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Original article

IN-VITRO ANTIFUNGAL POTENTIAL OF dsRNA MOLECULES ON FUSARIUM ACTIN RELATED PROTEIN 2/3, DNA POLYMERASE DELTA SUBUNIT AND ADENYLASE CYCLASE ESSENTIAL GENES ON COLLAR ROT AND WILT PATHOGENS OF PASSIONFRUIT

Florence Nassimbwa[a,b,*], Enock Matovu[a], Andrew Kiggundu[b], Charles Changa[b], Francis Mumbanza[b], John Adriko[b]

[a] *School of Biosecurity, Biotechnical and Laboratory Sciences Makerere University. P. O. Box 7062 Kampala, Uganda.*
[b] *National Biotechnology Research Center/ Tissue Culture, National Agricultural Research Laboratories. P. O. Box 7065 Kampala, Uganda.*

Abstract

BACKGROUND:

In Uganda two fungal diseases have become economically important; Fusarium wilt which is caused by.Fusarium oxysporum. f.sp passiflorae (Fop) and collar rot caused by Fusarium solani (Fs). The aim of this study was to determine antifungal activity of synthetic dsRNA constructed to silence essential genes; Actin Related Protein 2/3 (D6), DNA Polymerase delta subunit (J6) and Adenylase cyclase (K6), in Fusarium from banana on Fusarium sp from passionfruit.

METHODS:

In this study, pathogenic samples which were previously isolated from 3 districts in Central Uganda i.e. Wakiso, Mpigi and Mukono were tested to determine and explore RNAi as an anti-fungal control strategy for spore germination. Sample materials obtained were subjected to DNA extraction from pure mycelia and PCR was performed. Antifungal dsRNA assays were run to test the efficiency relying on the reduction in number of fungal colonies on PDA.

RESULTS:

Previous results showed that wilting was associated with one specific species of Fusarium oxysporum and collar rot with one species of Fusarium solani. Anti-fungal assay tests in this study included PCRs targeting the Fusarium actin related protein 2/3 (D6), DNA polymerase delta subunit (J6) as well as adenylase cyclase (K6) results showed that, % spore germination inhibition on Fop ranged from 61.9-93.9% and Fs ranged from -69-87.3%.

CONCLUSION:

These results show that an environmentally friendly control measure for the above diseases is possible through development of resistant plants which express dsRNA for a specified gene_ Fusarium Actin Related Protein 2/3.

RECOMMENDATION:

RNAi would be a promising target gene for RNAi –mediated resistance in passion fruit against the target fungal diseases.

Keywords: Antifungal potential, actin related protein, adenylase cyclase, dsRNA, PCR, polymerase delta subunit, Submitted: 2023-03-31 Accepted: 2023-04-20

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1. INTRODUCTION

In Uganda two fungal diseases have become economically important; *Fusarium* wilt which is caused by.*Fusarium oxysporum. f.sp passiflorae* (Fop) and collar rot caused by *Fusarium solani* (Fs) (Emechebe, 1976) . (Fischer, 2008). They are widespread and limit longevity of established orchards to between 2-5 years. They cause yield loses of 40-100% (Wangungu, 20-24 September 2010). A study on RNAi to latter development of host resistance in passionfruit cultivars through transformation for selected genes may reduce major losses in plant yield and quality. Range of diversity in pathogenic *Fusariums* from farmers' orchards was determined in an earlier study where samples were collected from 3 districts in Central Uganda which included Wakiso, Mpigi and Mukono to identify the *Fusarium sp* associated with collar rot and wilting of passion fruit (*Passiflora spp*). The study concluded that *Fusarium* wilt is caused by *Fusarium oxysporum f.sp passiflorae* (Fop) and collar rot caused by *Fusarium solani* (Fs) (Nassimbwa, 2015).This was done so that control measures that are effective against the predominant pathogenic variants in Central Uganda could be developed for most of the popular cultivars such as 'small purple' which are highly susceptible to wilt and collar rot.

