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Rapid Screening of *In-Vitro* Regenerated Plantlets of Four Nigerian Cowpea Varieties to Artificially-Inoculated Fungal Wilt Pathogen

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Abstract
Tissue culture technique provides a rapid means of studying plant-pathogen interaction in a controlled environment with further advantage of saving time and resources. Embryonic axes of three local cowpea (*Vigna unguiculata* (L.) Walp) varieties (Oloyin, Ife-Brown and Erusu) and one elite line IT86D-1010 were cultured *in-vitro* and plantlets challenged with spore inoculum of Fusarium wilt pathogen – *Fusarium oxysporum* f.sp. *tracheiphilum in order to* determine the response of the plantlets to the pathogen infestation. A solid MS medium amended with growth hormone, benzylaminopurine BAP at 1, 2, and 3 mg BAP/L was used for shoot induction. Mean disease severity score of 4.9 was recorded for Oloyin variety. There was no significant difference between the response of Ife-Brown cowpea variety and IT86D-1010 to Fusarium wilt pathogen. Least disease severity score of 2.9 was recorded for Erusu variety. Erusu was also consistently low in disease incidence and severity all through the assessment period. It was suggested that local Erusu variety be screened on the field for confirmation of this *in-vitro* observation.

Keywords: cowpea, *in-vitro* regeneration, hormones, Fusarium wilt, protocols.
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Introduction

Cowpea, the grain legume most traded and consumed in tropical Africa (Armstrong and Armstrong, 1980), is a major source of protein for rural and poor urban people (Singh *et al.,* 2002). Overall grain yield of cowpea in Nigeria traditional system has been identified to be between 0 – 300 kg/hawhich is considered low and has been attributed to a complex interaction of biotic and abiotic factors (Singh *et al*., 2002).

The increasing importance of cowpea production in some regions of Africa and the wide distribution of the Fusarium wilt described by Oyekan (1975) had necessitated further work in this unique plant-pathogen interaction. Race 1 of *Fusarium oxysporum* f. sp. *tracheiphilum* (E. F. Sm.) Snyd. & Hans. (Fot) had been identified to cause the vascular wilt of cowpea in Nigeria (Armstrong and Armstrong, 1980).

Tissue culture techniques had been used successfully to identify and select plants with desirable traits, resistance to disease inclusive (Daub, 1986). Thus, investigation of host-pathogen interactions *in vitro* is an efficient way of gaining a faster and comparatively cheaper method of studying plant response to pathogen invasion (Huang, 2001).

This study was aimed at screening *in-vitro* regenerated plantlets of four Nigerian cowpea varieties for resistance to artificially-inoculated fungal wilt pathogen, *Fusarium oxysporum* f.sp. *tracheiphilum*.

Materials and Methods

Source of cowpea varieties:- Seeds of Nigerian local varieties of cowpea (Erusu and Ife-Brown) were obtained from the Institute of Agricultural Research and Training (IAR & T), Ibadan, Nigeria. Oloyin, another local variety, was obtained from National Centre for Genetic Research and

Biotechnology, NACGRAB, Ibadan, Nigeria. Seeds of an elite line, IT86D-10-10, were obtained from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria.

Preparation of cowpea explants:- Embryonic axes excised from the seeds were the explants used in this study. Cowpea seeds were separately soaked in 1 liter of sterile water containing 0.3% sodium hypochlorite (NaOCl) overnight. They were rinsed three times with sterile water and then soaked again in a freshly prepared sodium hypochlorite solution. The seeds were allowed to stay for one hour in this solution after which the seeds were washed three times with sterile water. These activities were performed under a sterile laminar hood.

Embryonic axes from forty-five seeds in each cowpea variety were excised, and the apical segments of plumules and radicles were removed to obtain decapitated embryonic axes. The decapitated embryonic axes were subjected to shoot and root elongation, acclimatization and later transferred to screenhouse.

Shoot proliferation and elongation:- The basal medium (4.43 g/L MS, 5 ml/L B5 vitamins, 0.1 g/L inositol, 30 g/L sucrose, pH 5,7, 7 g/L agar) was supplemented with 0.1, 0.3 and 0.5 mg BAP/L. This constituted shoot proliferation medium. Shoots were kept in the medium for 20 days with transfer into fresh medium after the first 10 days. For shoot elongation, the same basal medium as above was supplemented with 0.05, 0.10 and 0.15 mg BAP/L. The shoots stayed in this medium for 20 days.

Root induction:- Regenerated shoots were transferred into basal medium containing IAA (an auxin) supplementation at 0.01 and 0.05 mg IAA/L.

