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Factors affecting biofilm formation as seed transmission mechanism of cowpea bacteria blight induced by *Xanthomonas axonopodis* pv *vignicola* (Burkholder) dye

Amodu U. Simeon[1]*, Kenneth C. Shenge[2], Akpa A. Dominic[3]

[1,3]Department of Crop Protection PMB 1044, Ahmadu Bello University Zaria, [2]Food and Animal Health Research Program/Dept. of Plant Pathology, Ohio Agricultural Research and Development Center, The Ohio State University1680 Madison Ave. Wooster, OH 44691 USA

ABSTRACT

Bacteria are carried in/on seeds by biofilm formation. However relatively few studies have been focused on the factors affecting biofilm formation ability of *Xanthomonas axonopodis* pv. *vignicola* (*Xav*) as a mechanism of transmission. Knowing the factors that enabling plant-pathogenic bacteria to form biofilm as a means to move and establish on/in the hosts provides the necessary basis to set up appropriate management approach. The study was conducted to determine factors affecting biofilm formation as mechanisms of seed transmission of *Xanthomonas axonopodis* pv. *vignicola* by growing bacterial cells in maize, millet, sorghum, Ife brown, Sampea 7 extracts and extracts of Ife brown + 0.5 g nutrient glucose agar (NGA) in a 96 microlitre wells. The seeds extract were prepared by soaking one hundred seeds each of Ife brown, Sampea7, millet, sorghum and maize in a 250 ml flask containing 100 ml SDW and for 20 h. Bacterial suspension adjusted to *ca*. 4.5 x 10^7 cfu/ml was suspended in each of the extracts and the media and filled twelve wells each of the treatment. These were incubated for 72 h, 96 h and 120 h. afterwards, the wells were emptied and the wells were stained with 1 % crystal violet (CV) solution in 33 % (V/V) acetic acid for approximately 20 minutes. Excess CV was washed with SDW. The bound CV to the wells were solubilized with 200 µl of 33 % acetic acid or acetone – ethanol and quantified spectrophotometrically using Well Reader (GF 3000 microplate Reader –Bran scientific and Instrument Company England). Specific Biofilm formations (SBF) was calculated. The experiment was replicated two times and repeated 3 times. There was statistical difference between the biofilm formation induced by the different extracts and NGA. All the seeds extract induces biofilm formation and the level of biofilm formation varies with time and the nutrient status of the media or medium.

KEYWORDS: Mechanism, biofilm, adhesion, attachment, extract, nutrient

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***Corresponding Author:**
Amodu U. Simeon
Email: amodusimeon@yahoo.co.uk; simeonumoru@gmail.com

INTRODUCTION

Biofilm are common in nature, as bacteria commonly have means in which they can attach to surfaces and to one another. It is very important to note that biofilms are simply a survival strategy of bacteria cells [1]. Individual bacterium comes together in order to become stronger. As it is natural, there is strength in numbers, with bacteria being no exceptions [2]. In general, Gram-negative bacteria use acylated homoserine lactose as auto-inducers or to communicate, and Gran-positive bacteria use oligopeptides as a means to communicate [1]. For infection to take place adhesion is an essential step required for colonizing a new host [3-5]. The bacterial surface structures important for adhesion were fimbrial and non fimbrial structures commonly known as adhesins [6, 7]. Essentially, bacteria may established on any surface exposed to some amount of water and nutrients. Once bacteria is attached to surface, it carry out a variety of detrimental or beneficial reactions depending on the species and on the surrounding environmental conditions [8-10]. Bacteria commonly have means in which they can adhere to surfaces and to each other [11]. (ii). Specific attachment: Permanent attachment of the microorganism to the surface sometimes called "anchoring". Specific adherence involves permanent formation of many specific lock-and-key bonds between complementary molecules on each cell surface [11]. Observation of plant pathogenic bacteria associated with hosts increasingly reveals biofilm-type structures that vary from small clusters of cells to extensive biofilm [12, 13].

Most of the bacteria that cause us problems are sessile – attached to a surface and they live in biofilm. Most researchers study extensively on planktonic cells (Free moving cell), while the actual problems involve biofilm bacteria. So new strategies based on a better understanding of how bacteria attach, develop biofilms and detach (spread) are urgently needed so as to develop affective control strategies [14]. In view of the forgoing, the current study aims at investigating factors affecting biofilm formation of *Xanthomonas axonopodis* pv. *vignicola* as seed transmission mechanism.