The RNA-mediated gene silencing is naturally occurring post-transcriptional gene silencing phenomenon evolutionarily conserved and sequence specific, this silencing mechanism recognizes double stranded RNA (dsRNA) as a signal to trigger the sequence-specific degradation of homologous mRNA. Today this phenomenon has emerged as a powerful reverse gene tool, especially organisms where gene targeting is inefficient or time consuming as well as a genetic tool for engineering plant resistance against various pathogens including fungi (Mumbanza, 2012).

Application of RNAi in crop biotechnology has shown that host resistance has long been identified as the most feasible and sustainable way of managing diseases in crops. This is achievable through conventional cross breeding and or genetic modification (GM) approaches. Among these is antifungal protection strategy and RNAi. This inhibits expression of one or more target genes in a phytopathogenic microorganism leading to cessation of infection, growth, development and reproduction and eventual death of the pathogen (Mumbanza, 2012). The RNAi has been successfully applied in controlling pest insects in the western corn rootworm in transgenic corn (control of Coleopteran insect pests through RNAi) (Baum, 2007)

2. MATERIALS AND METHODS

2.1. study design

An experimental study design was used to determine antifungal activity of synthetic dsRNA constructed to silence essential genes; Actin Related Protein 2/3 (D6), DNA Polymerase delta subunit (J6) and Adenylase cyclase (K6), in Fusarium from banana on Fusarium sp from passionfruit. Sample materials obtained were subjected to DNA extraction from pure mycelia and PCR was performed. Antifungal dsRNA assays were run to test the efficiency relying on the reduction in number of fungal colonies on PDA.

2.2. Study area:

National Agricultural Research Laboratories, (National Biotechnology Research Center/ Tissue Culture) Kawanda, Wakiso District, Uganda.

2.3. DNA extraction from Microorganism

Sample material was obtained from a previous survey of pathogenic fungal material collected from Central Uganda where fungi from infected plants were isolated by culturing twig sections from suspected plants on $\frac{1}{4}$ potato dextrose agar, sub-culturing to establish single colonies and selection for *Fusarium* pure colonies done on pentachloro nitro benzene (Nassimbwa 2015).

DNA extraction from pure mycelia of six fungal colonies, including 3 field isolates, two pathogenic re-isolates (purple and cream) and a positive control Foc (*Fusarium oxysporum cubense*) fungal

*Corresponding author.
Email address: nassimbwaflorence@gmail.com (Florence Nassimbwa)

colonies was done using sterile sea sand to aid maceration of mycelia as well as cause cell lyses. 200µl of TES extraction buffer (0.2 M Tris-HCL pH 8, 10mM EDTA pH8, 0.5M NaCl, 1% SDS) was added to the sample in a motor. Macerated tissue suspension was transferred to 1.5mm microcentrifuge tubes. Samples were then vortexed for 30 sec and then placed in a water bath at 65˚C for 30 min. One half volume (250µl) of 7.5M Ammonium acetate was added to the samples which were then mixed and incubated in a refrigerator for 10 min,then centrifuged (table top centrifuge 5415D;Eppendorf) at 13,200rpm for 15 min, supernatant was then transferred to a new tube. To 500µl of the sample, 500µl of ice-cold isopropanol was added and sample centrifuged for 10 minutes at 13,200rpm. Supernatant was decanted and each DNA pellet was washed with 800µl of cold 70% ethanol and centrifuged for 2 min at 13,200rpm. Pellets were air dried on clean sterile paper towels for 40 min. 50µl of RNASE free water was added to resusped the pellets, which were then incubated at -20˚C for 30 min (Mumbanza, 2012). Quality of extracted DNA was then assessed by electrophoresis on an Agarose gel (0.6g of agarose powder and mixing it in 50ml of 1X TAE buffer). 1.5µl of DNA loading dye was mixed with5µl of the RNASE free water and 5µl DNA, these were thoroughly mixed and were loaded into wells of the gel (5.5µl of the sample was loaded in each well). Gel was then run in the electrophoretic tank for 50 minutes and stained with ethidim bromide for 10 min. Bands on the gel were documented using a gel documentation system. (Gene Snap Product version 7:09; Syngene) (Nassimbwa 2015). Fig 1

