of Crop Soil and Pest Management, Federal University of Technology, Akure, Ondo State, Nigeria. Identities of the phytopathogenic fungi were authenticated at the Federal University of Technology, Akure (FUTA) Microbiology laboratory, by assessment of macroscopic and microscopic fungal features.

Detection of Antagonistic Activity

In order to detect antagonistic activity of bacterial isolates towards the growth of the fungal cultures, Fokkema (1973) method was employed. Using the conventional streak method, a 40mm streak was made from 24hr-old culture of the tongue isolates, 23mm away from the centre of a Petridish containing solidified Malt extract agar. Using a 7mm diameter sterile cork borer, the growing edge of a 4-day old test fungal culture was aseptically cut and placed at the centre of the plate already inoculated with the test antagonist.

Plates were incubated at 25°C and monitored for 5 days. Observations were made every 24hrs for 5 days on the inhibition of mycelial growth of the fungal pathogen by the antagonist under test. Control plates were not streaked with the antagonist. Only the fungal pathogen was placed at the centre of the Petridish. Percentage inhibition was calculated using the formula:

$$I = (r1 - r2 / r1) * 100\%$$

where I = percentage of inhibition, r1 = radius of the pathogen away the test antagonist, r2 = radius of the pathogen towards from the test antagonist

RESULTS

A total of four (4) isolates; *Micrococcus luteus*, *Streptococcus mutans*,

The antagonistic result of *Corynebacterium* sp. as shown in Figure III showed the highest percentage of inhibition against *Colletotrichum capsici* at day 1, but reduced at day 5; whereas, there was no antagonism between the same bacterium against *Fusarium oxysporum* at day 2 and *Sclerotium rolfsii* at day 2 and 3. *Streptococcus mutans* showed the highest percentage inhibition against *Colletotrichum lindemuthianum* at day 3, and stable antagonism between this microorganism against *Fusarium oxysporum* from days 3 - 5 as presented in Figure IV.

Corynebacterium sp. and *Aspergillus* sp. were subjected to inhibitory/antagonistic study. *Micrococcus luteus* against the pathogenic fungal species (Figure I), two negative percentage zones of inhibition were observed after the first day with *Sclerotium rolfsii*, and after the first and third day with *Colletotrichum capsici*. A neutral interaction was observed with the bacterium against *Colletotrichum pisi* and *Fusarium oxysporum* at day 1. In both cases of neutral and negative, there was a positive result at day 5 (Plate 1). The opportunistic fungus, *Aspergillus* sp. showed a high percentage inhibition (Figure II) against *Colletotrichum lindemuthianum* and *Colletotrichum capsici*, inhibition was however slightly reduced at day 5.

