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Table of Content: Volume 15 Number 19 11 May, 2016

ARTICLES

An efficient protocol for *in vitro* organogenesis and antioxidant studies in *Melia dubia* Cav. 768
Syed Naseer Shah, Tareq Ahmad Wani, Bhimi Ram, Monika Koul, Praveen Awasthi, Deependra Singh Rajput and Gillela Ravi Shanker Reddy

A novel pig feed formulation containing Aspergillus niger CSA35 pretreated-cassava peels and its effect on growth and selected biochemical parameters of pigs 776
Nyerhovwo J. Tonukari, Egbune E. Oliseneku, Oghenetega J. Avwioroko, Eferhire Aganbi, Osuvwe C. Orororo and Akpovwehwee A. Anigboro

Inhibition of cell death as an approach for development of transgenic resistance against Fusarium wilt disease 786
Betty Magambo, Khanna Harjeet, Geofrey Arinaitwe, Sali Tendo Ivan Kabiita Arinaitwe, Jerome Kubiriba, Wilberforce Tushemereirwe and James Dale

Evaluation of potential bio-control agents on root-knot nematode *Meloidogyne incognita* and wilt causing fungus *Fusarium oxysporum* f.sp. *conglutinans in vitro* 798
Rajinikanth Rompalli, Sreenivasa Rao Mehendrakar and Pavani Kantabathini Venkata

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African Journal of Biotechnology

Full Length Research Paper

An efficient protocol for *in vitro* organogenesis and antioxidant studies in *Melia dubia* Cav.

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***Melia dubia* Cav. (Meliaceae) is a multipurpose tree of tropical and subtropical regions mainly cultivated for its medicinal and industrial importance. Due to its versatile properties, it has been depleted in its natural environment. Moreover due to sluggish and poor seed germination, there is a threat of its gene pool exclusion from the natural habitat. The alternative method for conservation and efficient mass propagation is thus need of the hour. As per the extensive literature survey there is no report on efficient protocol for mass propagation of *M. dubia* through callus organogenesis. Therefore, the present work was aimed to develop *in vitro* organogenesis protocol for rapid and large scale production of planting material. From our results, maximum callus percentage, callus weight and fragile callus was observed on 1.0 mg/l benzylaminopurine (BAP) in combination with 0.5 mg/l naphthalene acetic acid (NAA). The callus differentiation was achieved at different concentrations of BAP and indole acetic acid (IAA). Multiple Shoot number per callus propagule 5.30 was observed on 0.5 mg/l BAP and 1 mg/l IAA concentration. The maximum rooting percentage (78.5%), root number per explant (4.33) and root length per explant (4.41 cm) was observed at 0.5 mg/l indol butyric acid (IBA) after 30 days of inoculation. Further the total flavonoid content, phenolic content and antioxidant properties of leaves of *in-vitro* regenerated plants where studied. Total flavonoids and phenolic content in leaves of *in vitro* Melia dubia* was 0.56 ± 0.8 mg quercitin equivalent (QE) and 2.97 ± 0.17 mg gallic acid equivalent (GAE) respectively. The antioxidant property was further assed through measurement of DPPH radical scavenging activity. The *in-vitro* regeneration protocol can be exploited for commercial cultivation and fulfilling the growing demand for fresh explant material through mass propagation of *M. dubia* an economically important plant species.**

Key words: *Melia dubia*, antioxidant, indole-3-butyric acid, flavonoids and phenolics.

INTRODUCTION

Melia dubia, a dicotyledonous multipurpose tree belonging to family Meliaceae, has huge commercial and industrial potential. The species is native to southern Asia (India-Pakistan-Iran) and has been introduced to South Africa, Middle East, America (Bermuda, Brazil and Argentina), Australia, Southeast (SE) Asia-Pacific Islands and

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southern Europe (Ram et al., 2014). Due to its multi-purpose uses like bioenergy production, paper and pulp manufacturing, furniture making, building constructions, making musical instruments etc., it has gained a great deal of attractiveness and elevated demand (Mandang and Aristien, 2003; Suprapti and Hudiansyah, 2004; Parthiban et al., 2009; Chinnaraj et al., 2011). Other than its industrial and commercial importance, the plant has proficient medicinal properties. The various extracts from different parts of the plant are known to have pharmacological importance which includes, antiviral, antibacterial, antifungal, antidiabetic, antineoplastic, antihelmintic and antileprosy properties (Kiritkar and Basu, 1999; Pettit, 2002; Nagalakshmi et al., 2003; Vijayan et al., 2004; Gerige and Ramjaneyulu, 2007; Susheela et al., 2008; Sukumaram and Raj, 2010; Sharma and Arya, 2011). Due to its elevated demand for commercial, industrial and therapeutic basis, *M. dubia* trees growing naturally have been indiscriminately logged which resulted in significant decline in its population. Conventionally, *M. dubia* is propagated through seeds, which have very poor (14-34.3%) germination rates because of hard stony seed coat, which makes it difficult to germinate without any treatment (Nair et al., 2005; Manjunatha, 2007; Anand et al., 2012). Therefore, it is imperative to use an efficient plant regeneration system under *in vitro* conditions for large scale production of planting material of the species from superior genotypes for quick rejuvenation. For large scale production, efficiency of propagation method is imperative. The purpose of micropropagation is to develop physiologically stable plantlets which can be acclimatized in a reduced time period and at a lower cost. In this backdrop, we have established a well developed *in vitro* regeneration system for *M. dubia* for rapid and large scale production of planting material. There are many reports of *in vitro* regeneration of the plant through axially bud proliferation (Ram et al., 2014), but it is the first report of regeneration through *in vitro* callus organogenesis. The ethanolic extract of field grown plants has promising antioxidant activity with IC_{50} (16.89 µg/ml) value (Valentina et al., 2013). In this direction, the present study was envisaged to evaluate the antioxidant activities of leaf tissue from *in vitro* regenerated plants of *M. dubia*. This activity can be ascribed to the phenolic and flavonoid compounds present in the species. Consequently the study was extended to determine total flavonoid and phenolic content of leaf tissue. The present protocol can serve as an important tool for commercial and industrial supply of bulk plant material of *M. dubia*. The tissue culture raised plants were further evaluated for their total phenolic and flavonoid content viz-a-viz its antioxidant property.

MATERIALS AND METHODS

Explants collection

Experiments were carried out in plant tissue culture laboratory of the Institute of Wood Science and Technology (IWST), Bangalore. Four Simple Randomized experiments were carried out. Explants of *M. dubia* were collected from established cultures grown under aseptic *in vitro* conditions. The leaf explants were inoculated on MS medium (Murashige and Skoog, 1962) supplemented with different doses of N^6-Benzyladenine (BA; 0.25, 0.5 and 1.0, 2 mg/l) and α-Naphthalene acetic acid (NAA; 0.1, 0.25, 0.5 and 1.0 mg/l) for callus initiation in first experiment. In second experiment callus multiplication was observed on MS medium supplemented with BA and NAA at their best combination. In the third experiment, healthy shoots were developed on benzylaminopurine (BAP; 1.0, 1.25, 0.5 and 0.25 mg/l) and Indole-3-acetic acid IAA (0, 1.0 2.0 mg/l). In fourth experiment *in vitro* raised micro-shoots were transferred to MS/2 strength rooting medium supplemented with different doses of Indole-3-butyric acid (0.1, 0.25, 0.5 and 1.0 mg/l).

Culture conditions

The inorganic salts used for preparation of culture medium were obtained from Qualigens Pvt. Ltd., India and phytohormones and B vitamins from Sigma Chemicals Pvt. Ltd., India. The medium contained 3% (w/v) sucrose, 0.6% (w/v) agar (Hi-Media chemical Ltd., India). The pH of the medium was adjusted to 6.0 before autoclaving for 15 min at 1.06 kg cm^{-2} (121°C). Explants were cultured in a 150 ml Boatel Borosil® Boatels containing 40 ml semi-solid medium. For *in vitro* shoot multiplication and rooting experiment, the cultures were incubated at 25 ± 2°C under 16 h illuminations with fluorescent light (50 µmol $Em^{-2} s^{-1}$).

