ARTÍCULO DE INVESTIGACIÓN

COMPARING INOCULATION METHODS TO EVALUATE THE GROWTH OF *Xanthomonas axonopodis* pv. *manihotis* ON CASSAVA PLANTS

Comparación de métodos de inoculación para evaluar el crecimiento de *Xanthomonas axonopodis* pv. *manihotis* en plantas de yuca

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RESUMEN

Xanthomonas axonopodis pv. *manihotis (Xam)* es el agente causal del tizón bacteriano de la yuca, una de las principales enfermedades de los cultivos de yuca en América del Sur y África. Hasta ahora, el desarrollo de la enfermedad se mide a través de AUDPC *(Area Under Disease Progress curve)*, pero no hay disponibles métodos cuantitativos fiables, esto debido posiblemente a la alta variabilidad del crecimiento bacteriano en la planta. Para establecer un método exacto para la cuantificación bacteriana durante el curso de la infección *Xam* dentro de los tejidos del huésped, se analizaron las poblaciones de bacterias sobre tallo y hojas, así como corte de hojas de las variedades de yuca MCOL1522 y SG107-35 con la cepa virulenta CIO151 *Xam*. En esta investigación se muestra que el movimiento de las bacterias a lo largo de los tejidos y especialmente en las hojas es estocástico. Por otra parte, hemos podido demostrar el crecimiento diferencial de la cepa virulenta *Xam* CIO151 tras la punción al tallo y la cuantificación de la bacteria a 6 cm de distancia del punto de inoculación de dos variedades que presentan niveles contrastantes de susceptibilidad.

Palabras clave: crecimiento bacteriano, métodos de inoculación, yuca.

ABSTRACT

Xanthomonas axonopodis pv. *manihotis* (*Xam*) is the causal agent of cassava bacterial blight (CBB), a major disease for cassava crops in South America and Africa. Until now the development of the disease is measured via AUDPC (Area Under Disease Progress Curve) but no reliable quantitative methods are available probably due to high variability of bacterial growth *in planta*. To establish an accurate method for bacterial quantification during the course of *Xam* infection within the host tissues, we analyzed bacterial populations upon stem and leaf-puncturing as well as leaf-clipping of cassava varieties MCOL1522 and SG107-35 challenged with the virulent *Xam* strain CIO151. Here, we show that the movement of bacteria along the tissues and especially in leaves is stochastic. Moreover, we were able to demonstrate differential growth of virulent *Xam* strain CIO151 upon stem-puncturing and quantification of bacteria 6 cm. away from the inoculation point of two varieties displaying contrasting levels of susceptibility.

Keywords: bacterial growth, cassava, inoculation methods.

INTRODUCTION

Cassava Bacterial Blight (CBB) is one of the most prevalent bacterial diseases of cassava, which can severely compromise its production, and therefore food security for more than 1000 million people, who depend on cassava starch as main

source of calories (FAO, 2011; López and Bernal, 2012). CBB is caused by the gram-negative bacteria *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) which enters plant tissues via wounding and colonizes the vascular tissues (Verdier, 2002). *Xam* is a highly diverse pathogen in Latin America, as well as in Africa, albeit to a lower extend (López and Bernal, 2012). The losses caused by *Xam* can reach 100 % of cassava production after three cycles of production if the environmental conditions are favorable (Verdier, 2002). Only a few resistant cassava varieties have been reported, which are mainly non-commercial varieties (Restrepo *et al.,* 2000a). Histological and molecular defense responses of cassava against *Xam* have shown to be activated in both resistant and susceptible varieties, but are faster and more intense in resistant plants (Kpémoua *et al.*, 1996; López *et al.*, 2005). As an important tool for improving the study of the cassava-*Xam* pathosystem, the cassava genome sequence was recently released (www.phytozome.net, Prochnik *et al.*, 2012). Also, the draft genome sequences of 65 *Xam* strains as well as the complete genome sequence of the reference *Xam* strain CIO151 were recently reported and are available (Bart *et al.,* 2012; Arrieta *et al.,* 2013), These important genomic resources will be crucial for the identification of key cassava and *Xam* genes determining resistance and susceptibility of cassava plants challenged with *Xam*.