Table 1. Concentration of cytokinin and auxin in growth media

Growth media	Hormone concentration (mg/L)		
Cytokinin (mg BAP/L)	First Application	Second Application	Third Application
Shoot Induction	1.00	2.00	3.00
Shoot Proliferation	0.10	0.30	0.50
Shoot Elongation	0.05	0.10	0.15
Period of transfer(days)	20	20	20
Auxin (mg IAA/L)			
Root Induction	0.01	0.05	
Period of transfer (days)	20	20	

Acclimatization:- In vitro rooted plantlets were removed from the rooting medium and washed to remove adhering gel and transplanted into plastic pots containing sterilized soil and coconut shaft at ratio 20:1 and covered with white nylon tied in an upright condition on a pole under shade. The well grown plants were removed after two weeks and then transferred to the screen house to allow for further growth. Plantlets were grown in three replications for each treatment, each replication having 10 plants.

Evaluation of *in vitro*-regenerated cowpea plantlets for resistance to *Fusarium* wilt

Preparation of inoculums:- Fusarium oxysporum f. sp. *tracheiphilum (Fot)* Race 1 was obtained from International Institute for Tropical Agriculture, IITA, Ibadan. The culture was resuscitated on Potato Dextrose Agar (PDA) at 28^{o}C for 48 hours, and sub-cultured on fresh PDA to obtain a pure culture. Pathogenicity test was performed to confirm the pathogen as the causative organism for cowpea vascular wilt.

Ten (10) ml of sterile distilled water was poured over a 3-day old culture plate of *Fot.* Sterile rod was used to dislodge the conidia and the conidial suspension poured into a beaker. It was standardized to 10^{7} conidia/ml and kept for use.

Inoculation of cowpea plantlets:- Transplanted plantlets in screenhouse were inoculated with 10 ml of 10^7 conidia/ml *Fot* applied to the base of each plant (Adhikari et al*.,* 1993).

*Disease severity rating:-*A modified scale of Lebeda and Buczkowski (1986) was used.

Table 2. Scale for disease severity rating

Disease Severity*	Inference
1. Symptomless, stems and leaves free of any visual symptoms	Immune (I)
2. Very limited wilting, 5% plant tissue wilted.	
3. Limited wilting, 6 – 10% plant tissue wilted	Resistant(R)
4. Moderate wilting, 11 – 20% plant tissue wilted	Moderately Resistant (MR)
5. Severe wilting, 21 – 50% plant tissue wilted	Moderately Susceptible (MS)
6. Very severe wilting, above 50% plant tissue wilted	Susceptible (S)
	Highly Susceptible (HS)

Disease assessment using AUDPC (Area Under Disease Progress Curve):- The area under disease progress curve (AUDPC) was calculated for plantlets from the disease severity score, according to the technique of Shanner and Finney (1977). Mean values were then obtained per variety.

AUDPC formula used for calculation was:

$$\Sigma_i [(D_i + D_{i-1})*(t_i - t_{i-1})]/ 2$$

where D = Disease score using the 1 – 6 severity scale,

t = days after inoculation, and i = 3, 7, 14, 21, 28 and 35 days after inoculations.

Cumulative AUDPC was calculated from the individual values obtained for each variety.

Data analysis:- Results on the number of successfully transferred plantlets, number acclimatized, number of surviving plants, and rate of disease severity were recorded. Data collected were subjected to analysis of variance and means separated by Fisher's Protected Least Significant Difference, LSD, at 5%.

Results

Number of cowpea plantlets acclimatized and number of survival:- IT86D-1010 had the highest percentage survival of 82.83%. The local variety that was closest to this was Erusu with percentage survival of 71.49%.

Table 1 : Percentage survival of acclimatized plantlets.

Variety	% Survival
Oloyin	44.33
Ife-Brown	55.67
Erusu	69.28
IT86D-1010	75.05
Lsd (5%)	13.2

Response of cowpea plantlets to infection by F. oxysporum f. sp. ttracheiphilum:- Table 2 showed the disease severity score of cowpea varieties 35 days after the inoculation. Oloyin was the most susceptible with severity score of 4.93. Erusu with disease severity score of 2.93 was moderately resistant. There was no significant difference in the response of Ife-Brown and IT86D-1010 to Fusarium wilt pathogen.

Table 2: Disease severity score for *in vitro* plantlets of four cowpea accessions.

Varieties	Disease severity score	Inference*
Oloyin	4.93	Susceptible, S
Ife-Brown	4.13	Moderately susceptible, MS
Erusu	2.93	Moderately resistant, MR
IT86D-1010	4.07	Moderately susceptible, MS
LSD (5%)	0.28	

Evaluation of area under disease progress curve (AUDPC):- The reactions of the four cowpea varieties to the *Fusarium* wilt of cowpea inoculum, evaluated as Area Under Disease Progress Curve (AUDPC) on five assessment days at 3, 7, 14, 21, 28 and 35 days after inoculation are shown in Fig. 1. Erusu was consistently low in AUDPC all through the assessment.