Hardening and transplantation

Tissue culture raised microshoots of 3 to 4 cm in length with 2 to 3 nodes were tested with various concentrations of IBA, indol butyric acid (IAA) and NAA either alone or in combinations for in vitro rooting. The rooted plantlets of *M. dubia* were removed from agar-agar and washed with distilled water to remove the traces of agar. Plantlets were transferred to root trainers containing autoclaved potting mixture of vermiculite, sand and soil (1 : 2 : 1 *v/v/v*) and placed under 4 weeks in mist chamber at 30 ± 5°C temperature and > 60% relative humidity (RH). Later on the plants were kept in polythene bags containing autoclaved soil and then shifted to a shade net (50% shade) for another 2 weeks before placing it in open nursery. After successful acclimatization plants were finally transferred to natural condition.

Quantification of total flavonoid content

Total flavonoid content was quantified using spectrophotometer. Dried crude extract (prepared from 100 mg of dried plant material) dissolved in 500 µl of distilled water was mixed with 30 µl of a 5% $NaNO_2$ solution, followed by 5 min of incubation at room temperature. After the incubation, 300 µl of 10% $AlCl_3.H_2O$ solution was added and the sample was further incubated for 6 min incubation. Finally 200 µl of 1 M NaOH and 200 µl of distilled water were added to the sample and absorbance was read at 510 nm. Total flavonoids were calculated using quercetin as standard (10 to 100 µg; R2 = 0.998). The results were expressed as mg quercitin equivalent (mg QE) per gram dry weight of the plant material. The experiment was repetitively performed in triplicates.

Quantification of total phenolic content

Total phenolic content in leaves of *M. dubia* was measured using the Folin–Ciocalteu reagent method as described by Pinelo et al. (2004). The total phenolic content was expressed as mg of Gallic

Table 1. Effect of of BA, NAA and their interaction on callus induction (%), callus weight and texture of callus induction in *M. dubia* at 30 days after inoculation. Values in the parentheses are arc sine transformation.

Treatment numbers	30 Days after inoculation			
	BAP +NAA (mg/l)	Callus formation (%)	Fresh weight (g)	Callus texture
T_1	BAP (0.25) + NAA (0.1)	69(56.5)	1.84±0.20*	Compact
T_2	BAP (0.5) + NAA (0.25)	83(70.08)	2.13±0.32	Compact
T_3	BAP (1.0) + NAA (0.5)	100(85.84)	2.44±0.36	Fragile
T_4	BAP (2.0) + NAA (1.0)	66(54.33)	1.72±0.19	Compact
LSD (0.05)		0.60	0.08±0.01	

*S.E; (LSD= Least Significant Difference).

Table 2. Effect of BAP and NAA on in vitro callus multiplication of *M. dubia* at 30 days after inoculation.

Treatments	30 Days after inoculation	
	BA and NAA (mg/l)	Fresh weight (g)
T_1	BAP	3.47±1.03*
T_2	BAP (0.25) + NAA (0.25)	4.60±1.65
T_3	BAP (1.5) + NAA (0.5)	5.62±1.80
T_4	BAP (2.5) + NAA (1.0)	4.23±1.55
$LSD_{(0.05)}$		0.19±0.05

*SE (LSD= Least Significant Difference).

acid equivalent (GAE) per gram of dry weight of the sample. The experiment was repetitively performed in triplicates.

DPPH radical-scavenging activity

Measurement of radical scavenging property of *M. dubia* was carried out according to the method described by Blois (1958). Ascorbic acid was used as positive control and % inhibition was determined according to the following equation:

$$\%Inhibition = [(A_{DPPH} - A_S) / A_{DPPH}] \times 100$$

Where, A_S is the absorption of the solution when the sample extract was added at a particular concentration and A_{DPPH} is the absorbance of the DPPH solution. Three experimental replicates were taken for the assay. The IC_{50} values were calculated as the concentration of extracts causing 50% inhibition of DPPH radical; a lower IC_{50} value corresponds to a higher antioxidant activity of sample.

Statistical analysis

Each experiment had three replicates for *in vitro* shoot multiplication and rooting. Each replicate had 10 propagules. The data were subjected to one way analysis of variance (ANOVA). All the experiments ascertained with “F” test for level of significance. The significance at $p \leq 0.05$, $LSD_{0.05}$ was computed for comparison of treatment means.

RESULTS

Callus induction

The experiment was aimed to investigate the different doses of BA and NAA (0.25, 0.5, 1.0, 2, 0.1, 0.25, 0.5, and 1.0 mg/l) on callus induction weight and callus texture at 30 days after incubation (Table 1). The effect of different doses of BA and NAA and their interaction on callus induction (%), weight and callus texture was found to be significant at the stage of sampling. Maximum callus induction, callus fresh weight and the best callus texture was observed on 1.0 mg/l BA and 0.5 mg/l NAA which was 57.99 and 41.86% in comparison to BAP 2.0 mg/l + NAA 1.0 mg/l. Fragile callus was observed on 1.0 mg/l BA and 0.5 mg/l NAA and compact callus was observed on other treatments.

Callus multiplication

The experiment was carried out to investigate the different doses of BA and NAA (0, 0.25, 1.5, 2.5, 0, 0.25, 0.5, and 1.0 mg/l) on callus multiplication at 30 days after incubation (Table 2). Various doses of BA and NAA significantly influenced callus multiplication at the stages of sampling Figure 1a. Fresh weight of callus was found to be maximum on 1.5 mg/l BA and 0.5 mg/l NAA which was 51.6% more in comparison to 2.5 mg/l BAP + 2.5 mg/l NAA at the stage of sampling.

Callus differentiation

Various doses of BA and IAA (1.0, 1.25, 0.5, 0.25, 0, 1.0 and 2.0 mg/l) significantly influenced callus differentiation

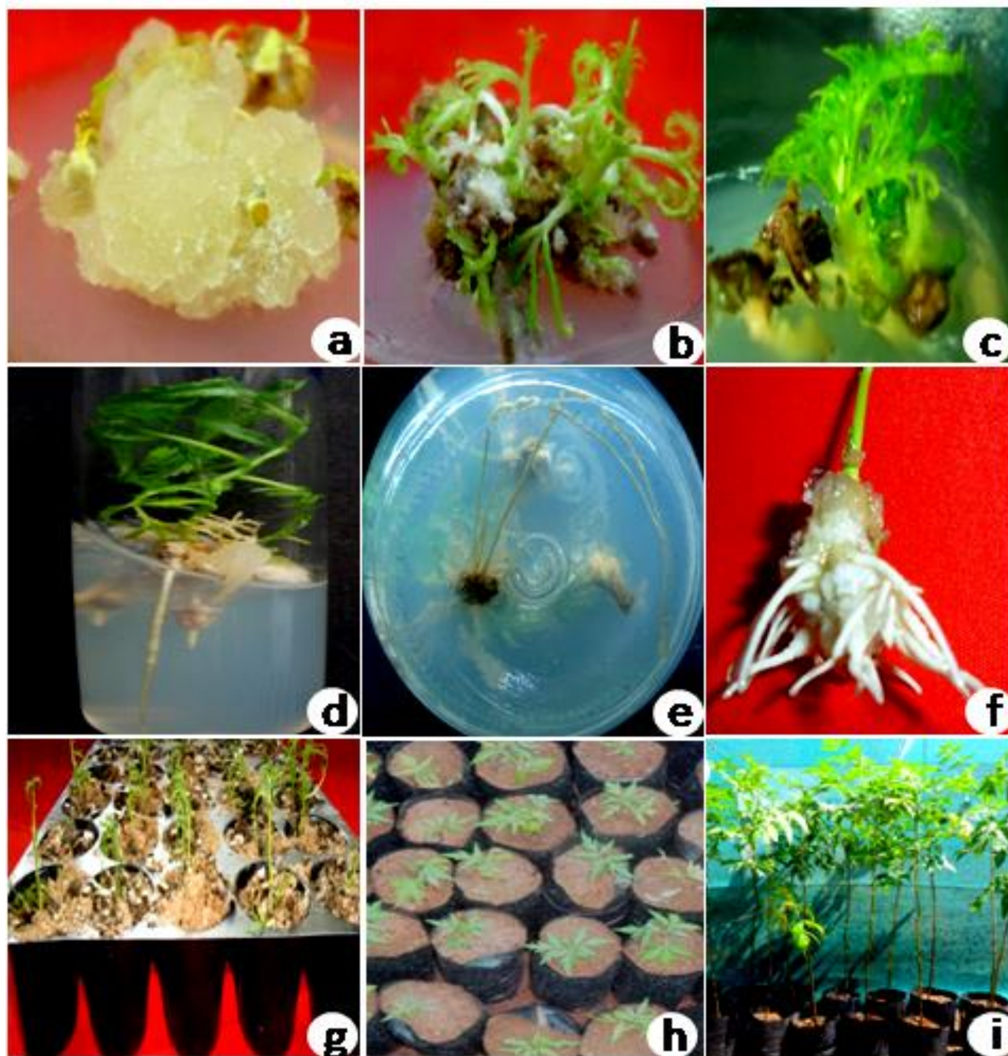

Figure 1. Effect of different phytohormones on different developmental stages: Callus multiplication **(a)**, Callus differentiation (b), shoot induction (c), Efficient rooting (d-f), Hardening and acclimatization of in vitro raised plants transferred in root trainers containing potted mixture for four weeks in mist chamber **(g)**, Plants transferred to polythene bags containing autoclaved soil **(h)** and full grown hardened plants in open environment **(i)**.