To phenotypically validate the function of putative cassava resistance genes, it is important to evaluate their effect on the growth of *Xam* populations *in planta*. Similarly, to evaluate the role played by *Xam* virulence/pathogenicity candidate genes, it is essential to evaluate the effect of the inactivation of these genes by following the growth of relevant mutant strains in susceptible cassava plants. Thus, the quantification of bacterial populations *in planta* is pivotal to demonstrate the function of plant and bacterial genes during infection, and complementary to the observation of macroscopic plant phenotypes. Traditionally, bacterial populations are quantified by evaluating a dilution series of infected plant tissues extracts plated on selective medium, but the inoculation methods vary depending on the studied pathosystem. The target tissues that are naturally colonized by the pathogen as well as the plant anatomy are key criteria to chose the most appropriate inoculation method (Meyer *et al.,* 2005). For example, in the Arabidopsis-*Pseudomonas syringae* pathosystem depending on the inoculation method, resistance mechanisms may or may not be triggered (Melotto *et al.,* 2006). In other pathosystems, such as in the interaction of *Xanthomonas campestris* pv. *campestris* with *Arabidopsis*, several inoculation methods are reported, but some are more prone to generate variability in plant responses and bacterial growth (Meyer *et al.,* 2005). To tackle this problem, Meyer *et al.,* (2005) reported on wound inoculation as the optimal method to avoid variability Interestingly, the function of the avirulence gene $avrAC_{Xcc8004}$ in this pathosystem could only be evidenced

upon puncturing but not by leaf infiltration, revealing that $avrAC_{Xcc8004}$ is specifically recognized within vascular tissues (Xu *et al.,* 2007). Hence, the method of inoculation plays an essential role in the study of a pathosystem of interest and it is, therefore essential to find what is the best one mimicking the pathogen infection under natural conditions.

Wounding, syringe-infiltration and spray inoculation are a few of the most prevalent methods to inoculate bacteria. In tomato, *Xanthomonas axonopodis* pv. *vesicatoria,* which colonizes the apoplast of the leaf mesophyl, is often inoculated by leaf infiltration (Kim *et al.,* 2008). *Xanthomonas oryzae* pv. *oryzae* colonizes the vascular tissues of rice and the most common inoculation method for quantitative assessment is leaf-clipping, which consists in cutting leaf tips with scissors previously immersed in a bacterial suspension. In cassava, several methods of inoculation of *Xam* into the plant have been evaluated in material grown from stem cuttings or *in vitro* (Jorge and Verdier, 2002). These methods include clipping leaves with contaminated scissors (Lozano and Laberry, 1982), spraying foliage, depositing bacterial suspensions on mechanically generated leaf holes and stem puncturing (Restrepo *et al.*, 2000b; Jorge and Verdier, 2002). Stem inoculation of plants grown *in vitro* was used to describe *Xam* colonization inside the plant through histological approaches (Kpémoua *et al.,* 1996). Interestingly, this study reported the presence of *Xam* agglomerates randomly distributed along the tissues. This could explain why most of the methods employed to quantify bacteria *in planta* produce a high variability, thereby making them inadequate for resistance screening and loss of pathogenicity assays. Additionally, microarray studies demonstrated that cassava gene expression upon *Xam* infection is quite delayed as compared to other pathosystems (López *et al.,* 2005). This is also correlated to the observation that primary symptoms appear only 8 days-post inoculation of 4-month old susceptible cassava plants with a virulent strain of *Xam* (López *et al.,* 2005). At present, the reference method to evaluate cassava resistance is through area under the disease progress curve (AUDPC). However, this method is based on the visual inspection of disease intensity over time and does not measure bacterial populations *in planta*, which is particularly relevant considering the quantitative nature of the resistance to CBB (Jorge and Verdier, 2002).

The aim of this work was to evaluate different inoculation methods relevant to the *Xam*-cassava pathosystem, quantifying bacterial growth and following up the symptomatic development in order to establish an accurate method with reduced variability. Hence, despite the observation of high variability in bacterial populations, we show that the most accurate evaluation method is by determining bacterial growth away from the site of inoculation point in the stem of cassava plants grown in greenhouse.

MATERIAL AND METHODS
Bacterial strains
Xam strain CIO151 was streaked onto YPGA medium (5 % yeast extract, 5 % peptone, 5 % glucose and 15 % agar) and incubated at 28 °C during 48 hours. One colony was grown onto YPG medium and incubated at 28 °C for 18 h before inoculation. Liquid inoculums were calibrated at OD_{600nm}= 0.002, corresponding to 10^6 colony forming units (CFU).

Greenhouse plants assays
Cassava varieties MCOL1522 and SG10735 were grown in greenhouse conditions (day/night temperature = 28/19°C, 12h photoperiod, and 80 % relative humidity). Leaves of 8-weeks-old plants were used to test the puncturing and clipping inoculation methods. For puncturing, a sterile needle was used to make a the incision on the main nerve of the central lobe. Five microliters of calibrated inoculums (OD_{600nm}=0.002) were placed on the puncture. For clipping, scissors were immersed in the inoculum solutions and a transversal section was made 1 cm. below the tip of the leaf. For both methods, the bacterial population was quantified at 0, 7, 14 and 21 days post inoculation (dpi). Depending on the experiment, different sections of stem or leaves were collected.