2.4. *Preperationand purification of PCR products for preparation of dsRNA.*

PCR was performed using primers for specific and rapid detection of *Fusarium oxysporium f.sp cubensi* Foc genes for inhibition (Vengaza.USA). PCR involved 10minutes initial denaturation step 90˚C for 40 cycles consisting of 1 minute denaturation, 1minute primer annealing at 55 degrees Centigrade and a minute extension at 72 degrees followed by 10 minutes extension step at the same temperature. Amplified products were then size fractionated inclusive of a marker, on a 1.5%Agarose gel electrophoresis in 1XTAE buffer. Gels were then stained with ethidium bromide in an aqueous solution for viewing using agel documentation system(Gene Snap Product version 7:09; Syngene). PCR product was quantified using a nanodrop. Purification of PCR product was done using a GeneJET PCR purification kit and following the manufacturer's instructions. Genes corresponding to primer sets D6 (D-Actin Related Protein 2/3 (ARP23), J6 (J- DNA Polymarase delta subunit (DPD) and K6 (K-Adenylase cyclase (AC). 6 purple re-isolates from Mpigi were selected for purification and quantification. Preparation of dsRNA followed using a Megascript kit. Selected genes purified DNA was thawed and the RNA polymerase enzyme mix was placed on ice. Master mix at X3 concentration of DNA for D6, J6 and K6 was used. Vortexing of the 4 ribonucleotide solutions (ATP, CTP, GTP, UTP) was done. Unit mix was composed of 2µl of each of the 4 ribonucleotides, 2µl of 10X reaction buffer, 2µl of enzyme mix and this transcription reaction was assembled and vortexed at room temperature. Samples were then incubated at 37˚C overnight. The reaction was then stopped and RNA was precipitated by adding 30µl of nuclease free water and 30µl of Lithium chloride precipitation solution. The mixture was vortexed to mix thoroughly and then chilled at -20˚C for 40 minutes and centrifuged at 4˚C for 15min at 13000rpm to pellet the RNA. Supernatant was carefully removed and the pellet washed once with 1ml of 70% ethanol. The RNA was then resuspended in RNAse free water, consultation and research done before at Vengaza, Plant Biotechnology Consulting and Research (Florida, USA).

2.5. *Antifungal dsRNA assays*

Efficiency of the assay was decided relying on the reduction in number of fungal colonies on PDA, work initially done at Vengaza.USA and initially applied by Mumbanza F.Harvesting of spores was done by flooding plate cultures with sterile water and rubbing with a sterile spatula. The concentration of spores was determined us-

ing a haemocytometer (1/10mm deep, bright line; Boeco, Germany) under optic microscopy (Orthoplan, Germany) at 40X magnifiction. The concentration of spores was adjusted to the appropriate working inoculums dilutionof 5,000 spores/ml.To determine the optimum test concentration for the dsRNA to use in spore germination inhibition bioassays, 0.6 ng/µl was selected in reference to work done by Mumbanza, F. Mixed concentrations of 15ml dsRNA, 10ml spore suspension and double distilled water were prepared for all samples apart from the controls which had odsRNA, 10ml spore suspension.10ml water. All were incubated overnight at 24˚C after which individual colonies established on the media were counted. The colony count was done by observing the plates through a window light and marking the colony positions on the plate bottom. The experiments were conducted under complete randomized design (CRD) with three replicas of each treatment.

Optimization of dsRNA :To determine the optimum concentration for the dsRNA to use in spore germination inhibition bioassays, 0.6 ng/µl was selected in reference to previous work done by Mumbanza, F. 2012.