at 30 days after inoculation Figure 1b. The highest shoot number per callus propagule was observed on 0.5 mg/l BA and 1mg/l IAA. Shoot number per callus propagule was 133% more in comparison to treatment 1 Figure 1c. Shoot length per callus propagule was recorded at 30 days after inoculation. Shoot length per callus propagule was found to be maximum at 0.5 mg/l BA and 1 mg/l IAA which was 175% more in comparison to treatment 1 (Table 3).

In vitro rooting

The experiment was carried out to study the effect of four doses of IBA (0.1, 0.25, 0.5 and 1.0 mg/l) with MS/2 medium and their all possible interactions on *in vitro* rooting (%) and root number per explant at 30 days after inoculation (Table 4). Various doses of IBA significantly influenced rooting (%) at the stage of sampling Figure 1d-f. The effect of 0.5 mg/l IBA provide significantly maximum rooting % which was 71% more in comparison to 0.25 mg/l IBA.

Root number per explant

The effect of four doses of IBA significantly enhanced root number per explant at 30 days after inoculation. 0.5 mg/l IBA provide significantly maximum on root number per explant which was 270% more in comparison to 0.25

Table 3. Effect of BAP and IAA on callus differentiation in *M. dubia* at 30 days after inoculation.

Treatments	30 Days after inoculation		
	BAP+IAA (mg/l)	**Shoot number/explant**	**Shoot length/explant (cm)**
T_1	BAP (1.0)	2.27±0.32*	1.27±0.22*
T_2	BAP (1.25)	3.13±0.54	1.37±0.27
T_3	BAP (0.5) + IAA (1.0)	5.30±1.23	3.53±0.64
T_4	BAP (0.25) + IAA (2.0)	4.27±1.10	3.27±0.61
LSD (0.05)		0.28±0.03	0.28±0.02

*SE (LSD= Least Significant Difference).

Table 4. Effect of different concentrations of IBA and MS/2 medium on *in vitro* rooting %, root number and root length in *M. dubia* at 30 days after inoculation. Values in the parentheses are arc sine transformation.

Treatment numbers	30 Days after inoculation			
	IBA (mg/l)	**Rooting (%)**	**Root number per explant**	**Root length per explant (cm)**
T_1	IBA (0.1)	56 (49.2)	1.27±0.26*	1.37±0.32*
T_2	IBA (0.25)	53 (48)	3.22±1.25	3.23±1.30
T_3	IBA (0.5)	96 (78.5)	4.33±1.54	4.41±1.61
T_4	IBA (1.0)	60 (51.4)	1.17±0.21	1.13±030
LSD (0.05)		20.21	0.19±0.02	0.13±0.02

*SE (LSD= Least Significant Difference).

Table 5. Measurement of total flavonoids, phenolics contents and antioxidant activity in extracts prepared from leaves of tissue culture raised plant of *Melia dubia.*

Material	Total flavonoid content (mg QE)	Total phenolic content (mg GAE)	IC_{50} (µg/ml))
Leaves	0.56 ± 0.08*	2.97 ± 0.17*	242.88 ± 12.55*

*S.E, P-value < 0.05. IC_{50}: the concentration of extracts (µg/ml) causing 50% inhibition of DPPH radical.

mg/l IBA.

Root length per explant

Administration of different doses of IBA significantly enhanced root length per explant at 30 days after inoculation. 0.5 mg/l IBA provide significantly maximum for root length per explant which was 290% more in comparison to 0.25 mg/l IBA.

Hardening

Following hardening procedures, 6-weeks old hardened plants (Figure 1g-i) were successfully transferred to field conditions. The survival rate of *in vitro* raised plants under hardening conditions showed prominent growth with about 94% success. This gives the suitability of protocol.

Total phenolic and flavonoid content

Flavonoids significantly contribute to the total antioxidant property of the plants (Luo et al. 2002). Total phenolic content were found to be high in *M. dubia* in comparision to total flavonoids content (Table 5).

DPPH radical-scavenging activity

DPPH method is a simple, rapid and reproducible assay used for measuring the antioxidant activity of plant extracts (Mishra et al., 2012). The IC_{50} value for methanolic extract of leaves of *M. dubia* was found to be 242.88 ± 12.55 µg/ml (Table 5). The antioxidant activity (DPPH radical-scavenging activity) of all the extracts was lower than that of ascorbic acid ($IC_{50,}$ 6.1 ± 0.32 µg/ml), used as positive control; a lower IC_{50} value corresponds to a higher antioxidant activity of sample.

DISCUSSION

A culture medium is defined as a formulation of inorganic salts and organic compounds (apart from major carbohydrate sources and plant growth regulators) used for the nutrition of plant cultures (George, 1993). It usually consists of a balanced mixture of macro-and-micro elements together with vitamins and other organic nutrients, including a carbon source. Nutritional requirements for optimal growth of a tissue, *in vitro,* may vary with the species and parts of a plant. When starting with a new system, it is essential to work out a medium that will fulfill the specific requirements of the tissues (Bhojwani and Razdan, 1996; Smith, 2000). During the present investigations on *M. dubia* MS nutrient medium was used. There are many reports comparing different media for their effect on *in vitro* shoot multiplication (Rugini, 1984; Mehta et al., 2000; Lu, 2005; Jain et al., 2009). Most of the workers have routinely used MS medium for shoot multiplication (Reddy el al., 1998; Komalavalli and Rao, 2000; Devi and Srinvasan, 2008). Sha Valli Khan et al. (2002), reported the multiplication of white friable callus on MS medium with NAA (1.0 μM) in combination with BAP (1.0 μM) in *Bixa orelliana* L. Nirmalakumari et al. (1993) reported 6-7 shoots from one month old callus of leaf and stem on MS medium supplemented with BAP (2.0 mg/l) and IAA (0.5 mg/l) in *Azadirachta indica*. Chaicharoen et al. (1996) observed maximum 9 shoot from callus, when medium was supplemented with 1.0 mg/l BA in *Melia azedarach*. Chaturvedi et al. (2003) revealed that 5 µM BAP alone favored shoot induction from anther derived callus on MS medium. Srivastava et al. (2009) observed maximum (78%) shoot regeneration when callus was sub cultured on MS medium containing 5 µM BAP alone in *A. indica*. Contrary to the above observation, Islam et al. (1993) reported that shoot organogenesis was the best from cotyledons origin callus on MS medium with 2.0 mg/l BA and 0.2 mg/l NAA used in *A. indica*. Vila et al. (2003) in *M. azedarach* reported successful regeneration of plantlets from leaf derived callus by using BA (4.4 µM) and NAA (0.46 µM) in MS medium. Sharry et al. (2006) reported induction of multiple shoots from callus in MS medium with NAA (0.5 mg/l) and BAP (1.0 mg/l). In the present study, IBA was found to be the most excellent auxin for rooting in terms of number of roots and root length. In accordance to our result, IBA have been used extensively for rooting in a wide range of plant species of Meliaceae family such as; *M. azedarach* (Thakur et al., 1998; Sen et al., 2010), IBA 1.0 mg/l in MS/2 medium favoured the best root induction 83 to 90% respectively. Chemically MS medium shows variation in concentration of different components as compared to other media. It has been observed that IAA, IBA and NAA enriched medium was also responsible for root induction in *B. tulda* (Saxena, 1990) and *D. strictus* (Mascarenhas and Murlidharan, 1989). In case of *M. dubia* IBA is best which play a key role in root induction. The procedure offers an efficient and rapid method which can be adopted commercially for mass multiplication of *M. dubia*. MS medium supplemented with NAA 0.5 mg/l + BAP1.0 mg/l on callus multiplication, BAP 0.5 mg/l + IAA 2 mg/l for shoot regeneration and MS/2 with IBA 0.5 mg/l for *in vitro* rooting and has been selected for efficient and rapid multiplication of *M. dubia* 30 days after inoculation. Also the technology developed may be used to get sustained supply of *M. dubia*, which requires raising them on a mass scale for plantations and forestation purposes. Commercial exploitation of this protocol for multiplication of this economically important species is possible as demand for fresh explant can meet easily.