For stem inoculation, eight week-old plants were used. Puncturing with a sterile toothpick was made on the third internode from the apical region. Ten microliters of calibrated inoculums were placed on the puncture. Different leaf or stem sections of were collected at 0, 7, 14 and 21 dpi, depending on the experiment.

AUDPC
Eight week-old cassava plants were inoculated in the stem with the strain CIO151. Plants were inoculated and evaluated at 0, 7, 14, 21 and 28 dpi following a severity scale 0-5 (Jorge *et al*., 2000) where 0- healthy plant, no reaction observed, 1- dark area or necrosis around the inoculation point; 2- gum exudates on the stem; 3- wilting of one or two leaves and exudates; 4- more than two leaves wilted and 5- complete wilting and dieback. Ten biological replicates were used for each variety AUDPC was calculated as described previously (Jorge *et al.,* 2000).

Quantification of bacterial population
For every experiment, three technical repetitions from three distinct plants of each variety were used. Leaves and stem sections were collected, cleaned in 70 % ethanol and sterile water and disinfected with 2 % sodium hypochlorite and ground in sterile water. Serial dilutions were plated on YPGA medium and incubated at 28 °C. After 48 hours *Xam* colonies formed were counted. Non-inoculated plants were used as controls.

All the assays were performed with three biological repetitions.

Statistical analyses
The statistical Wilcoxon test was used as a non-parametric test to compare related samples. For AUDPC, a t-test was employed. Data from three biological repetitions (consisting of three technical replicate) were generated to complete statistical analyses.

RESULTS
Susceptibility level
In order to compare the susceptibility level of the two cassava varieties that were used in our study, we applied a qualitative standard method (AUDPC), to discriminate between resistant and susceptible, which relies on the use of a symptoms scale. Through this approach the phenotype of a given variety can be quantified in a rank from 0 to 122.5, 0 indicating the plant to be highly resistant and 122.5 highly susceptible (Jorge and Verdier, 2002). As a result of the analysis of three independent biological replicates, the obtained average AUDPC value were 13.8 and 55.5 for the varieties SG107-35 and MCOL1522, respectively (Fig. 1A and B, indicative of a significant difference in the susceptibility level of the two cassava varieties to *Xam* strain CIO151 (t-test with p-value=0.0474). Interestingly, symptoms observed in MCOL1522 appear earlier than in SG10735, where the plants remain healthy at 7 dpi and symptoms increase quickly between 14 and 21 dpi. More than one plant of COL1522 variety showed a symptom scale superior to 4, which is the criterion employed by Restrepo *et al*., (2000b) to be considered as susceptible. On the other hand, none SG107-35 plants showed this symptom scale and in consequence following the same criterion this variety is considered as resistant. Overall, these data demonstrate that MCOL1522 and SG10735 are useful varieties for our purpose to evaluate the sensitivity of different inoculation methods.

Leaf-clipping inoculation
To establish a reliable method with reduced variability to quantify bacterial growth in cassava, we first evaluated leaf-clipping inoculation. As shown in Figure 2A, bacterial numbers at the inoculation point were similar in both cassava varieties MCOL1522 and SG10735, which is in opposition to what was expected from the AUDPC analysis. Inspection of bacterial growth at 2 cm. away from the inoculation point also showed a similar trend in both varieties (Fig. 2B), indicating that there is no differences in bacterial numbers .

Leaf puncturing inoculation
Because *Xam* is a vascular pathogen, we next investigated the effect of puncturing the main vein of 8 week old cassava leaves. As shown in Fig. 3A, bacterial growth was monitored over time within a 5 mm^2 leaf fragment comprising the inoculation point in the main vein. Again, measurements performed at 7, 14 and 21 days post-infection indicated that bacteria multiplied at similar level in both MCOL1522 and SG10735 varieties.

Similarly, when bacterial numbers were monitored 2 cms away from the point of inoculation no difference could be observed between the two varieties (Fig. 3B). Hence, neither leaf-clipping nor leaf puncturing inoculation revealed differences in *Xam* growth within leaves of the two cassava varieties.

