IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 3, Issue 5 (Sep.- Oct. 2017), PP 77-82
www.iosrjournals.org

Developing Preliminary *In-Vitro* Disease Assay for Cassava Resistant to Bacterial Blight Disease

[1]Ihuoma C. Okwuonu, [2]Joseph T. Onyeka, [3]ChiedozieN. Egesi
National Root Crops Research Institute Umudike, Nigeria
Corresponding Author: [*]Ihuoma C. Okwuonu

Abstract: *In-vitro disease assay was developed as a preliminary tool for screening cassava resistance to Xanthomonasaxonopodispv.*manihotis *(Xam), the causative agent of cassava bacterial blight disease. Detached petiole-borne leaf was cultured in cassava rooting medium and inoculated with 0.1 µl of bacterial suspension(Xam transformed with pLux)at* OD_{600}*0.5. Inoculated plantlet were incubated at 30ºC and monitored daily for bacterial growth via bioluminescence imaging. Root and shoot induction was observed 7 and 14 days after inoculation respectively. Bioluminescence imaging was scored on the scale of 0-3 with scores of 0, 1, 2 and 3 representing absence of growth, limited, moderate and severe bacterial growth. Six-weeks-old greenhouse grown plants from different scoring scale were inoculated with Xam and evaluated for resistances. Data derived from in-vitroassay corresponded with the result obtained from greenhouse evaluated plantlets. This confirms the potential of in-vitro assay as a preliminary tool for evaluating disease resistance.*

Keyword: *Cassava, in-vitro disease assay, cassava bacterial blight, bioluminescence, Xanthomonasaxonopodis*

Date of Submission: 02-10-2017 Date of acceptance: 10-10-2017

I. Introduction

Methods for assessing, screening and selecting for disease resistant plants in the greenhouse or field are time consuming, costly and subject to environmental conditions in terms of concentration of inoculum and weather variations [1]. In other to fast track screening procedure, and thus reduce time and cost associated with this process, there is need to develop rapid evaluation methods for selecting events intended to proceed to greenhouse or field evaluation. Over time, in-vitro technology had proven efficient in enhancing most plant breeding operations such as virus elimination, embryo rescue and rapid multiplication of plantlets. This could be extrapolated to methods for rapid screening and selecting for disease resistant plants. *In-vitro* screening methods have been employed in evaluating disease resistance in fruit crops [2], barley [3], pear [4], wild grapes [5] and potato [6].

Selection of resistant candidate species in fruit crops was attained by inoculating explants in a culture medium amended with cultural filtrate of fungal or bacterial pathogen. Resistant plantlets were then selected and regenerated from surviving explants [2]. This tool provides a rapid and efficient method of screening and selecting for disease resistant candidate compared to traditional field methods which requires a long time for a fruit tree to germinate and mature [1]. Similarly, the efficiency of in vitro assay for rapid evaluation of disease resistance was illustrated by Paprstein*et al.*[4] who was able to determine the level of resistance of eight pear cultivars to *Erwiniaamylovora* using *in-vitro* plantlets. Also the reliability of this technique was further confirmed by Sim*et al*. [6] who showed that the result of greenhouse evaluation of potato cultivars to *Pectobacteriumatrosepticum*, the causative agent of black leg disease was in agreement with that obtained from in vitro assay of same cultivars.

The major advantages of using *in-vitro* assay in determining resistance or susceptibility of selected plant genotypes includes the precise control of the chemical and physical environmental conditions in the medium and the ability to screen very large samples in the shortest possible time while excluding possible interfering microorganisms. However this is definitely not a true representation of field condition where multiple factors play a role in determining resistance in the field. The aim of this study is not to necessarily replace the traditional greenhouse and field screening methods but to develop a preliminary screening method to determine susceptibility and resistance of selected genotypes.