Plants produce a diverse number of secondary metabolites in response to changing oxidative environment. As such plant extracts are expected to contain a diversity of molecules with different structures and functions. *M. dubia* is being previously reported for the presence of flavonoids and phenolic compound (Murugesan et al., 2013; Gopal and Manju, 2015). In the present study total flavonoids content, phenolic content and antioxidant potential were determined from the leaves of tissue culture raised plant of *M. dubia*. In this study, flavonoid content was found to be 0.56 ± 0.08 mg quercitin equivalent (mg QE) per gram dry weight of the plant material, while total phenolic content was 2.97 ± 0.17 mg gallic acid equivalent (mg GAE) per gram dry weight of the plant material. Flavonoid and phenolic content reported in other species of meliaceae family such as; *A. indica* was 0.2 to 1.07 mg/g of plant material (phenolic content) and 0.61 to 5.29 mg/g of plant material (flavonoids content). These report are comparable with the total flavonoids and phenolic content of *M. dubia* (Khamis Al-Jadidi and Hossain, 2015).

Conclusion

As such there is no report on efficient protocol for mass propagation of *M. dubia.* Therefore, the present work was aimed to develop *in vitro* propagation protocol for rapid and large scale production of planting material. The results demonstrate that the leaves may act as excellent source for isolation of potential antioxidants with significant amount of flavonoids and phenolics. They may serve as natural antioxidants in pharmaceutical preparations.

Conflict of Interests

The authors have not declared any conflict of interests.

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Abbreviations

BAP, 6-Benzylaminopurine; **NAA,** α-naphthalene acetic acid; **IAA,** indole-3- acetic acid; **IBA,** indole-3-butyric acid; **DPPH,** 2, 2-diphenyl-1-picrylhydrazyl; **GAE,** gallic acid equivalent; **QE,** quercitin equivalent.

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Full Length Research Paper

A novel pig feed formulation containing *Aspergillus niger* CSA35 pretreated-cassava peels and its effect on growth and selected biochemical parameters of pigs

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This study investigated the effects of Aspergillus niger CSA35 pretreated-cassava (Manihot esculenta Crantz) peel feed (CPFG) on the body weight gain and some selected biochemical parameters of pigs. Cassava peels treated with biomass of A. niger CSA35 for a period of three weeks to initiate enzymatic digestion of peels were dried, ground and used in varying proportions to formulate pig rations in combination with other feed ingredients. Twenty 85–95 days old pigs (5.85 ± 0.70 kg) were randomly divided into four experimental groups. Group 1 received 0% CPFG amidst other feed ingredients (control), while Groups 2, 3 and 4 received 40%, 60% and 100% CPFG respectively. At the end of the feeding trial (21 days), the animals were weighed and blood samples collected for biochemical analysis. Results showed that increasing the amount of the fungus-pretreated cassava peels in pig rations increased the protein, fat and carbohydrate contents of the experimental feeds. Conversely, the percentage fibre content was reduced. The weight gain of pigs fed the control diet was significantly (p<0.05) lower than those fed with 60% CPFG and 100% CPFG but did not differ from those fed 40% CPFG. Serum calcium and albumin levels were observed to be significantly lower (p<0.05) in control group than in treatment groups. The highest serum calcium level was, however, observed in 100% CPFG group. Activities of liver function enzymes and serum creatinine level of pigs fed the formulated diets did not significantly differ from those of control unlike their serum urea levels. It was concluded that pig feeds formulated with cassava peels pretreated with A. niger CSA35 enhanced feed's nutritive value and metabolisable energy, boosted serum albumin and calcium levels in pigs, increased pigs body weight and are health-friendly since the feeds did not pose threat of liver damage in the pigs investigated.

Key words: Cassava peels, *Aspergillus niger* CSA35, pig feed formulation, weight gain, biochemical parameters.

INTRODUCTION

The need to provide adequate animal protein for the growing population of third world countries is of major concern. It has been recognized over the years that the development of swine, poultry and rabbit sub sector of the animal industry is the fastest means of bridging the protein deficiency gap prevalent in most tropical countries (FAO, 1990). This is due to their short generation interval and high fecundity (Nkwengulila, 2014; John et al., 2014)

but, the major constraint against pig production is the high cost of feeds. In Nigeria, it could be as high as 75 to 80% in the fattening herd and 60 to 65% in the breeding herd (Tewe, 1997; Akinfala and Tewe, 2001).

Commercialized production of these animals' feeds involves use of ingredients that have prohibitive costs due to their comparative use between man and animals. Most of these ingredients are energy-based. Thus, improvement in animal production through the use of non-conventional feed ingredients or energy sources has long been advocated (Nnadi et al., 2010) and efforts to reduce the cost of production are being directed towards the use of affordable and available alternative sources of energy and protein in the diets of pig (Woyengo et al., 2014). The alternative feedstuff, therefore, must be ingredients with less competition by other secondary industrial users and producers which are readily available in commercial quantities and affordable prices. Also, pigs should be capable of converting these alternative feedstuffs (which will normally be discarded by humans) into wholesome animal protein (Adesehinwa et al., 2011; Adesehinwa, 2008).

Cassava, *Manihot esculenta* Crantz, is a major carbohydrate-rich tuber plant cultivated in the tropics (Avwioroko and Tonukari, 2014). Current world total production is about 157 million tons per annum with Nigeria accounting for about 16% (FAO, 1990). The first attempt at substituting cereals with cassava in commercial pig rations began during the Second World War where its use cushioned the effect of post war shortage of grains in Europe (Muller et al., 1974). Manner and Gomez (1973) and Iyayi and Tewe (1994) noted conclusively that cassava might replace maize and cereals without any negative effects. In Nigeria, in spite of cassava availability, its use as sole component of energy source in livestock feed has not been given due recognition (Avwioroko et al., 2016). However, there are many pioneering studies which highlighted on the suitability of cassava tuberous meal for swine feeding and its potential as a good substitute for maize for all classes of pigs (Job, 1975; Adegbola, 1977; Nghi, 1986; Tewe and Egbunike, 1992). Jiménez et al. (2005) had reported that pigs fed diets formulated to contain 40% of cassava root meal with other ingredients showed similar performance and carcass traits with those fed conventional diet. Cassava peel meal has, therefore, become one feed ingredient which has been consistently incorporated into the diets of pigs as alternative energy source (Iyayi and Tewe, 1988; Adesehinwa et al., 1998), but for its high fibrous content (a feature of most locally available agro-industrial by-products and wastes) which has limited its use by monogastric animals (Longe and Fagbenro-Byron, 1990).

Fibrousness of feedstuffs (mostly of by-product of plant origin) is important in relation to their feeding value to pigs (Adesehinwa et al., 1998). The addition of fibre to swine diets decreases the digestible energy (DE) and metabolisable energy (ME) concentration of the diet (Kennelly et al., 1978; Kennelly and Aherne, 1980) and often results in bulk feeds. The influence of crude fibre on organic matter digestibility varies from feed to feed, depending on the special characteristics of the crude fibre in individual feeds (Kidder and Manner, 1978). The fibrous portion of feed, being fairly indigestible to pigs, influences the digestibility of the other constituents by exerting a protective action, encasing these constituents in a digestion-proof shield, as it were, thereby obstructing the access of digestive enzymes (Mitaru and Blair, 1984). Hence, for efficient use of cassava peel in pig feeding, some form of physical treatment is essential to the breaking down of the fibre encapsulating the more soluble constituents so that digestive secretions can penetrate more completely (Kidder and Manner, 1978).

Another constraint to the use of these agro-industrial wastes is the dearth of affordable and sustainable local technologies to modify these products to forms acceptable to our livestock industries. A way out is to source for cost-effective and environmentally-friendly measures as exemplified by the biotechnological option of fermentation using generally recognized as safe (GRAS) microorganisms (US FDA/CFSAN, 2008) for the biological transformations of agro-industrial wastes to yield products with enhanced nutritional values (Israelides et al., 1998; Oboh and Akindahunsi, 2003; Nwafor and Ejukonemu, 2004; Aro, 2008). Microbial fermentation has been reported as an effective means of breaking down non-starch polysaccharides of agro-industrial wastes to increase their metabolisable energy and their nutritive value in general (Aro, 2008).