Stem inoculation

Xam naturally colonizes cassava stems and techniques have been previously established to inoculate stems under laboratory conditions (Restrepo *et al.*, 2000b; Jorge and Verdier, 2002). We therefore evaluated bacterial growth of *Xam* after stem puncture inoculation. In a first attempt, bacterial growth was monitored at 7, 14 and 21 dpi within a 3 cm long stem fragment including 1 cm below and 2 cm above the inoculation puncture, respectively. As shown in Fig. 4, bacterial number was similar to those

observed upon leaf-clip inoculation with no differences observed among varieties. In a second attempt, the same puncture inoculation method was employed but bacterial multiplication was monitored within different stem fragments. Stem fragments of 1 cm-long were collected at the puncture of inoculation, at 2 cm and 6 cm above it. As shown in Fig. 5A and 5B, bacteria grew similarly in both varieties when measurements were performed from the inoculation site and 2 cm away from it. Interestingly, as shown in Fig. 5C, quantification of bacterial at 6 cm above the puncture of inoculation highlighted a 4-log difference at 21 dpi of *Xam* populations in variety MCOL1522 as compared to SG10735. These differences are significant (W-test at p-value=0.01945) and confirm the results obtained by AUDPC that differentiate between the two cassava varieties.
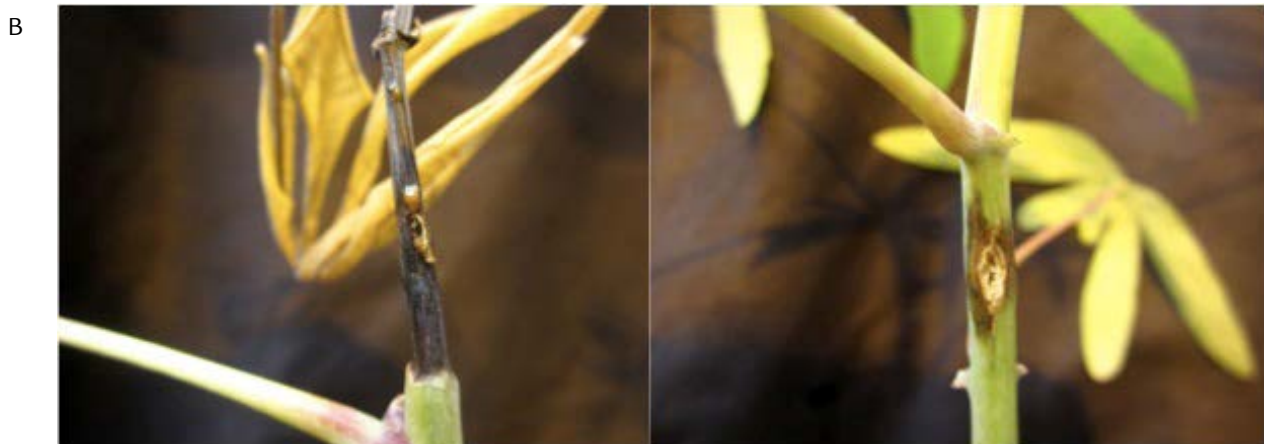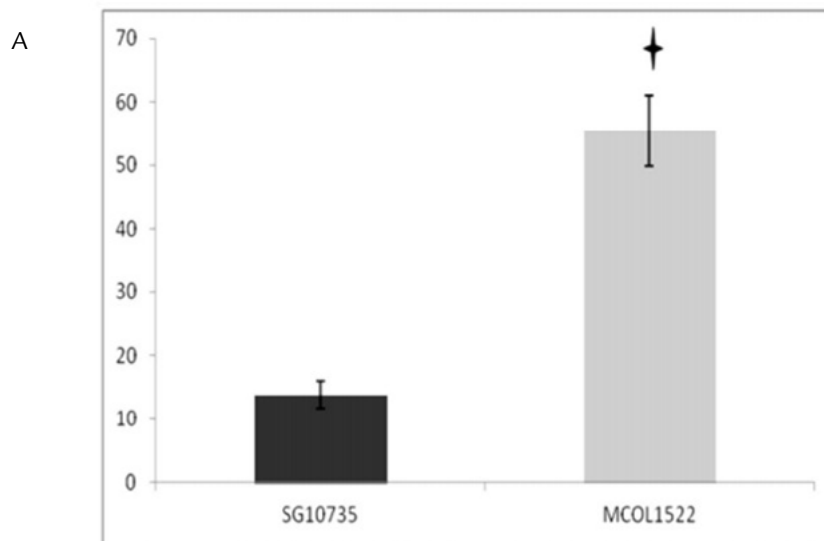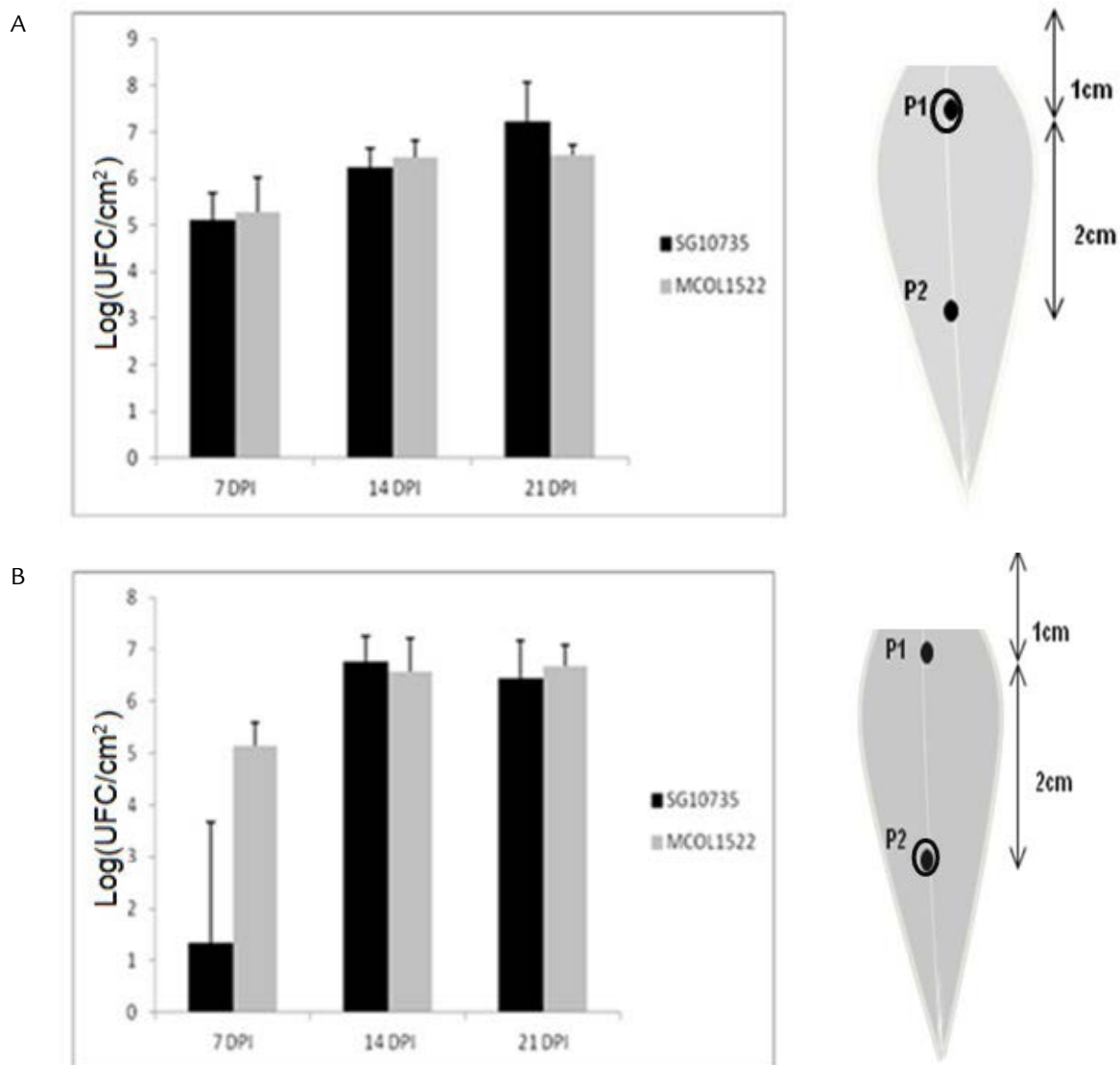