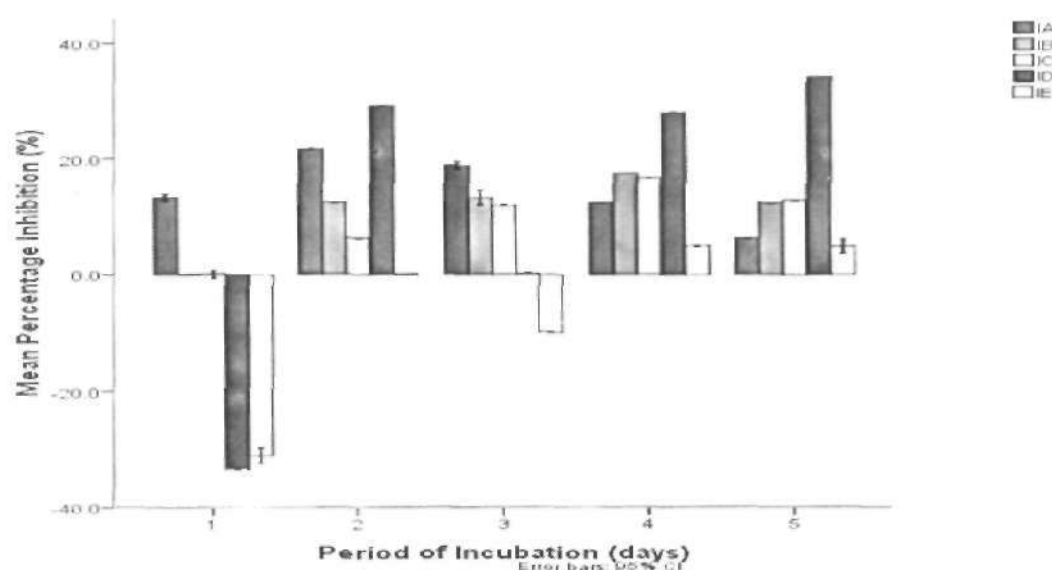


Fig. I: Antagonistic effect of *Micrococcus luteus* against pathogenic fungi
Key: IA= Percentage inhibition of *Colletotrichum lindemuthianum*, IB= Percentage inhibition of *Colletotrichum pisi*, IC= Percentage inhibition of *Fusarium oxysporum*, ID= Percentage inhibition of *Sclerotium rolfsii*, IE= Percentage inhibition of *Colletotrichum capsici*

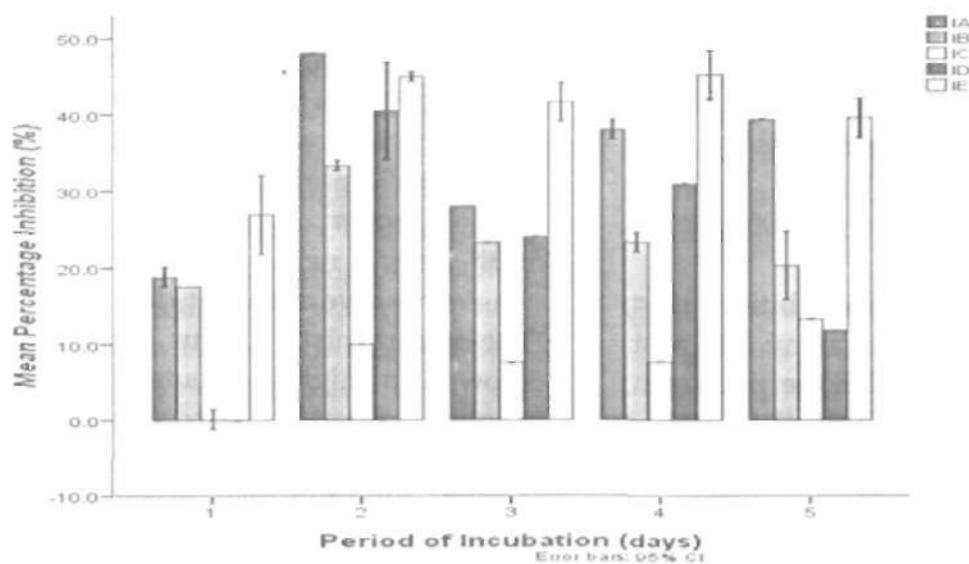


Fig. II: Antagonistic effect of *Aspergillus* sp. against pathogenic fungi
Key: IA= Percentage inhibition of *Colletotrichum lindemuthianum*, IB= Percentage inhibition of *Colletotrichum pisi*, IC= Percentage inhibition of *Fusarium oxysporum*, ID= Percentage inhibition of *Sclerotium rolfsii*, IE= Percentage inhibition of *Colletotrichum capsici*