The concept of using microorganisms in enhancing the nutritive value of plant and animal products is not entirely a new one. Aro (2008) had reported using a number of isolates from rotting cassava to enrich cassava and in recent years, considerable emphasis has been placed on the improvement of fibrous crops by the growing of non-toxic fungi on straw (Shrivastava et al., 2011). The ability of fungi to produce enzymes, which bring about catalytic transformations via a wide range of desirable reactions, makes them interesting to industrialists and agriculturists (Adrio and Demain, 2014). Recent advances in biotechnological applications of this sort are opening new frontiers in the bioconversion of agro-industrial wastes to products of significance in livestock production (Avwioroko et al., 2016). In this study, the effect of pig feeds formulated with cassava peels pre-treated with *Aspergillus niger* CSA35 on body weight gain and on

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Table 1. Composition of formulated experimental feeds.

Ingredients (kg)	Group 1 (0%CPFG)	Group 2 (40%CPFG)	Group 3 (60%CPFG)	Group 4 (100%CPFG)
BSG or wheat offal	20	20	20	20
PKC	50	50	50	50
Fresh cassava peels (Non-treated)	27	16.2	10.8	-
Cassava peels pre-treated with fungi (CPFG)	-	10.8	16.2	27
Bone meal	1.25	1.25	1.25	1.25
Limestone	1	1	1	1
Pig Grower Supermix	0.75	0.75	0.75	0.75
Total (100 kg)	100	100	100	100

BSG, Brewer's spent grain; PKC, palm kernel cake; CPFG, cassava peels pre-treated with fungus.

some selected biochemical parameters of pigs was investigated.

MATERIALS AND METHODS

Preparation of yeast peptone dextrose (YPD) agar

Yeast peptone dextrose agar (YPD) was prepared following the method described by Avwioroko et al. (2015). Briefly, 2.0 g of glucose monohydrate, 1.0 g of yeast extract, 2.0 g peptone and 1.5 g agar-agar powder were measured into a 250 ml conical flask. Little volume of distilled water was added to dissolve the flask contents and thereafter, the solution was made up to 100 ml with distilled water (H_2O). The solution was sterilized by autoclaving it for 15 min at 121°C, allowed to cool to about 50°C and thereafter poured into sterile petri dishes to solidify.

Growth of *Aspergillus niger* CSA35 on cassava peels

A fungus, *A. niger* CSA35, previously isolated and identified to be associated with cassava spoilage/degradation using 18S rRNA gene sequence (Avwioroko and Tonukari, 2014), was grown in sterile yeast peptone dextrose (YPD) agar in the dark for a period of 7 days. The fungal biomass was later sub-cultured using a cassava finished product (eba) to facilitate its massive growth and sporulation for one week (A sterile forcep was used as inoculating tool and about five scoops of fungal cells was collected from plate and spread all over the surface of the cassava finished product (eba) which weighed approximately 600 g]. At the end of one week incubation at room temperature, there was massive noticeable fungus growth on the 'eba' prior to use for treatment of cassava peels]. The fungus was finally transferred into 52.5 kg of fresh cassava (*M. esculenta*) peels and thoroughly mixed together to ensure growth on all the cassava peels. The medium was stirred twice daily to ensure even distribution of nutrients thereby facilitating the release of more digestive enzymes by the fungus to degrade the substrate. After three weeks, the fungus-treated cassava peels were dried, ground and used in varying proportions to produce pig rations in combination with other feed ingredients.

Experimental animals and experimental feed composition

Twenty pigs of the large white breed, between the ages of 85 and 95 days, with average body weight of 5.85 ± 0.70 kg, were used for the study. They were injected with Ivomec® (Ivermectin) subcutaneously against endo-and ecto-parasites and weighed using a cage and a weighing balance, before the experiment began. Table 1 shows the composition of the formulated feeds given to the experimental animals. The pigs were randomly divided into four treatment groups. Each treatment group had five pigs in a completely randomized design. All groups were fed with the same quantity of their respective experimental diet twice daily. The pigs were allowed *ad libitum* access to the diets and water throughout the duration of the study (21 days).

Blood sample collection

At the end of the feeding trial (21 days), the pigs were bled to obtain blood samples. The bleeding was done in the morning of day 22 before feeding and 2 ml of blood was collected into test tube via ear vein puncture method. The blood samples were allowed to clot and then centrifuged at 3000 *g* for 10 min. The supernatant (serum) was separated and used for biochemical analysis.

Biochemical assays

The proximate analysis of the compounded feed was carried out using standard laboratory methods (AOAC, 1995). Serum urea concentration estimation was carried out according to the method described by Weatherburn (1967). The principle is based on hydrolysis of urea in serum to ammonia in the presence of urease; thereafter, the ammonia was measured photometrically by Bethelot's reaction. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assayed by the method of Reitman and Frankel (1957). Estimation of serum creatinine was done by the principle of Bartels and Bohmer (1972). Creatinine in alkaline solution reacted with picric acid to form a coloured complex which is directly proportional to the creatinine concentration. Serum albumin concentration was determined by the method of Doumas et al. (1971) and serum calcium level was estimated according to the method described by Lothar (1998). All the assays were carried out in accordance with the instructions in their respective RANDOX assay kits.

Estimation of metabolisable energy

Metabolisable energy was calculated from the proximate chemical composition data using the AOAC (1995) formula:

Metabolisable energy (Kcal/kg) = (37 × CP) + (81.8 × CF) + (35.5 × NFE)

Where CP = crude protein (%), CF = crude fat (%) and NFE = nitrogen free extract (carbohydrate, %).

Statistical analysis

The values obtained from the different experiments were reported as mean ± SD. The significant differences between mean values were obtained by using One-way Analysis of Variance (ANOVA) and least significance test (LSD) procedure as described by Ogbueibu (2005).

RESULTS

Proximate composition of formulated pig diets and energy levels

The proximate compositions of the formulated feeds used in this experiment are presented in Figure 1a. The protein content of the experimental diet increased from 13.3% (in feed 0% CPFG) to 16.15% (in 100%CPFG feed). Similarly, the percentage fat content of the feeds increased from 3.3% in 0% CPFG feed to 3.7% in 100% CPFG feed. Likewise, the percentage carbohydrate content of the feeds also increased with increase in amount of fungus-treated cassava peels included in the feed. Conversely, the percentage fibre content was reduced by increasing the amount of fungus-treated cassava peels in the diets. An increase was observed in the metabolisable energy value of the pig diets as the percentage (%) of fungal-degraded cassava peels in the diet increased (Figure 1b). It ranged from 3112.14 kcal in the control diet (0% CPFG) to 3293.27 kcal in the last group (100% CPFG).

Effect of formulated pig diets on body weight

The weight gain of pigs fed the control diet (0% CPFG) was significantly ($p<0.05$) lower than those fed with 60% CPFG and 100% CPFG in their diets but not significantly ($p>0.05$) different from those fed 40% CPFG. Pigs maintained on diet containing 100% CPFG were, however, observed to have the highest weight gain of 1.23 kg (Figure 2).

Effect of formulated pig diets on some biochemical parameters

As shown in Figure 3, the activities of serum aspartate aminotransferase (AST) in pigs fed feed 60% CPFG and 100% CPFG did not significantly differ ($p>0.05$) compared to that of pigs fed the control diet (0% CPFG). However, the AST activity of the group fed with 40% CPFG diet was significantly ($p<0.05$) reduced compared with that of the control feed group. Also, the serum activities of alanine aminotransferase (ALT) in pigs maintained on diets formulated with the fungi-pretreated cassava peels were not significantly ($p>0.05$) different from that of the control group pigs.

The serum urea level in pigs fed the control diet was significantly lower ($p<0.05$) when compared with all the experimental groups (Figure 4). Serum creatinine level in pigs fed the control diet was, however, not significantly different ($p>0.05$) when compared to those of other experimental groups (Figure 4). Levels of serum albumin in pigs fed the formulated feeds containing the fungus-pretreated cassava peels were significantly higher ($p<0.05$) than those of the control group pigs (Figure 5). The value ranged from 3.4 mg/dl in the control diet group (0% CPFG) to 8.8 mg/dl in pigs maintained on diet formulated with 100% CPFG.