Figure 1. A). AUDPC in Cassava varieties SG10735 and MCOL1522 inoculated with *Xam* strain CIO151. Black star indicates statistycall differences at p-value = 0.0474. B). Cassava symptoms at 28 DPI. Left panel MCOL1522 variety, right panel SG107-35.

DISCUSSION

With the advent of cassava and *Xam* genome sequences it should be possible to identify resistance and pathogenicity candidate genes. The functional validation of both, bacterial and plant genes will require studying their phenotypes when these are overexpressed or mutated. To achieve this objective, a robust system of inoculation to observe the loss or gain on resistance to *Xam* is required. To establish a method for quantification of bacterial multiplication, we studied different methods previously reported in the literature for cassava and/or other pathosystems (Jorge and Verdier, 2002; Wydra *et al.,* 2004; Meyer *et al.*, 2005 Kim *et al.,* 2008).

Stem inoculation is a widely used method to evaluate the resistance/susceptibility of cassava plants and it represents the more natural way of *Xam* spread in the crop (Wydra *et al.,* 2004). However few studies have quantified the bacteria present in these tissues and the only optimal way to do it is the qualitative AUDPC approach (Jorge and Verdier, 2002). In the present study, we observed quantitative differences in the bacterial numbers between the two varieties used in this study only by taking a portion of stem, 6 cm away from inoculation point. The results obtained with this method were comparable with the AUDPC results. Hence, we argue that the methodology
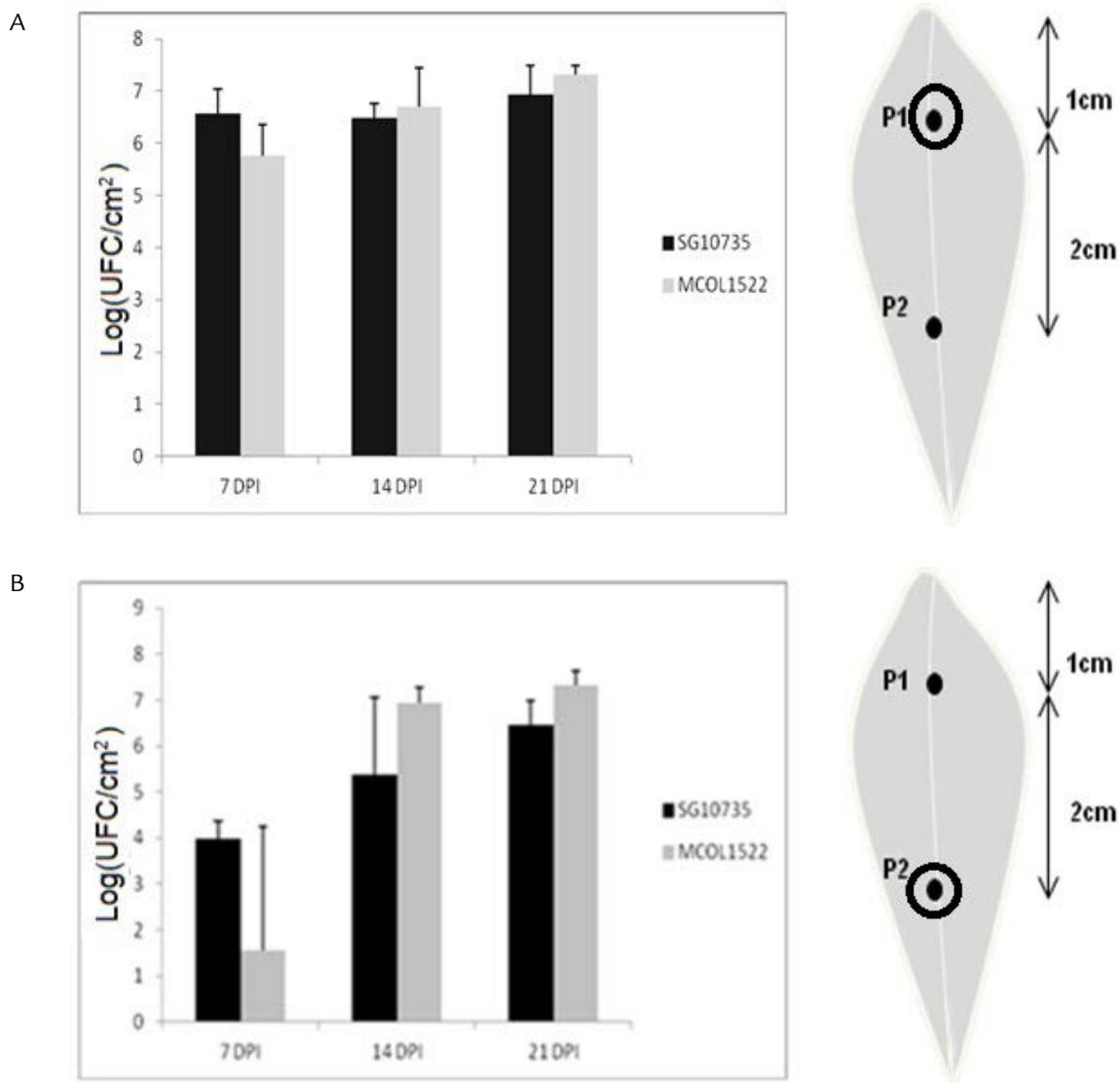

Figure 2. Bacterial growth analysis upon leaf clipping inoculation of Cassava varieties SG10735 and MCOL1522 at 7, 14 and 21 days post-inoculation with Xam strain CIO151, Bars represent standard deviation. (A): Growth at the inoculation point. and (B): 2 cm. away from inoculation point. Illustration on the right shows a 2-month old cassava leaflet where black circles represent the areas where bacteria were quantified. No statistical differences were obeserved.

presented here constitutes a sensitive method allowing detection of small resistance/susceptibility differences between varieties. Furthermore, this result suggests that *Xam*, as a vascular pathogen, moves preferentially across the stem and not as effectively on leaves, and supports the idea of non-regular bacterial aggregation across the tissue but only close to the inoculation point.