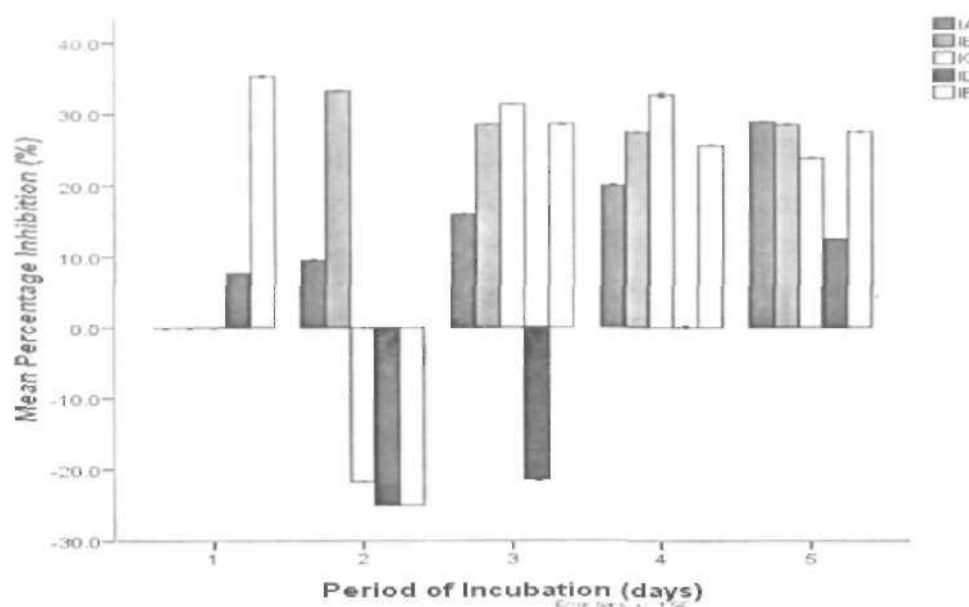


Fig. III: Antagonistic effect of *Corynebacterium* sp. against pathogenic fungi
Key: IA= Percentage inhibition of *Colletotrichum lindemuthianum*, IB= Percentage inhibition of *Colletotrichum pisi*, IC= Percentage inhibition of *Fusarium oxysporum*, ID= Percentage inhibition of *Sclerotium rolfsii*, IE= Percentage inhibition of *Colletotrichum capsici*

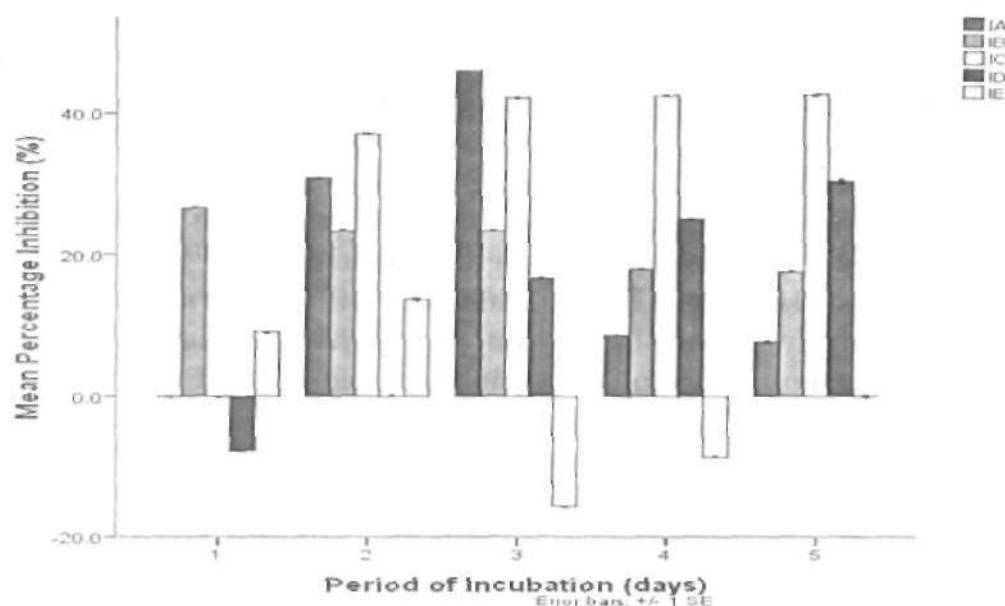


Fig. IV: Antagonistic effect of *Streptococcus mutans* against pathogenic fungi

Key: IA= Percentage inhibition of *Colletotrichum lindemuthianum*, IB= Percentage inhibition of *Colletotrichum pisi*, IC= Percentage inhibition of *Fusarium oxysporum*, ID= Percentage inhibition of *Sclerotium rolfsii*, IE= Percentage inhibition of *Colletotrichum capsici*