II. Materials & Methods

Inoculum preparation

*Xanthomonasaxonopodix*pvmanihotis (Xam), the causative agent of cassava bacterial blight diseasewas transformed with plasmid containing bacteria luciferase gene (pLux) and streaked on NYGA medium (containing5g of peptone (Oxoid), 3g of yeast extract (Difco), 20g of glycerol and 10 g of Agar (Lab My Salford, UK) supplemented with 100 µg/ml of Rifampicin and incubated at 30ºC for 48 hours. Cultures were scraped with a disposable plastic loop and suspended in 1 ml of 10mM Magnesium Chloride ($MgCl_2$). A 1:200 dilution of the bacteria suspension was made by aliquoting 5 µl of bacteria suspension into 995 µl of 10 mM $MgCl_2$.The optical density (OD_{600}) of bacteria suspension was determined using Nanodrop spectrophotometer (Thermo fisher scientific, USA). OD_{600} obtained was multiplied by the dilution factor to determine the actual OD in 1 ml of bacteria suspension. Volume of bacteria suspension required to obtain an OD_{600} of 0.5 was determined and diluted with the corresponding volume of 10 mM $MgCl_2$.

In-vitro establishment and assay

In-vitro leaves detached from the petiole were inoculated in rooting medium comprising of Murashige and Skoog medium (MS2 agar) supplemented with 2% sucrose. The leaves were inoculated with the petiole immersed into the medium, with the adaxialregion in direct contact with the medium (Fig.1). 0.02% of silwet, a surfactant was added to the bacterial suspension to aid easy dispersion on to leaf surface. 0.1 µl of bacteria suspension was dispensed onto the leaf surface and left for 15 minutes to dry. pLux negative Xam was used as negative control while 10 mM $MgCl_2$ was used as mock. Cultures were incubated at 30ºC and monitored daily for bioluminescence. Experiment was replicated thrice and mean value of the three experiment used for analysis.

Greenhouse establishment and disease assay

In-vitro derived plantlets were established in the green house (Fig. 2) as described by Taylor *et al*. (2012). The plantlets were inoculated with Xam as described by Bodnar*et al*. (2014) after a period of six weeks. The samples were monitored at 3, 5 and 7 days post inoculation (dpi).

Bioluminescence

Inoculated cultures were exposed for a period of 4 minutes using the chemiluminescence program of the Azure biosystem C300 gel documentation system (Fig.3). The total amount of light emitted from bioluminescence and area covered is indicative of bacterial growth and virulence on the sampled events. Efficacy of the assay was determined by setting up a mock experiment with 10mM $MgCl_2$, a negative control with Xam strain (pLux-negative Xam) and text experiment with Xam containing the pLux plasmid (pLux-positive Xam) as shown in Figure 1. Results were interpreted on the scale of 0-3, with 0 = No growth, 1= very limited growth, 2 = moderate growth and 3 = Severe growth.

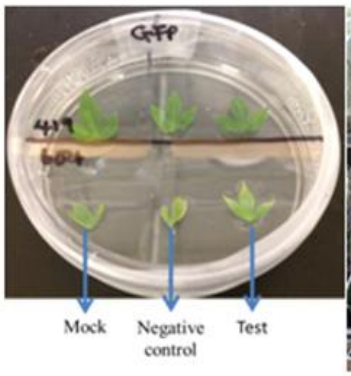

Fig. 1; *In-vitro* leaves detached from petiole and cultured on rooting mediumwere inoculated with Xam suspension at 0.5 OD600, incubated at 300Cobserved at different time

Fig. 2: Six weeks old *In-vitro* derived cassava plantlets established in the screen house ready for challenge with *Xanthomonasaxonopodis*pv. Manihotis

Fig. 3: Azure biosystem C300 gel doc. Gel documentation system for bioluminescence imaging

III. Result

Petiole regenerated plantlets were derived 14 days after inoculation on rooting medium at 30ºC. Factors like susceptibility to Xam andsize of leaf explant which provided the surface area for bacterial growth affected the germination and rooting of the explants (Fig. 4). Visualization of bacteria proliferation on leaf surface was aided

by the total amount of light emitted from bioluminescence and area covered as shown in Figure 5. The mock (Fig. 5AI) and negative control (Fig. 5AII) gave no background signal thereby validating the signal from the test experiment as an indication of the presence and growth of the bacteria on the leaf surface. Figure 5A shows the experimental set up at 0 dpi while Figure 5B shows the proliferation of bacterial and consequently disease development at 7 dpi. It took a period of 4 to 7 days to visualize bioluminescence on the surface of the text experiment. Figure 5BIII shows the proliferation and spread of Xam from the point of inoculation into the mid-rib and veins thus indicating the susceptibility of this event to Xam infection.