Evaluation of symptoms in leaves, as it is established in several pathosystems, would be more economical and practical for testing phenotypes in cassava plants. Nevertheless, probably because of the high content of polysaccharides present in the cell wall of cassava leaves, methods such as infiltration are very difficult and variable between cassava varieties. To do an accurate bacterial quantification, leaf puncturing and leaf clipping methods were evaluated. The results obtained using these methods, resulted in high variability, and lead us to evaluate bacterial movement in plant tissues. *Xam* is a vascular pathogen that colonizes the xylem (Verdier, 2002). Therefore, the variability reported here, can be explained considering the possibility that the flow of water and other substances through the vascular vessels could increase bacterial mobility. In consequence, bacterial aggregation varies in space and time resulting in sampling variability. Thus, the quantification of the bacteria at distant sections from the inoculation point will be a stochastic phenomenon appropriate for *Xam*.

Figure 3. Bacterial growth analysis upon leaf puncturing inoculation of cassava varieties SG10735 and MCOL1522 at 7, 14 and 21 days post-inoculation with *Xam* strain CIO151. Bars represent standard deviation. (A): Growth at the inoculation point and (A): 2 cm. away from inoculation point. Illustration on the right shows a 2-month old cassava leaflet where black circles represent the areas where bacteria were quantified. No statistical differences were observed.

Even if a high variability was observed, we did not observe any differences in the cassava varieties after using the inoculation methods on leaves, although by AUDPC, the traditional and standard method to evaluate resistance, the two varieties were different. Previous studies using other cassava varieties indicated that the cassava responses are similar in the resistant and susceptible varieties in particular in the mesophyll, despite not evaluating different regions of the plant or different times post inoculation.

Despite of these facts, we could observed the bacterium was not able to grow in distant regions from the inoculation point in stem. Interestingly, it has been reported that leaf inoculation could be a useful method to evaluate mutant pathogenicity (Castiblanco *et al.,* 2013). Nevertheless based on our results, we argue that this technique is not useful to evaluate resistant and susceptible cassava varieties. Consequently, the mobility assays could be important to compare the growth of pathogenic *Xam* strains in contrasting varieties. Recent PCR-based strategies have been developed to detect *Xanthomonas* in planta (Albuquerque *et al.*, 2012; Lang *et al.*, 2014). Further studies could be directed to quantify bacteria employing real time PCR.

CONCLUSIONS

In conclusion and taking together these results, we propose as a quantitative evaluation, complementary to AUDPC, to measure the bacterial growth taking 1 cm of tissue 6 cm away from the stem inoculation point at 21 dpi.