2.6. Statistical analysis

After 24- 48 hours individual colonies were counted and expressed as percentage of the control plate cultural colonies. Spore germination inhibition was determined based on the following formula.

Inhibition % = 100- Treatment (Av. No. of colonies)X100

Control (Av. No. of colonies)

3. RESULTS

Results determining antifungal activity of synthetic dsRNA constructed to silence essential genes; Actin Related Protein 2/3 (D6), DNA Polymerase delta subunit (J6) and Adenylase cyclase (K6), can be used on passionfruit, and other Fusarium related diseases in other plants, animal and human medicine.

3.1. DNA quality and quantity

3.2. PCR amplification of genes D, J and K for samples 1-6

Screening for the best genes to amplify for RNAi antifungal assays involved preliminary steps of PCR with nine *Fusarium* essential genes. This was also done to confirm that target samples had *Fusarium* essential genes. Samples showing bands of the same size of 600bp are suspected to be related or the same *Fusarium* species.Target genes included: A, B, C, D, E, F, J, K and L

A-Nuclear condensing, B- Coatomern alpha, C-DNA directed RNA polymerase, D- ARP 2/3, E- Coatomer zeta, F- Cap methyltransferase, J-DNA polymerase delta subunit, K- Adenylate-cyclase and L- Protein Kinase C. (Nassimbwa, 2015).

3.3. Anti-fungal assay

3.4. Table 1.In vitro spore germination ds RNA inhibition of fungal re-issolates from C-Kawanda and P-Mpigi .

Anti-fungal assays were performed on the cream and purple pathogenic re-isolates from P. edulis. Results bellow show single colony counts done after 48 hours, these were compared with the control. Colony numbers percentage inhibition was established using the formula in section 2.4. Reaction efficiency of the assay was determined relying on the reduction in number of fungal colonies on PDA.

4. DISCUSSION

Treatment CT1 (dsRNA-D6) showed an inhibition of 87.3% which was the highest of the three treatments subjected to suspected *F. solani* spores. This was followed by CT2 (ds RNA-J6) which had an inhibition of -40% and lastly CT3 which had an inhibition of -69%.(Table 1)

CT1 had a positive % inhibition implying that the D6 dsRNA in this treatment was able to reduce the growth of 87.3% of the spores from the cream re-isolated pathogenic fungus as compared to the water treated negative control CT4. Unlike CT2 and CT3 which had a negative %inhibition of -40% and -69% respectively which means dsRNA