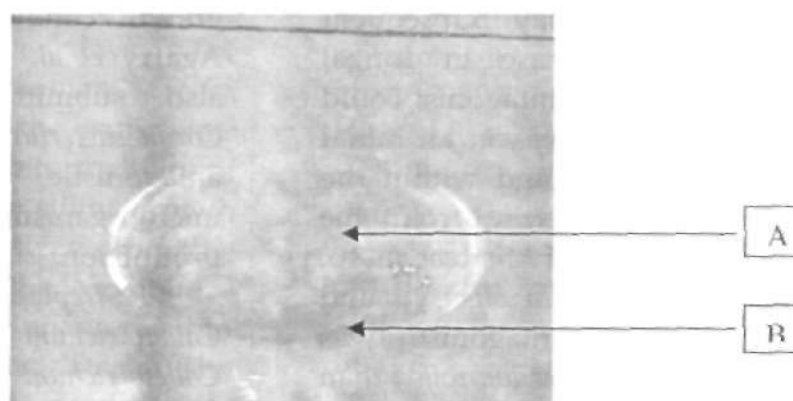


Plate 1: Antagonism of *Streptococcus mutans* against *Fusarium oxysporum* (Day 5)

Key: A= Pathogenic fungal plug, B= Isolated bacterial streak

DISCUSSION

The production of substances that inhibit other microorganisms in the microbial environment of the oral cavity could serve as aggressive by-product that may eliminate competitors and pathogens. Bacterial antagonism may be one of the mechanisms which regulate

the bacterial flora of the tongue. All oral isolates identified by phenotypic and biochemical characteristics were used. Oral microflora have been known to produce substances with inhibitory effects on other microorganisms (Marsh, 2009). Antagonism was measured

percentage inhibition between the pathogenic fungal plug and bacterial streak (for bacteria isolates), and the pathogenic fungal plug and fungal plug (for the isolated fungus).

Increase in percentage inhibition (growth of pathogen away from the antagonist) between first and second day for *Micrococcus* sp. (13.8 ± 0.1 and 21.7 ± 0.0 ; $P = .05$ respectively) against *Colletotrichum lindemuthianum* was an indication of antagonism of the bacterium against the fungus. However, in subsequent days fungus grew towards the antagonist in a synergistic relationship. The fungus could have better adjusted to the growth conditions within the medium, and produced some substances to counter the initial antagonism observed. For this same bacterium against *Colletotrichum pisi* and against *Fusarium oxysporum*, neither synergism nor antagonism was observed on the first day. Subsequent days with slow increase in fungal growth away from the antagonist could mean that the fungus was in an initial lag phase at day one, and within the interval grew more away from the bacterium. In the case of *Micrococcus* sp. against *Sclerotium rolfsii* and *Colletotrichum capsici*, antagonism was more obvious with *Sclerotium rolfsii* than with *Colletotrichum capsici* (Figure VI). The inhibition of pathogenic microorganisms by *Micrococcus luteus* could probably be due to the production of organic acids and bacteriocins. This is in agreement with the report of Agarry et al. (2005). *Colletotrichum* sp. is an important plant pathogens causing disease in cereals, legumes, vegetables, perennial crops and tree fruits (Pakdeevvaraporn et al., 2005).

Antagonism between *Corynebacterium* sp. and *Colletotrichum lindemuthianum* showed a steady antagonism (0.0 ± 0.0 to 16.0 ± 0.0 and 28.9 ± 0.1 ; $P = .05$ respectively) as the fungus grew further away from the bacterium. *Corynebacterium* sp. showed slight fluctuation in the percentage inhibition of *Colletotrichum pisi*. The fungus may have expressed metabolites that were able to resist the antagonistic action of the bacterium. A synergistic relationship seemed to exist between *Corynebacterium* sp. against *Fusarium oxysporum* (-21.7 ± 0.1 ; $P = .05$), and more with *Sclerotium rolfsii* (-25.0 to -21.4 ± 0.0). Growth of *Fusarium oxysporum* (32.5 ± 0.1 ; $P = .05$) away from the antagonist could be as a result of the antagonist being able to express certain antifungal biological compounds (e.g. toxins, bacteriocins of the corynecin-linocin type etc.) at latter days after adjusting to growth conditions within the media. Agarry et al. (2005) and Marsh (2009) also submitted that members of *Corynebacterium* species may also exert antagonistic effect on other microorganisms via bacteriocins production.