Representative events illustrating the 0 – 3 scoring scale is shown in Figure 6. Each level comprises of three independent events assigned to the same level. Score 0 is representative of events showing little or no bacterial growth while score 1 and 2 is representative of events showing limited and moderate bacterial growth respectively. Events assigned the score of 3 showed severe bacterial growths covering a wider area of the leaf surface. Data was collected from a series of three replicates and the mean of the three experiments used to determine the scoring scale assigned to each event as shown in Figure 7. Out of the 27 events evaluated, 6, 13 and 8 events fall within the scoring scales of 0-1, 1-2 and 2-3 respectively. Four randomly selected 6-weeks-old greenhouse grown events,604-wt, M7, M9 and M18 infiltrated with 0.1 ml of Xam suspension of OD_{600} 0.5 showed a corresponding disease phenotype as indicated by the bioluminescence assay (Fig. 8).

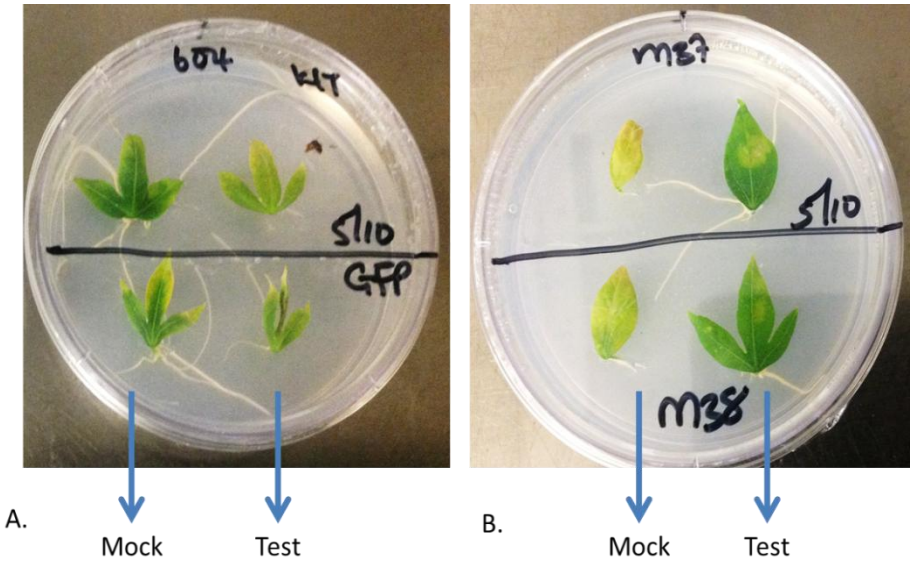

Fig. 4: Petiole regenerated cassava plantlet derived after 14 days incubation at 30^0C. (A).Regeneration of Xam inoculated plantlets were retarded compared to the mock inoculated plantlets (B) Plantlets regeneration retarded by size of plantlet.

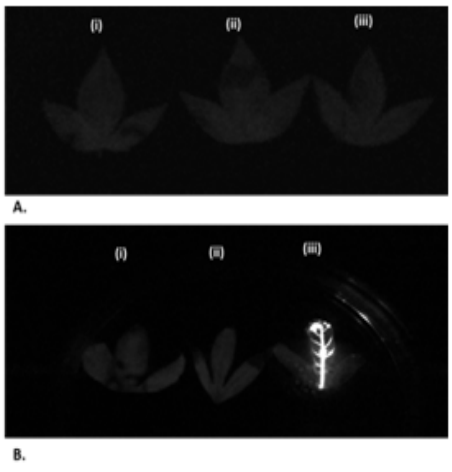

Fig. 5: *In-vitro* assessment of mutants to observe performance in relation to Xam: (a)) Samples inoculated for bioluminescence assay at 0 dpi (i) mock sample inoculated with $MgCl_2$ (ii) sample inoculated with pLux negative Xam_{668} and (iii) sample inoculated with pLuxXam (B) bioluminescent of inoculated samples at 7 dpi