MATERIALS AND METHODS

Determination of Nutrient Factor Affecting Biofilm Formation

These nutrient source from two cowpea varieties (extract of Ife brown and Sampea 7), millet, maize, sorghum, extract Ife brown + 0.5 % glucose and Nutrient Growth Agar (NGA) were used to determine the nutrients factor affecting biofilm formation. One hundred seeds each of Ife brown, Sampea7, millet, sorghum and maize were surface disinfected in 3 % sodium hypochloride. These were put in a 250 ml volumetric flask containing 100 ml SDW and incubated for 20 h. [15]. The resulting extracts were filtered under sterile conditions. X. *axonopodis* pv. *Vignicola* were grown on NGA until a log phase [16]. Bacterial suspension adjusted to *ca*. 4.5 x 10^7 cfu/ml was suspended in each of the extracts and Ife brown extracts + 0.5 g glucose. Biofilm formations were determined using 96 – well microtiter plates filled with 200 μl bacterial suspension/well. Twelve wells were filled for each of the seven treatments. The control plates were their corresponding extracts without inoculation. The plates were incubated at 27^{0}C without shaking for 72 h. After which the planktonic cells were removed and rinsed the well with SDW five times and the cells biomass attached to the surface of the well were washed with physiologic

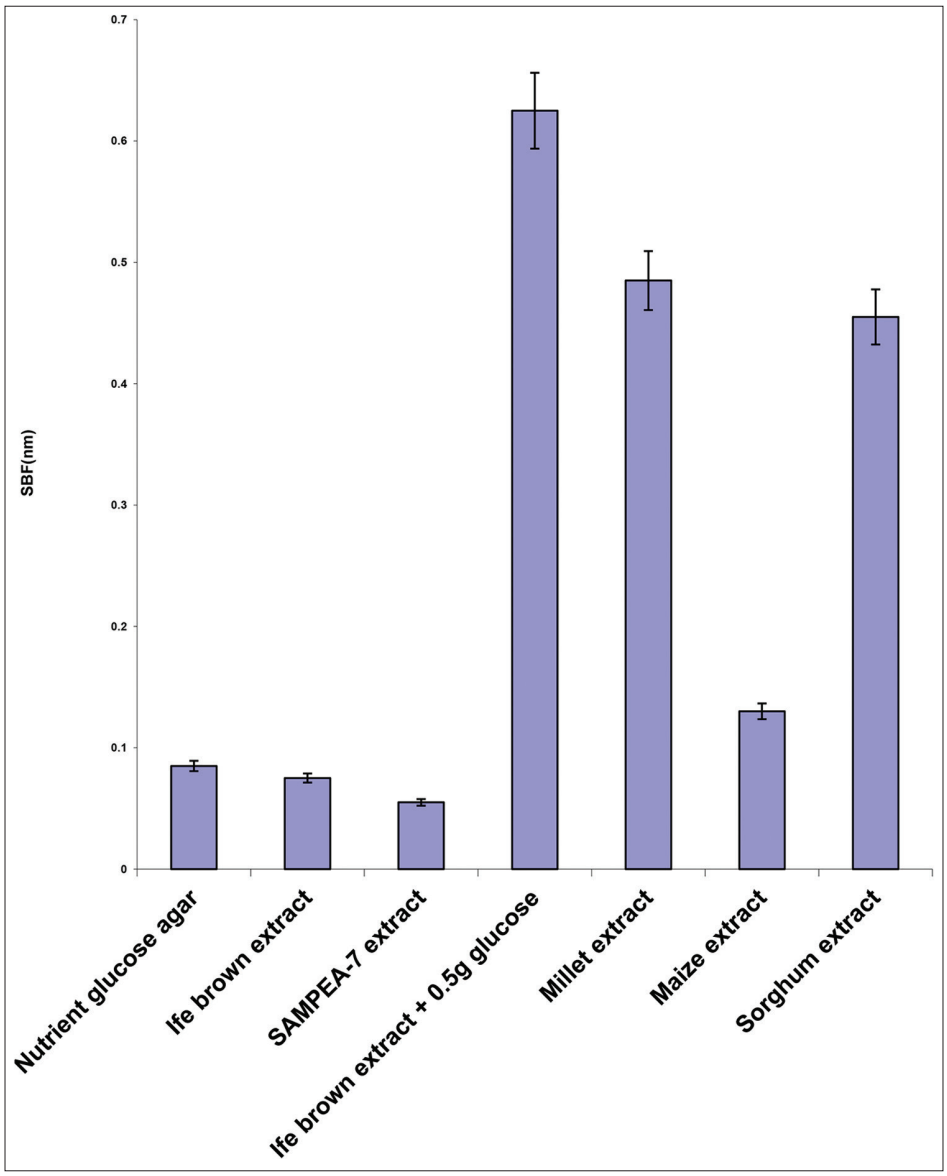

Figure 1: Nutrient factor affacting Biofilm Formation

solution and allowed to dry overnight at 25 ^{0}C. The wells were stained with 1 % crystal violet (CV) solution in 33 % (V/V) acetic acid for approximately 20 minutes. Excess CV was washed with several change of SDW. The bound CV to the wells were solubilized with 200 μl of 33 % acetic acid or acetone – ethanol and quantified