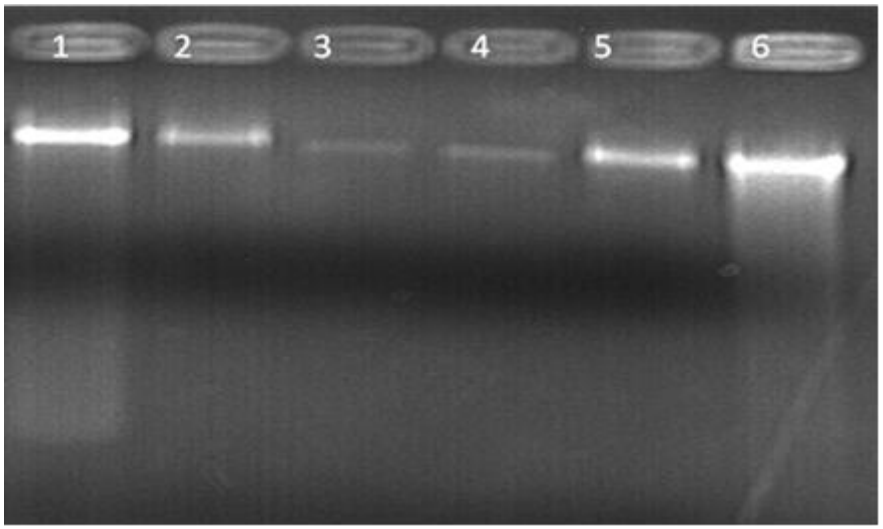

Figure 1: Fig 1.Loaded with 4μl of sample in each well, a representative0.6% agaroseGel electrophoresis profile of DNA extracted from various fungal mycelium sampled for collar rot and wilted passionfruit from farmer's fields in Central Uganda. Single bands imply that the DNA quality was good. Quantification of isolated fungal DNA (using a Nanodrop 2000C spectrophotometer. Producer; Thermal Scientific)1 Cream reisolate from Kawandaat a concentration of 448.8 ng/μl, 2 Purple isolate from Mukono 292 ng/μl, .3 purple isolate from Mpigi 168.3 ng/μl, 4 Purple isolate from Wakiso 64.9 ng/μl, 5 Foc (positive control Fusarium oxysporium f.sp cubensi) 112.4 ng/μl and X.6 Purple re-isolate from P.edulis from Mpigi514.5 ng/μl same as 3. Anti-fungal assays were only done for samples 1 and 6 which had already been proved to be pathogenic re-iolates by earlier studies by Nassimbwa 2015.

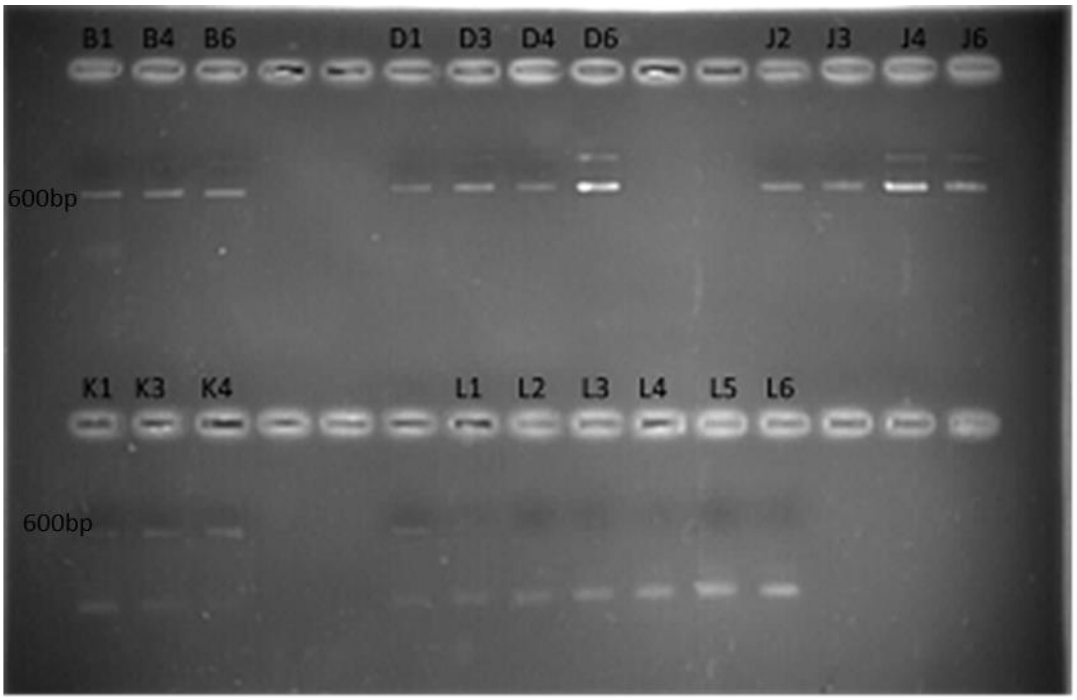

Figure 2: Gene snap of a 1.5% agarose gel stained in ethidium bromide forPCR Products with band size of 600bp. After a series of other PCR reactions on several genes (not shown), 3 genes were selected beased on the above gel i.e. D, J and K.1-6. representative samples are 1- Cream re-isolate from Wakiso, Kawanda, 2- Purple Mukono, 3-PurpleMpigi, 4-PurpleWakiso, Entebbe, 5- Purple (positive control Fusarium oxysporium f.sp cubensi) and 6-Purple re-isolate from Mpigi. NB. K6 is not represented but was also positive.