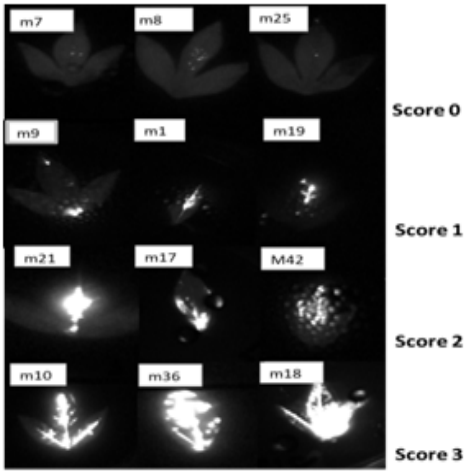

Fig. 6: Bioluminescence assay showing the scoring scale for bacteria spread on leaf surface: Representative samples of events evaluated illustrating the scoring scale of 0 = no growth, 1 = limited growth, 2 = moderate growth and 3 = severe grwoth for bacteria growth on leaf surface

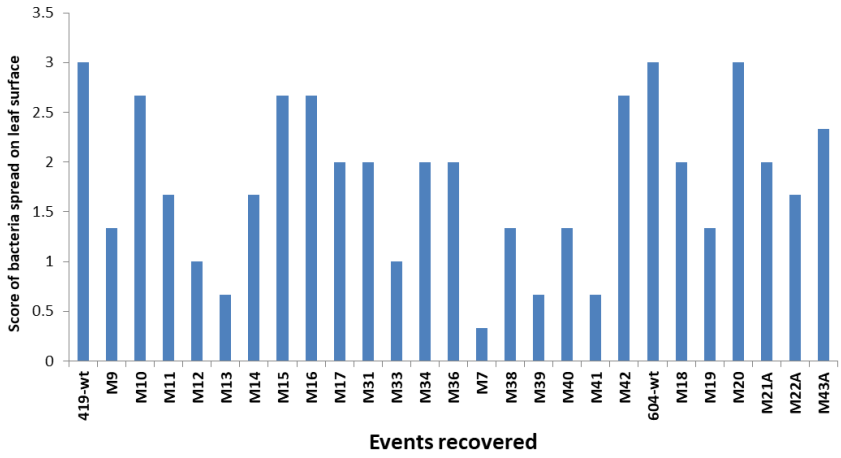

Fig. 7: Average score for bioluminescence assay: Average value of bioluminescence scores based on intensity and surface leaf areas covered by bacterial growth as preliminary indication of susceptibility, tolerance and resistance.