Similarly, serum calcium level was observed to be significantly higher ($p<0.05$) in pigs fed the respective formulated experimental diets compared to the control group (0% CPFG). The highest serum calcium level was observed in pigs fed with feed containing 100% CPFG (Figure 5).

DISCUSSION

The ability of fungi to degrade cassava fiber has been reported in literature (Ofuya and Nwajiuba, 1990; Iyayi and Losel, 2001). The report of Ofuya and Nwajiuba (1990) revealed successful biodegradation of the fibrous by-products of cassava tuber processing (cassava peels) by *Rhizopus* species. In their study, over 35% of the original cellulose content of the substrate was lost in solid state fermentation. *A. niger* grown on rye grass straw was also reported by Han (1978) to produce similar results. The increase in crude protein value of the degraded cassava peels in the present study was partly due to the ability of the fungal digestive enzymes to increase the bioavailability of the protein hitherto encapsulated by the cell. The increased protein content in feeds containing cassava peels pretreated with *A. niger* CSA35 is also in agreement with the findings of Bachtar (2005) who reported an increase in crude protein when *A. niger* was inoculated on sago fibre and cassava fibre, resulting to 16.5 and 18.5% protein increase, respectively. The increase in the energy value of the diets observed in this study may be due to the ability of the fungus to breakdown the starch and non-starch polysaccharide contents of the cassava peels into monomeric sugars, thereby making the diets easily metabolisable (Iyayi and Aderolu, 2004; Balagopalan, 1996). The percentage increase in carbohydrate content and decrease in fibre content observed in the formulated diets was in consonance with the report of Olowofeso et al. (2003) who described the effects of *S. cerevisiae* (yeast) and dietary fiber sources on the diets of growing pigs. Another study by Oboh and Akindahunsi (2003) reported biochemical changes in cassava products (flour and gari) after subjection to *S. cerevisiae* solid media fermentation. A short-term (21 days) feeding trial carried out on 85 to

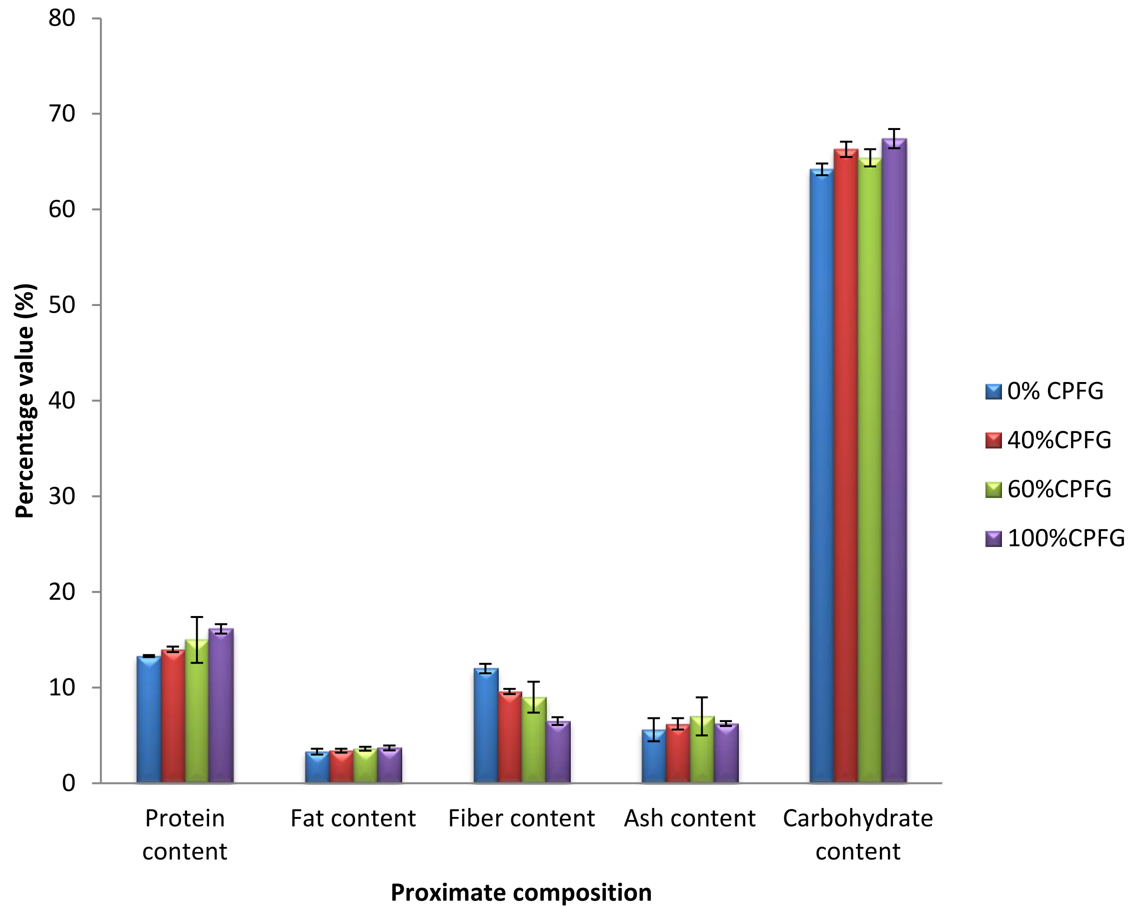

Figure 1a. Proximate composition of the formulated experimental pig feeds. CPFG denotes cassava peels pre-treated with fungus.

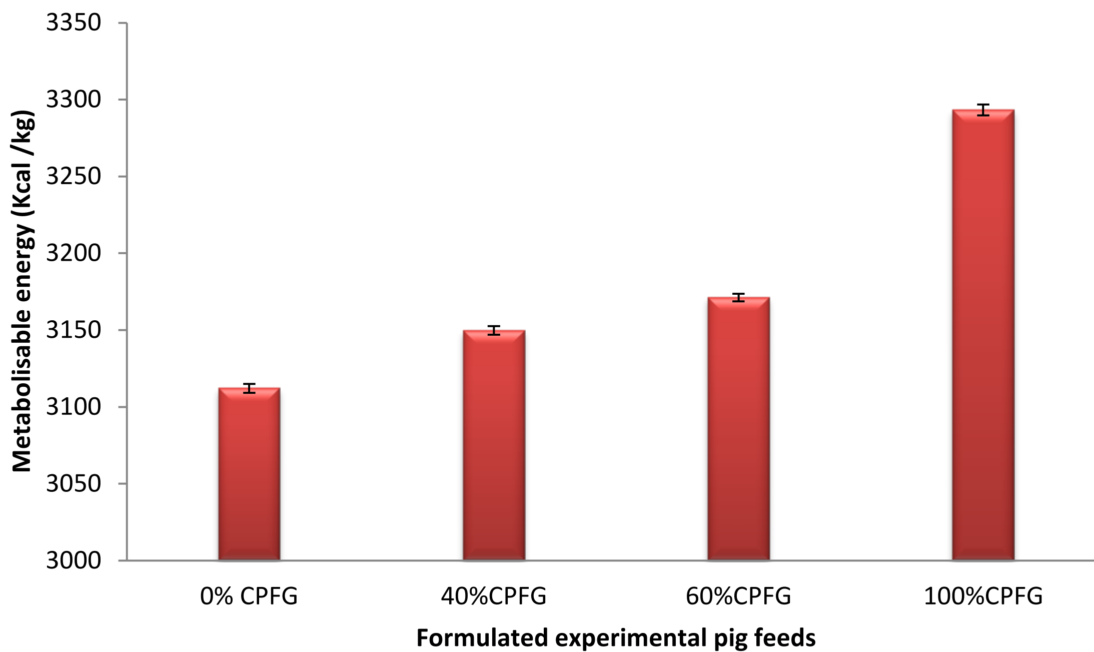

Figure 1b. Metabolisable energy (Kcal/kg) of the formulated pig feeds. CPFG denotes cassava peels pre-treated with fungus.