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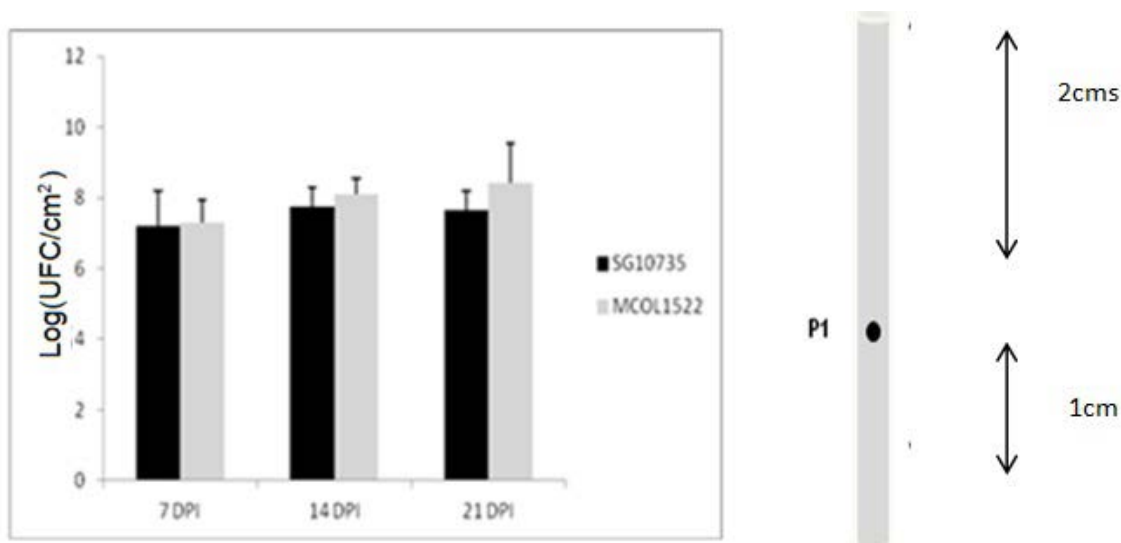
Figure 4. Bacterial growth analysis upon stem puncturing inoculation of cassava varieties SG10735 and MCOL1522 at 7, 14 and 21 days post-inoculation with *Xam* strain CIO151. Bars represent standard deviation. Bacterial growth in 3 cm. of stem composed of 1 cm. below and 2 cm. above the inoculation point. Illustrationon the right shows a 2-month old cassava stem where black dot represent the inoculation point. No statistical differences were evidenced.
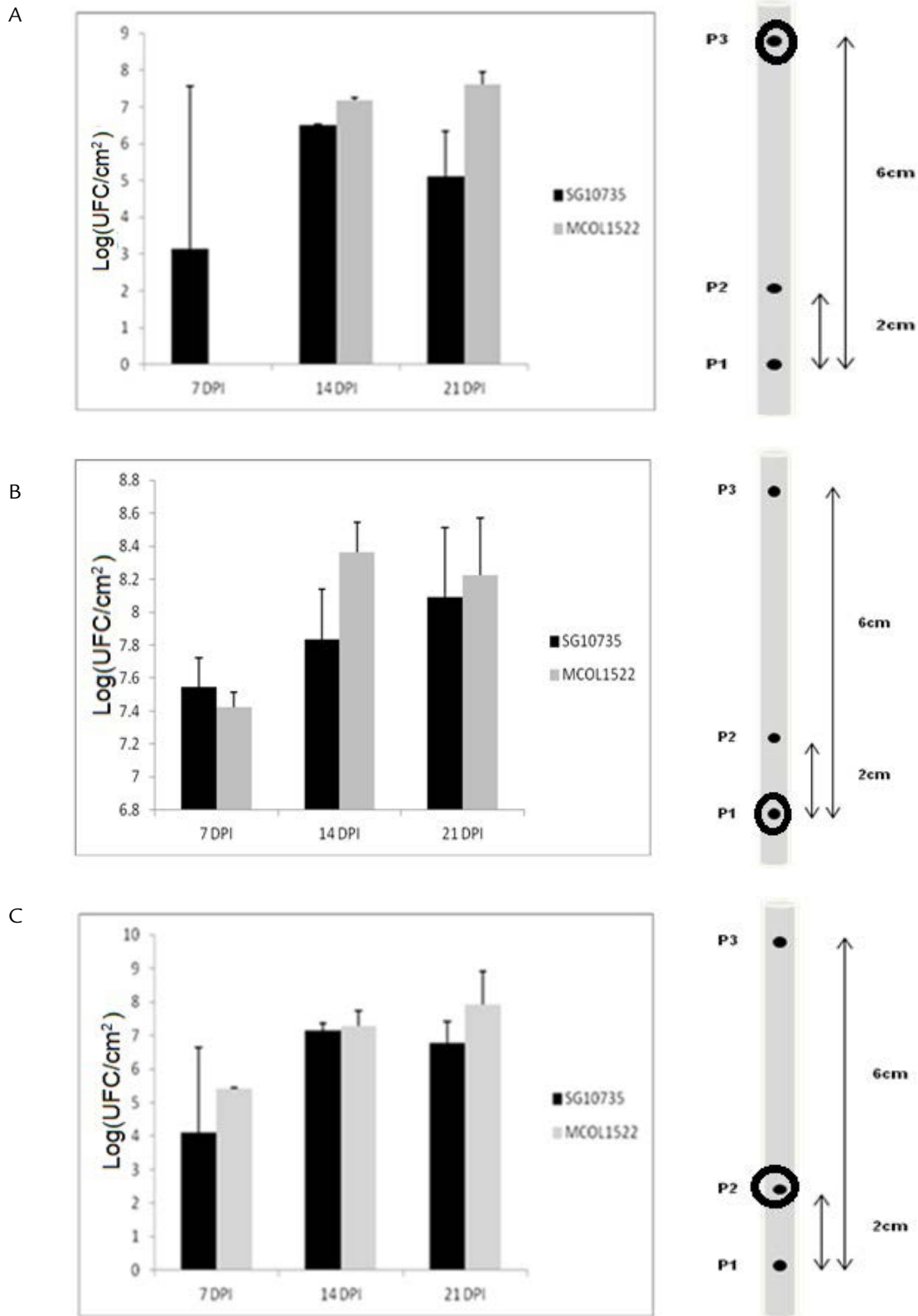
Figure 5. Bacterial growth analysis upon stem puncturing inoculation of cassava varieties SG10735 and MCOL1522 at 7, 14 and 21 days post-inoculation with Xam strain CIO15. Bars represent standard deviation. (A): Growth at the inoculation point and (B): 2 cm. away from inoculation point. (C): 6 cm. away from inoculation point. Illustration on the right shows a 2-month old cassava stem where black dots represent the areas where bacteria were quantified. SG107-35 and MCOL1522 at 21 DPI presented significant differences. (Wilcoxon test p-value = 0.01945).

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