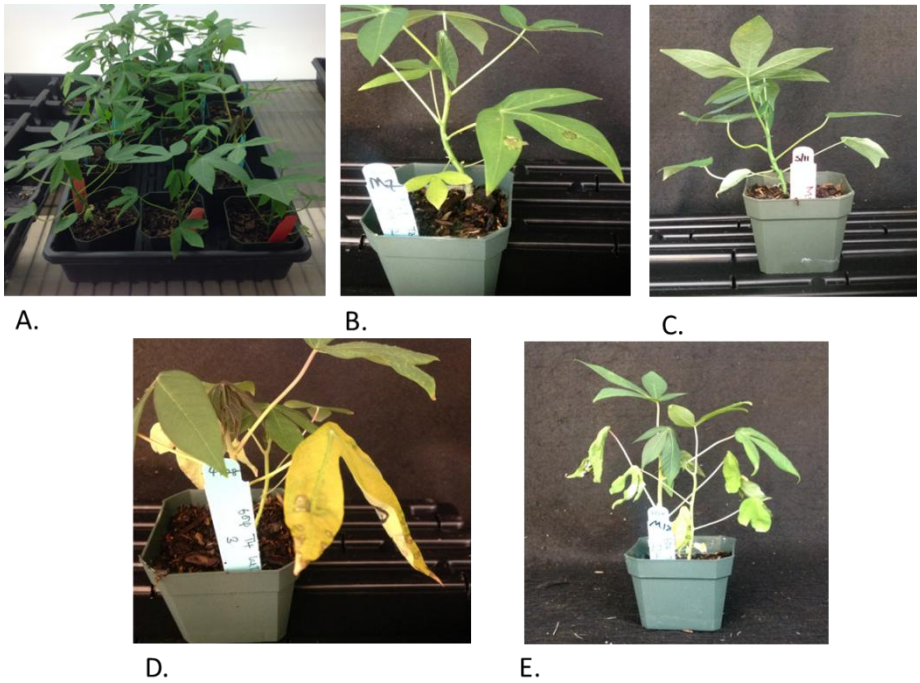

Fig. 8: **Greenhouse evaluated of selected events showing tolerance and susceptibility:** (A). Xam inoculated greenhouse events at 0 dpi, (B) Event M7 showing tolerance to Xam infection corresponding to score 0 (C). M9, showing tolerance and corresponding to a score of 1,(D) 604-WT showing susceptibility and corresponding to a score 3 (E) M18 showing advanced symptoms of wilting and dieback also corresponding to a score of 3

IV. Discussion

In-vitro cassava regeneration has mostly been through somatic embryogenesis using shoot apical meristem and immature leaf lobes [8], apical and axial nodal culture [9] as well ascallus induction. Alternative method for *in-vitro* cassava regeneration for preliminary disease assay was explored by evaluating the totipotency of petiole-borne leaf explants. Detached petiole-leaf explants were inoculated into rooting medium and exposed to 0.1µl of bacterial suspension at the OD_{600}0.5. This was required to facilitate regeneration process as well as providing a medium to test for the reaction of candidate events to pathogens. Similar, experiment was carried out by Fokunang*et al*. [10] who developed an *in-vitro* method for assessing resistance to cassava anthracnose disease (CAD). His approach however differs from that employed in this study, in that culture medium for the causative agent was amended with cortex extracts of candidate cassava lines and assayed for pathogen inhibition[10]. This method even though effective entails the destruction of test materials and thus limits its availability especially where limited numbers are available.Other *in-vitro* inoculation methods employed in other plants include piercing healthy *in-vitro* potato plantlets [6], application of bacterial suspension at 10^6 CFU/ml to detached 3mm shoot apex of pear [4], inoculating leaflets of *in-vitro* cultivated potato with 1000 µl droplet of fungal culture [11]. For our specific situation, petiole-borne leaf was preferred for easy assessment of bacterial growth on leaf surface.

Root initiation from the petiole was observed 5 to 7 days after inoculation and whole plantlets derived after 14 days. This might be attributed mainly to the rooting medium used for the inoculation. Shoot regeneration can be achieved earlier than 14 days by incorporating shoot inducing hormone such as benzylaminopurine (BAP) into the medium. It was also observed that regeneration/germinationefficiency of the explants varied in the experiment and could be attributed to factors such size of explant, time of exposure to pathogen and volume of inoculum used. Since uniform sizes of explant were not used due to insufficient quantity of test materials, the volume per surface area of leaf was not uniform across the experiment. It is therefore recommended for optimum regeneration efficiency that candidate lines should be multiplied at the same time to get uniform explants and that the explants are allowed to grow for a period of 7 days before exposing them to the pathogen.

Bioluminescence imaging was employed to aid visualizationof bacteria growth and spread on leaf surface by transforming *Xanthomonasaxonopidis*pv. manihotis, with plasmid containing bacteria luciferase gene

(pLux). This method was adopted because this kind of chemiluminescencethat occurs in living microbial cells does not require external optical excitation and thus excludes background luminescent signal [12]. Selected events based on the result of bioluminescence assay scored on the scale of 0 to 3, with the score of 0 being tolerant and 3 being highly susceptible were greenhouse tested to validate the data derived *in-vitro*. Result obtained showed disease scores generated from bioluminescence assay of *in-vitro* experiment corresponds to the result obtained from the greenhouse. This finding agrees with the report of Sima*et al.* [6] who confirmed the potency of laboratory techniques to screen potato genotypes to *Pectobacteriumatrosepticum* the causative agent of black led disease. Their result showed that data generated *in-vitro* was in accordance to data derived from the greenhouse. In the same manner, Fokunang*et al.* [10] showed that *in-vitro* screening assay of cassava for resistance to CAD could serve as a preliminary screening technique to determine CAD resistant cassava cultivars. Use of *in-vitro* technique for preliminary assay has also being confirmed in pears against *Erwiniaamylovora*[4], potato, against *Alternaria alternate*[11] and Barley against *Fusariumgraminearumor* F. culmorum[3].