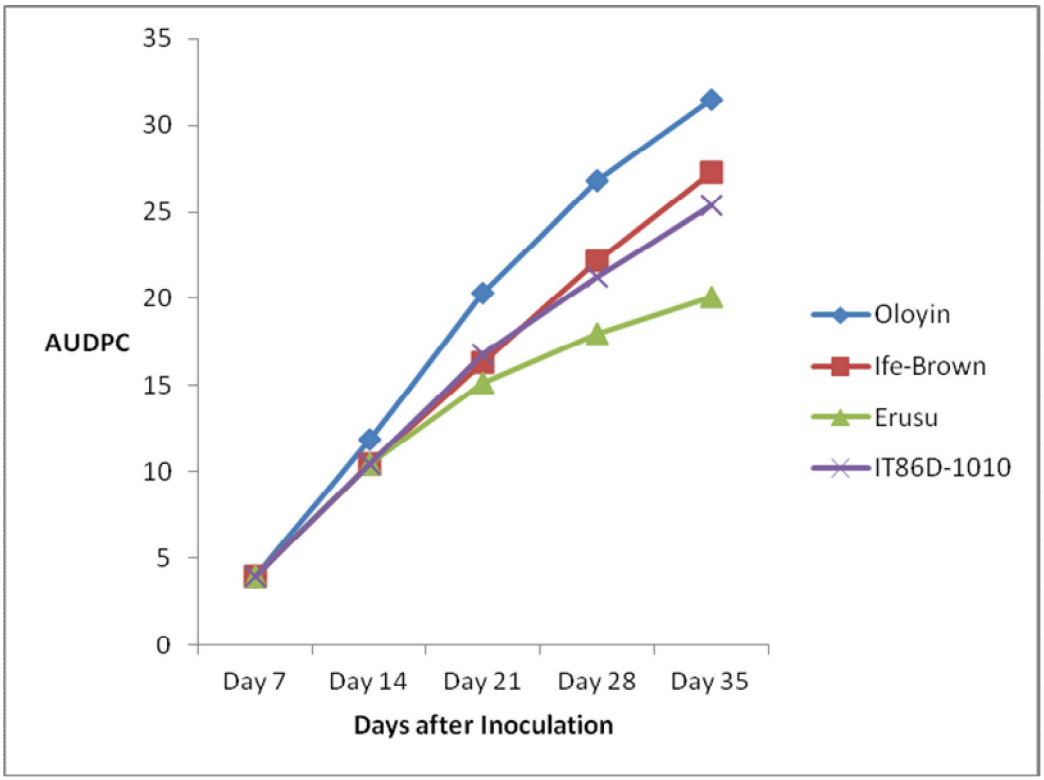

Fig. 1: Pattern of disease progress in four cowpea varieties

Discussion

Tissue-cultured derived variants of Erusu showed improved resistance to Fusarium wilt when compared with study where local cowpea varieties propagated from seeds succumbed to Fusarium wilt invasion (Fawole, 2010). The use of tissue culture-induced variability for obtaining desirable genetic variation for crop improvement had been recorded over two decades ago (Nickell, 1977; Daub, 1986). In more recent studies, researchers found that plants regenerated from callus and protoplast cultures varied in a number of traits, including morphological characteristics, maturity date, yield, and response to pathogens (Rajeswari et al., 2009; Chamandoosti and Azad, 2012). The varieties of crops that exhibit desirable somaclonal variation has lead Scowcroft and co-workers to conclude that the phenomenon is ubiquitous (Scrowcroft et al., 1983). Workers who had carefully compared variation between culture-derived and asexually or seed-propagated plants concluded that more variation occurred with plants regenerated from culture (Biswas et al., 2009; Wang and Wang, 2012).

Report by Aigbe and Fawole (2010) indicated that Ife – Brown was susceptible to *Fusarium* seed rot pathogen. Oyekan (1975) also reported *Fusarium* wilt incidence of 21 percents in Ife – Brown variety of cowpea. Another important factor in evaluating plant genotype for disease resistance is the adequate timing of disease assessment. The strong significant effect of time-variety interaction in this research work indicates that varieties have different disease rating curves over time. The use of multiple point assessment takes into account the onset and the progression of disease for field evaluation.

In this study, evaluation at 28 days after inoculation showed a high correlation with evaluation at 35 days after inoculation which appear to be the optimal critical stage for single point disease assessment. Haynes and Weingarten (2004) indicated that it is often impossible or difficult to make several sequential disease assessments that enable the calculation of AUDPC due to constraint of time, finances and logistics. However, theoretical research by Jeger and Viljamen-Rollinson (2001) asserted that with as few as two assessments, much information could be generated from AUDPC as with many sequential assessments.

In conclusion, this work had shown that tissue-cultured Erusu was moderately resistant to Fusarium wilt. It is left to plant breeders to investigate the heritability of this positive trait. But as advances are made in mutation genetics, biotechnology and molecular plant pathology, more rapid and cost efficient screening methods shall continue to be needed to enhance the efficiency and success rate of a robust resistance breeding programmes. This work had provided a reminder to this area of research.

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