spectrophotometrically using Well Reader (GF 3000 micro plate Reader –Bran scientific and Instrument Company England). Specific Biofilm formations (SBF) was calculated using the formula: SBF = B –NC/BG, Where B is the amount of CV bound to the cells attached to the surface of the wells, NC is the negative control, and BG is the OD_{630} of bacterial growth [17]. The experiment was repeated three (3) times and data obtained and means separated by Least Significant Difference (LSD).

Determination of Time factor affecting Biofilm Formation

Media that were used for this investigation were: Nutrient glucose Agar (NGA), Ife brown seed extract, Ife brown seed extract plus 0.5 g glucose, maize extract, sorghum extract, and Sampea7 extract. Seeds extracts were prepared as described previously. Bacteria suspension adjusted to *ca* 4.5 x 10^7cfu/ml was suspended in each of the media while their corresponding extracts without inoculation were used as control. Biofilm formations were determined using 96 well micro titer plates as described previously at 72 h, 96 h and 120 h. Data obtained were analyzed and mean data were used to plot graphs for appropriate representation of the results.

RESULTS

Figure 1 shows the result of biofilm formation induced by seed extracts. Ife brown extract + 0.5 g glucose induced the highest SBF followed by millet extract, followed by sorghum extract, and maize extract. Biofilm formation induced by nutrient agar and two cowpea varieties were not statistically different ($P<0.05$). The biofilm formation did not significantly increase with time except Ife brown extract + 0.5 g glucose where biofilm formed at 96 h was higher than that formed 72 h but did not increase at 120 h (Figure 2).