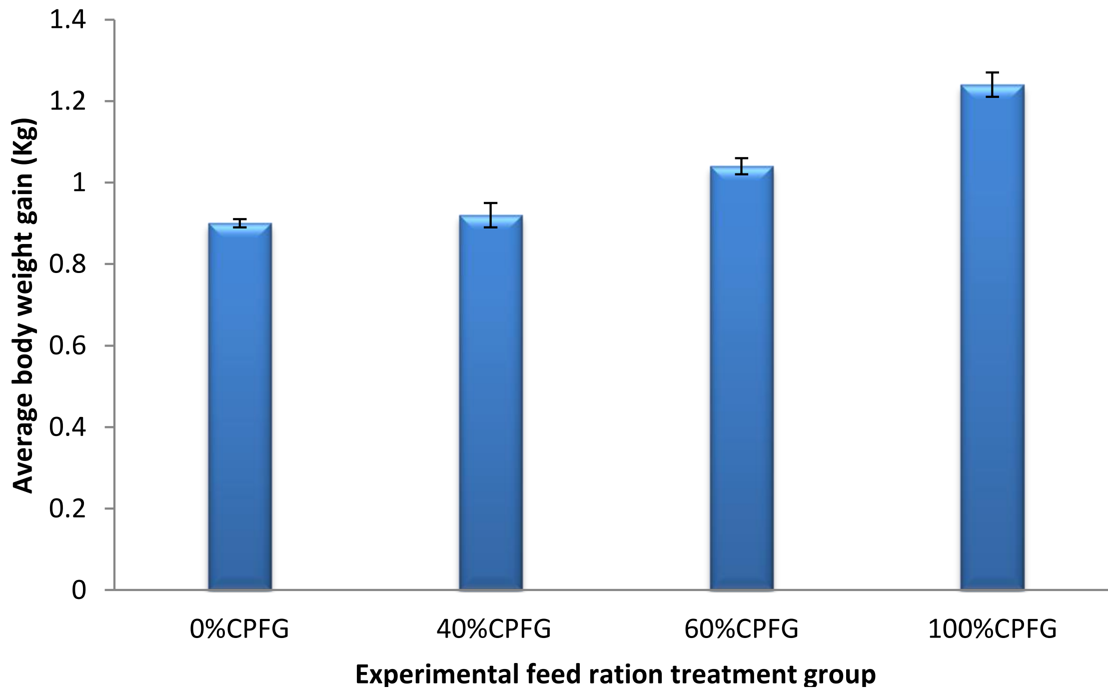

Figure 2. Effect of feed pretreatment on the average body weight gain (Kg) of the experimental pigs. CPFG denotes cassava peels pre-treated with fungus.

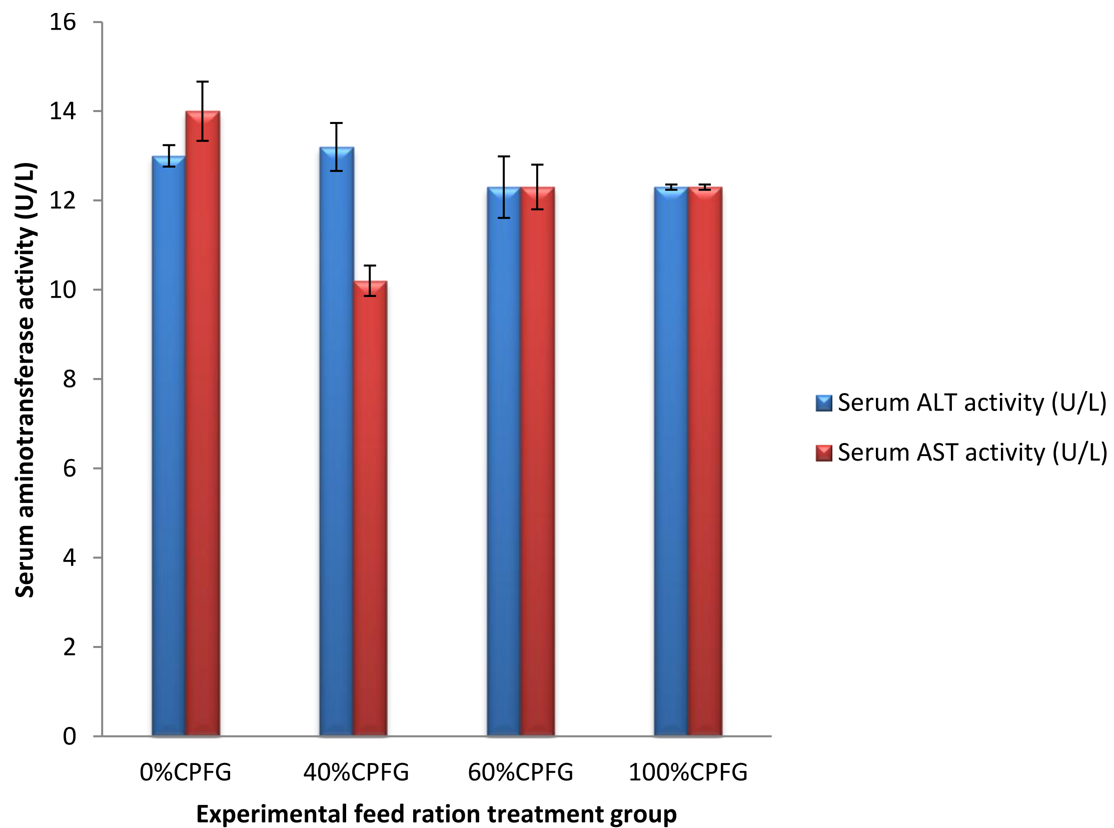

Figure 3. Effect of feed pretreatment on serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities (U/L) in the experimental pigs. CPFG denotes cassava peels pre-treated with fungus.

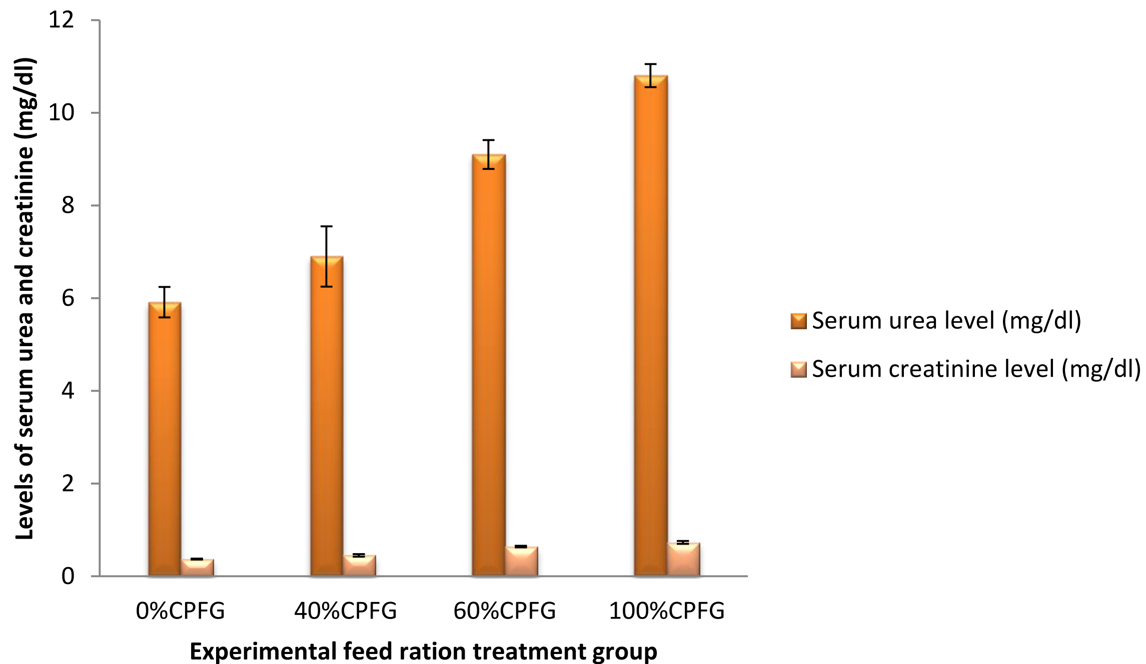

Figure 4. Effect of feed pretreatment on serum urea and creatinine levels (mg/dl) in the experimental pigs. CPFG denotes cassava peels pre-treated with fungus.