Streptococcus mutans against *Colletotrichum lindemuthianum* and *Colletotrichum capsici* showed slight fluctuation in the percentage inhibition and antagonism. Both the antagonist and fungal pathogen may be able to express metabolites that counteract themselves at incubation intervals. This could be responsible for the antagonistic ability of *Streptococcus* sp. and also the resistant ability of the fungus. The bacterium against *Fusarium oxysporum* and *Sclerotium rolfsii* showed a reasonable antagonism in terms of growth away from the antagonist. The

antagonistic activity observed in this bacterium could be attributed to its ability to produce organic acids and other variety of metabolites with antifungal capabilities (Tharmila et al., 2013).

Aspergillus fumigatus (48.0 ± 0.0) appreciably antagonized the growth of *Colletotrichum lindemuthianum*. The pathogen was however able to slightly resist the inhibitory ability of the fungus at latter days. Antagonism was not strong between *Aspergillus fumigatus* and *Fusarium oxysporum* (7.6 to 13.3 ± 0.0). There was however, an initial neutral interaction of *Aspergillus* sp. against *Sclerotium rolfii* and *Fusarium oxysporum*. Percentage inhibition and antagonism reduced at latter days after the second day with *Sclerotium rolfii*. Metabolites produced by the antagonist could not withstand the possible biocompounds produced by the pathogenic fungus, and were counteractive at latter days. The reduction with *Sclerotium rolfii* indicated the ability of the pathogenic fungus to resist the inhibitory ability of *Aspergillus* sp., while the slight growth of *Fusarium oxysporum* away from the antagonist indicated the inhibitory ability of the fungal antagonist to overcome the pathogen's resistance. The antagonistic ability of *Aspergillus* sp. could be related to its unique ability to produce two compounds (fumigacin and gliotoxin), both chemically unrelated compounds possessing strong antibiotic activities (Marsh, 2009; Arthur et al., 1944). The relevant metabolites produced by the oral microflora isolated in this work however, could be further elucidated and exploited for possible applications, especially against the fungal pathogens.

It is pertinent however, to note that despite the many research efforts dealing with biological control of plant diseases, application of microbiological control remains limited, the main criticism being the lack of consistency. Several reasons can account for this. One claimed advantage of biological control is the narrow specificity of the biocontrol agents (BCAs). Their application will not affect the non-target organisms and therefore will respect the environment better than large spectrum molecules. However, in practice, the population of a pathogenic organism presents certain diversity and a single given strain of a BCA might not have the same efficacy on all the pathotypes present in the population (Schisler et al., 2000). Moreover, the prevailing conditions most favourable for the development of the pathogen might not be the same as the conditions required for maximum expression of the antagonistic activities of the BCA (Nicot et al., 2002). Hence, this makes it necessary to carefully study the effect of inoculum type, application rate and time of application to ensure efficacy of biological control (Jones et al., 2004a, b). Finally, both the diversity of the natural population of the pathogen and the climatic conditions in nature are much more variable than those used in the laboratory to study BCAs and their mode of action. Other constraints result from the diverse modes of action of the BCAs which have different consequences on the population density and activity of the target pathogens (Alabouvette et al., 2006).

CONCLUSION

In the *in vitro* antagonistic assays carried out in this research on four microorganisms isolated from the

human tongue (*Micrococcus luteus*, *Streptococcus mutans*, *Corynebacterium* sp. and *Aspergillus fumigatus*) against five species of pathogenic fungi (*Colletotrichum lindemuthianum*, *Colletotrichum pisi*, *Fusarium oxysporum*, *Sclerotium rolfsii* and *Colletotrichum capsici*), all isolates had varying antagonistic effects against the pathogens. Isolates, such as *Aspergillus fumigatus* and *Streptococcus mutans* showed the highest percentage inhibition with the plant pathogens. These metabolites produced by these isolates could thus be elucidated and exploited for use in the control and management of plant diseases. They could also be used not only against plant pathogens but also on other human pathogens. However, further research is required to identify the biological compounds which could have been responsible for the antagonism observed.

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