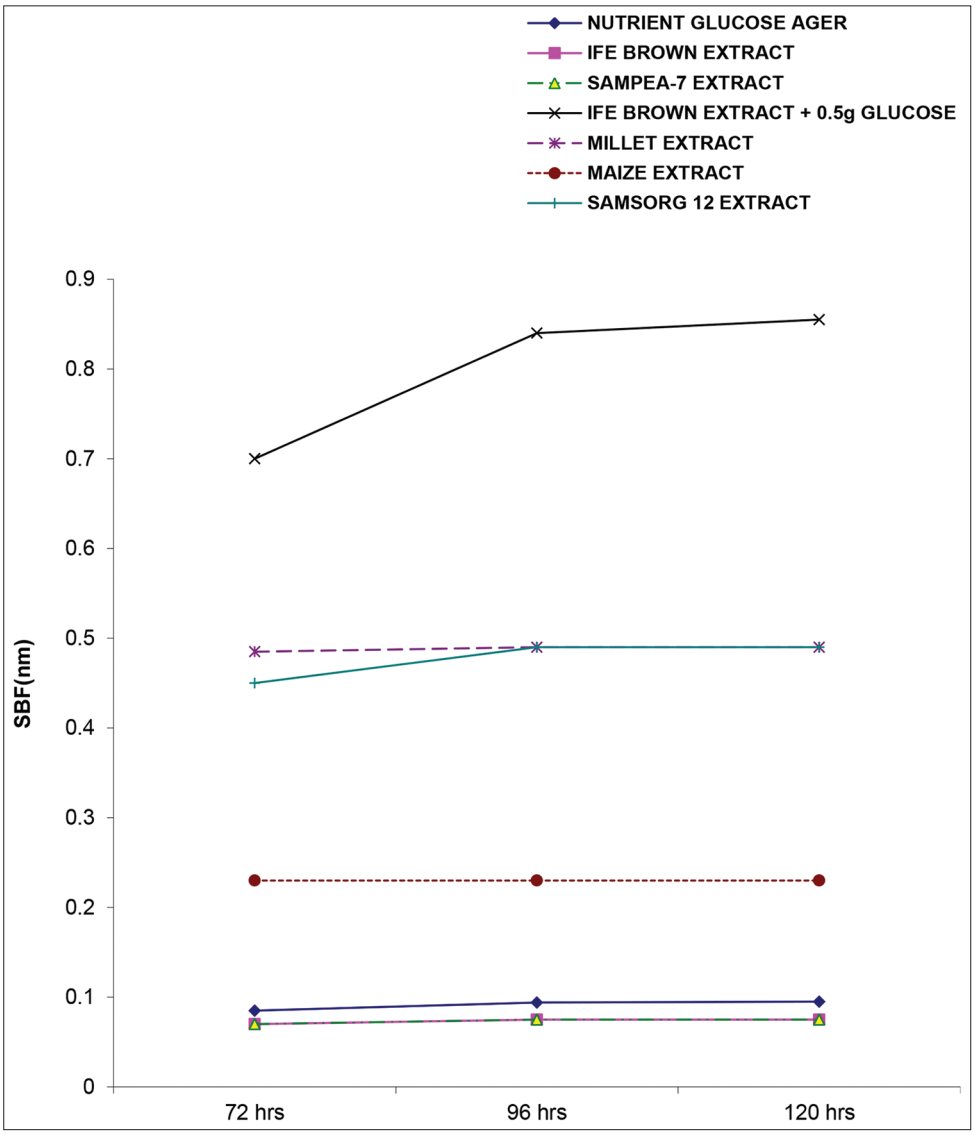

Figure 2: Time factor affecting biofilm formation

DISCUSSION

Bacteria cell surface is polarize and this polarity is what leads to surface adhesion and cell cohesion to wells as observed in the work [2]. It is clear that medium/media that are rich in nutrients support the production of SBF than the medium/media that lack nutrients as control well did not produce any SBF. Bacterium coalesces by linking extracellular polysaccharides on their cell walls. This result is in agreement with the report of Huber *et al*. [18]. Bacteria interactions are due to exchange of metabolites with plant and microorganisms [19]. From the work Ife-brown extract plus 0.5 g glucose produced highest biofilm, and this shows that bacteria growth/biofilm formation is dependent on external source of carbon and nitrogen provided by the host plant or the surrounding environment [20]. Biofilm are formed over reasonable time and its part of mechanism for bacterial colonization [21,22]. Bacteria attachment to surface and subsequent biofilm formation over time and this phenomenon constitute a strategy for bacteria to survive desiccation or other environmental stresses and actively participate in defense mechanisms of the pathogens [16].

The rate at which the biofilm grows beyond the initial attachments is influenced by both the time, and the amount of nutrient within the aqueous medium [2, 23]. Observation of bacteria associated with plants increasingly reveals biofilm-type structures that vary from small clusters of cells to extensive biofilm. The five stages of biofilm development are: initial attachment (planktonic cell attachment), irreversible attachment, maturation I, maturation II and dispersal which are dependent on available nutrient and time. Biofilms have been found to be involved over 80 % of all infections [24,2]. Once anchored to surface, bacteria carry out a variety of detrimental or beneficial reactions depending on the species and on the surrounding environmental conditions [25, 8].

CONCLUSION

The study of factors affecting biofilm formation of bacterial cells to seed or plant phyllo sphere surfaces demonstrated that the different nutrient types influences biofilm formation by the phytopathogenic bacteria. All the seeds extract induces biofilm formation and the level of biofilm formation varies with time and the nutrient status of the media or medium. Aggregation followed by biofilm formation is a strategy used by pathogenic bacteria during colonization of plants phyllo sphere for its protection from stresses and maintenance of inoculum reservoirs.

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