Table 1: **In vitro spore germination ds RNA inhibition of fungal re-issolates from C-Kawanda and P-Mpigi .**

TREATMENT	ds RNA	MEAN	% INHIBITION
1	CT1- D6	6.3	87.5
2	CT2- J6	38.0	-40.0
3	CT3- K6	45.7	-69.0
4	CT4- Control	27	-
5	PT1- D6	8	93.9
6	PT2- J6	42.7	67.7
7	PT3- K6	50.3	61.9
8	PT4- Control	132	-

from J6 and K6 respectively instead facilitated the germination of more spores i.e. by 40% and 69% respectively as compared to the water treated negative control CT4, this could be attributed to the fact that the spores recovered from the inhibition after 24 hours and were even more aggressive after this. dsRNA activity on purple fungus re-isolate originally from Mpigi and Wakiso

Treatment PT1 (dsRNA-D6) showed an inhibition of 93.9,% which was the highest of the three treatments subjected to suspected *F. oxsporium* spores. This was followed by PT2 (ds RNA-J6) which had an inhibition of 67.7% and lastly PT3 which had an inhibition of 61.9% (Table 1).

PT1, PT2 and PT3 had positive % inhibition implying that the dsRNA from D6, J6 and K6 respectively in these treatments was able to reduce the growth of 87.3%, 67.7% and 61.9% respectively of the spores from the purple re-isolated pathogenic fungus as compared to the water treated negative control PT4.

Usually a difference in the spore germination inhibition is an RNAi response and can be attributed to siRNAs silencing efficacy and level of complementarity between the target genes. https ://onlinelibrary.wiley.com/doi/10.1002/ps.3480

Based on percentage spore germination inhibition, this study showed that synthetic dsRNA molecules homologous to many of the selected target genes could be used as transgenes to engineer transgenic passion fruit with host induced RNAi system hence the potential to enhance resistance to *Fusarium oxysporum f.sp passiflorae* and *Fusarium solani*.

As suggested by Mumbanza (2012) this study also helps to establish a method which can be used as a possible way of identifying candidate target genes for RNA-mediated resistance against fungal pathogens. A key challenge for designing RNAi-based crop protection strategies is the identification of effective target genes in the pathogenic organism. In his study, in vitro antifungal activities of a set of synthetic double-stranded RNA molecules on spore germination of two major pathogenic fungi of banana, *Fusarium oxysporum Schlecht f. sp. cubense* WC Snyder & HN Hans (Foc) and *Mycosphaerella fijiensis Morelet* (Mf) were evaluated. Results: All the tested synthetic dsRNAs successfully triggered the silencing of target genes and displayed varying degrees of potential to inhibit spore germination of both tested banana pathogens. When Foc dsRNAs were applied to Foc spores, inhibition ranged from 79.8 to 93.0%, and from 19.9 to 57.8% when Foc dsRNAs were applied to Mf spores. However, when Mf dsRNAs were applied on Mf spores, inhibition ranged from 34.4 to 72.3%, and from 89.7 to 95.9% when Mf dsRNAs were applied to Foc spores. Conclusion: The dsRNAs for adenylate cyclase, DNA polymerase alpha subunit and DNA polymerase delta subunit showed high levels of spore germination inhibition during both self- and cross-species tests, making them the most promising targets for RNA-mediated resistance in banana against these fungal pathogens. (Mumbanza, 2012).

5. CONCLUSSION

Anti-fungal assay results show that Actin Related Protein 2/3 (ARP) gene would be the best for further transformation of passionfruit for resistance against both *Fusarium* collar rot and wilt pathogens. The observation can lead to the speculation that DNA sequences of target genes in these two fungi present some similarities and share homologous genes. For this reason it would be a promising target gene for RNAi –mediated resistance in passion fruit against the target fungal diseases. Furthermore as suggested by Mumbanza (2012) this study also helps to establish a method which can be used as a possible way of identifying candidate target genes for RNA-mediated resistance against fungal pathogens.