V. Conclusion

Petiole-borne leaflet has been confirmed as an excellent explant for *in-vitro* regeneration of cassava for disease assay. Shoot regeneration medium supplemented with BAP could be used to facilitate shoot regeneration before 14 days as obtained using rooting medium. Bioluminescence imaging was excellent in visualizing bacterial growth and spread on the leaf surface. Result obtained from bioluminescence imaging of the *in-vitro* assay corresponded to the result obtained from the greenhouse. However, to optimize the *in-vitro* technique, uniform explant should be used and explant allowed for a period of 7 days after inoculation before exposing to pathogen.

References

[1]. M.E. Ostry, In vitro screening and selection for disease resistance, USDA Forest Service Gen. Tech. Rep. 297. 1997. 155–160.
[2]. R. Chandra, M. Kamle, A. Bajpai, M. Muthukumar, and S.Kalim , In vitro selection: A candidate approach for disease resistance breeding in fruit crops. Asian Journal of Plant Sciences.http://doi.org/10.3923/ajps, 2010.437.446.
[3]. K. Kumar, K. Xi, T. K. Turkington, A. Tekauz, J. H. Helm and J. P. Tewari, Evaluation of a detached leaf assay to measure fusarium head blight resistance components in barley, Canadian Journal of Plant PathologyVol. 33 , Iss. 3,2011 Pages 364-374
[4]. F. Paprstein , J. Sedlak , J. Sillerova , J. Korba, In Vitro Evaluation of Cultivar Resistance to Fire Blight ActaHorticulture, 2014.1056.43
[5]. S.J.Kim, and Y.S Park, In Vitro Evaluation of Korean Wild Grapes for Disease Resistance, Journal of Agriculture and life science, 2(1), 2015, 2006–2009.
[6]. A. Sima,J. Mozafari, H. Nader, and M. Cobra, In vitro evaluation of resistant of potato cultivars against black leg disease (Pectobacteriumatrosepticum). Biological Forum-An International Journal, 7(2), (2015). 1087–1094.
[7]. A. M. Bodnar , L. M. Cruz Gómez, A.Bernal, B.Szurek, C. E. L.Carrascal,Comparing inoculation methods to evaluate the growth ofXanthomonas, Artículo De Investigación, 20(2), 2014, 47-55.
[8]. K.Mongomake, O. Doungous, B. Khatabi, and V.N. Fondong, Somatic embryogenesis and plant regeneration of cassava (ManihotesculentaCrantz) landraces from Cameroon. SpringerPlus, 2015. 1–12.
[9]. R.Shiji, G. James, S. Sunith, R. Muthuraj, Micropropagation for Rapid Multiplication of Planting Material in Cassava (ManihotesculentaCrantz), Journal of Root Crops, Vol 40, No 1 2014)
[10]. C.N. Fokunang, A.G.O. Dixon, T. Ikotun, C.N. Akem and E.A. Tembe, Rapid Screening Method of Cassava Cultivars for Resistance to Colletotrichumgloeosporioidesf.sp. manihotis. Journal of Phytopathology, 150: 2002 6–12.
[11]. H.R. Mirkarimi, A. Abasi-moghadam, and J. Mozafari, In vitro and greenhouse evaluation for resistance to early blight of potato isolated from Alternariaalternata, Agricultural Sciences4(9), 2013, 473–476.
[12]. K. Jia, and R.E. Ionescu, Measurement of Bacterial Bioluminescence Intensity and Spectrum : Current Physical Techniques and Principles, 2015), 19–45. http://doi.org/10.1007/10

Ihuoma C. Okwuonu. "Developing Preliminary In-Vitro Disease Assay for Cassava Resistant to Bacterial Blight Disease." IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB) , vol. 3, no. 5, 2017, pp. 77–82.