6. RECOMMENDATION:

Based on percentage spore germination inhibition, this study showed that synthetic dsRNA molecules homologous to many of the selected target genes could be used as transgenes to engineer transgenic passionfruit with potential to enhance resistance to Fusarium oxysporum f.sp passiflorae and Fusarium solani.

7. LIST OF ABBREVIATIONS

ATAAS: Agricultural Technology and Advisory Services
C: Kawanda isolate
ds RNA: double stranded Ribo Nucleic Acid
Fop: Fusarium oxsporum f.sp passiflorae
Foc: Fusarium oxsporum f.sp cubensi
Fo: Fusarim oxysporum
Mf: Mycosphaerella fijiensis Morelet
MDI: Mean Disease Index
NABC: National Agricultural Biotechnology Center
NARO: National Agricultural Research Organization
P: Mpigi isolate
PDA: Potato Dextrose Agar
RNAi : Ribo Nucleic Acid interference

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Author biography

Florence Nassimbwa (MSc. Molecular Biology) is a Biologist (Lecturer) and an instructor (FOO) at DAS Aviation School, Entebbe, and a Ph.D. candidate in Biological sciences at Kyambogo University. I have knowledge and experience in Biological sciences and Aviation Human Performance and Limitations(Human Factors).

Enock Matovu Ass. Prof. Enock Matovu obtained his Ph.D. in Molecular Parasitology from the University of Bern, Switzerland in 2001, while he worked as a Research Officer at the Livestock Health Research Institute, Tororo, Uganda. Since then has continued his work on drug resistance and later diagnostics for African Trypanosomiasis. In 2004, he relocated to the Makerere University School of Veterinary Medicine, where he was first employed as a Lecturer. In 2008, Enock received the prestigious Royal Society Pfizer Award in recognition of his work on molecular mechanisms of drug resistance in African trypanosomes. The year (2007) he had obtained the Joint Third World Academy of Science Award for Young Scientists, for his contribution to the field of Molecular Parasitology. Enock Matovu has vast experience in HAT ranging from surveillance,diagnostics, drug resistance, and clinical trials.

Andrew Kiggundu Senior research scientist with National Agricultural Research Organization (NARO). University of Pretoria, South Africa. Forestry and Agricultural Biotechnology Institute (FABI) and the Department of Botany, Ph.D. in Plant Biotechnology.

Charles Changa National Biotechnology Research Center/ Tissue Culture, National Agricultural Research Laboratories. P. O. Box 7065 Kampala, Uganda.

Francis Mumbanza Researcher at the University of Ghent: Contact (voluntary lab member). CAVElab - Computational & Applied Vegetation EcologyFrancis Mumbanza Mundondo Department of Environment, Faculty of Bioscience Engineering.

John Adriko Is a Crop Scientist with experience in both field agriculture and research. Dr. Adriko holds a Ph.D. in Plant Pathology from the University of Copenhagen, a Masters's Degree in Crop Science, and a bachelor's Degree in Agriculture, both from Makerere University. He has valuable skills and experience in agriculture and food security obtained during his work with District Local Government Production Department, National Agricultural Advisory Services (NAADS), and National Agricultural and Research Organization (NARO). He also worked as Agricultural Consultant for NilePro Consult Limited and is now Arise Development Consultant playing a key role in training farmers on agriculture production for income and food security for the Arua District Local Government, World Vision, CARE Uganda, and Eastern Archdiocesan Development Network (EADEN). He also worked as Field Extension Agent for Farm Talk/ Tree Talk in West Nile (Straight Talk Foundation). He has carried out farmer training in improved crop production for Governmental as well as Non-governmental institutions. Dr. Adriko was the agriculture technical expert in a market access survey done for

ZOA Uganda and the Lead Consultant in agriculture,food security, and child nutrition analysis for Plan Uganda.