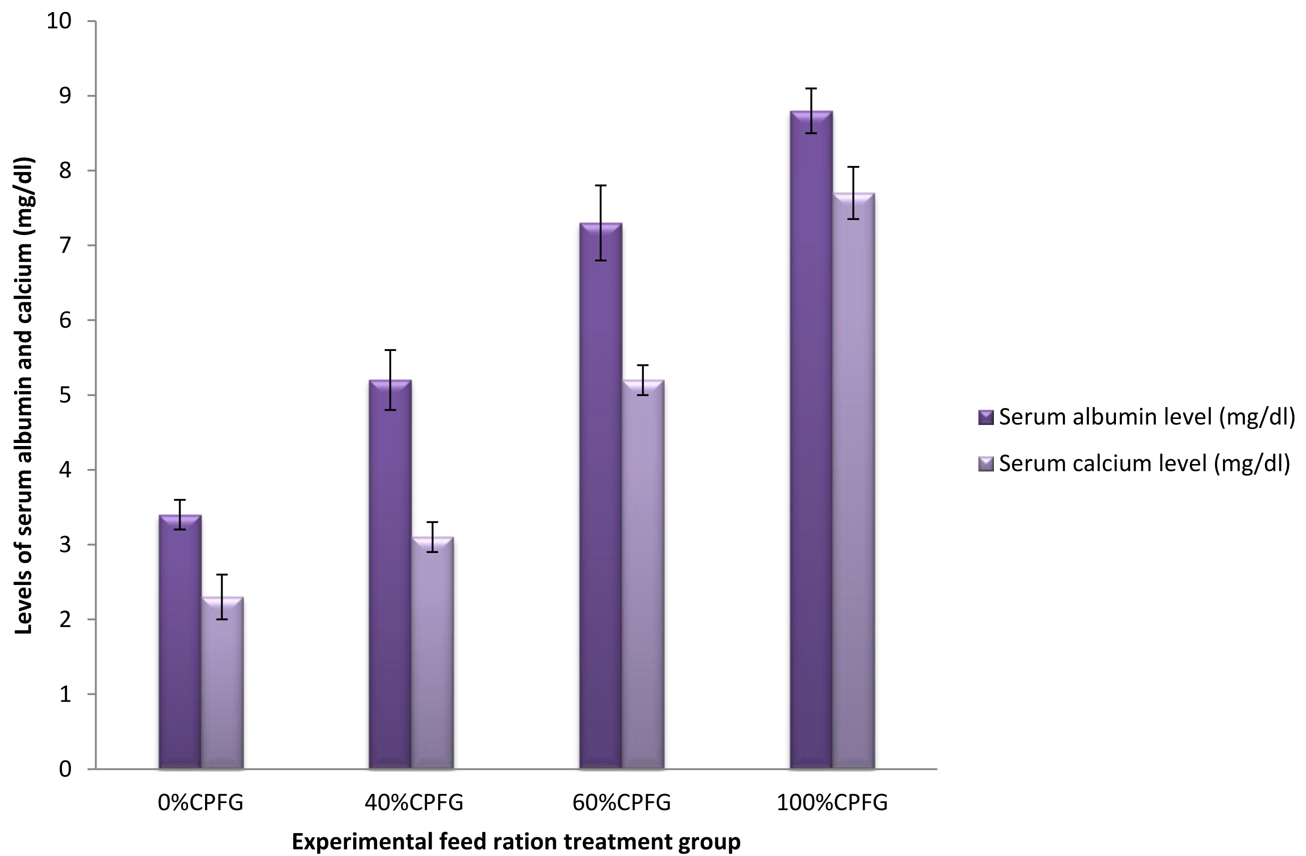

Figure 5. Effect of feed pretreatment on serum albumin and calcium levels (mg/dl) in the experimental pigs. CPFG denotes cassava peels pre-treated with fungus.

95 day-old swine using the formulated feeds showed that pigs fed diets containing cassava peels pretreated with *A. niger* CSA35 had significantly higher weight gain than those fed control diet. The efficiency of protein and feed utilization was reported to decrease with increased fibre content in diets (Adesehinwa, 2007). Thus, the greater weight gain of the pigs maintained on diets formulated with fungal-degraded cassava peels compared to the control in the present study supports this fact since the formulated feeds containing fungus-pretreated cassava peels had lower fibre contents relative to the control feed and that led to their efficient utilization than the control. The increase in weight of the pigs could also be attributed to the fact that the viscosity of the feed along the gastro-intestinal tract of the birds was reduced. Undegraded agro-industrial by-products are able to increase viscosity of the digesta and the transit time in the gastro-intestinal tract which can lead to increase in the size and stability of the unstirred layer at the mucosal surface of the digestive tract (Bedford, 1995). This reduces the contact between the feed and the digestive enzymes and slows the uptake of sugars, amino acids and lipids in the foregut, resulting in the impaired digestibility of the major nutrients (Bedford, 1995; van der Klis et al., 1995). This may result in poor weight gain by livestock fed undegraded feed materials by-products like cassava. There is also cumulative evidence that increasing viscosity of the digesta promotes bacterial proliferation, which is to the detriment of overall digestive efficiency and by implication, may affect the body weight gain of a livestock (Choct et al., 1995). The improvement in the utilization of the biodegraded cassava peels may ultimately have resulted in improvement of the body weights of the fed pigs.

The influence of diets on haematological and serum biochemical variables has been established (Mafuvadze and Erlwanger, 2007). Feeding of pigs with the experimental diets formulated in the present study did not induce elevation of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the pigs. This indicated that the integrity of the liver as a delicate internal organ in the pigs was not jeopardized. Usually, elevated activities of these aminotransferase enzymes (ALT and AST) in the serum are used as indicators or biomarkers of hepatocellular injury or damage (Emenalum et al., 2009; Adeyemo and Sani, 2013). The results of serum aminotransferase activities obtained in the present study showed that the formulated pig diets using *A. niger* CSA35 pre-digested cassava peels were not only able to meet the requirement of the growing pigs but also did not pose toxicity threat to the pigs' internal organs especially the liver within the duration of the study. Further researches on clinical effect would, however, be required since serum urea level in pigs maintained on the experimental diets was observed to be significantly higher ($p<0.05$) compared to control group pigs (0% CPFG).

Serum calcium and albumin levels were observed to be significantly lower ($p<0.05$) in control diet group pigs compared to the respective formulated-diet group pigs. Fermentation has also been shown to increase the mineral (ash) content of cassava peels (Olowofeso et al., 2003; Oboh and Akindahunsi, 2003). The increased serum calcium could be channeled into pathways involving bone formation (osteogenesis) thereby resulting to production of swine with healthy and strong bones over a long period of time (Dawson-Hughes, 2015; Peacock, 2010). High calcium and albumin levels have also been severally reported to be implicated in many physiological reactions required for general wellbeing of living organisms (Roche et al., 2008; van der Vusse, 2009; Bose and Tarafder, 2012; Carmeliet et al., 2015). Specifically, serum albumin is required for transport of short-chain free fatty acids from the blood stream to cells for β-oxidation (during starvation) (van der Vusse, 2009; Shinawi and Abu-Elheiga, 2014) or to adipose tissues (in a well-fed state) where they are stored intracellularly in the form of triacylglycerol molecules or triacylglycerides in lipid droplets, thereby leading to the production of pigs with relatively high adiposity (Dunning et al., 2014). This could be a possible biochemical rationale for the higher body weights observed in pigs fed diets containing cassava peels pretreated with the fungus compared to those fed the control diet.

Conclusion

Pretreating cassava peels with *A. niger* CSA35 could be used to enhance its nutritive value and metabolisable energy. The fermented peels in addition to other feed ingredients could be a source of nutrient for growing pigs which would bring about increase in weight of the pigs without any adverse effect on their serum biochemical parameters. A 60 to 100% inclusion of pretreated cassava peel is recommended given its high effect on the weight gain of growing pigs as recorded in the present study.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Inhibition of cell death as an approach for development of transgenic resistance against Fusarium wilt disease

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***Fusarium oxysporum* f. sp. *cubense* (Foc) is one of the major threats to dessert banana (*Musa* spp.) production. In Uganda, 'Sukali Ndiizi' is one of the most popular dessert banana cultivars and it is highly susceptible to Fusarium wilt. Development of resistant cultivars through transgenic approaches has shown to offer one of the most effective control options for most diseases. The transgenic approaches for providing plant disease resistance have mainly been through either enzymatic destruction of pathogen structures, neutralization of pathogen and its products or production of metabolites that eventually kill the pathogen. However in recent years, methods that prevent cell death of host plant after infection especially for necrotrophic pathogens like *F. oxysporum* have registered success in providing resistance in several crops. We investigated whether the transgenic expression of a programmed cell death inhibition gene in Sukali Ndiizi could be used to confer Fusarium resistance to Foc race 1. Embryogenic cell suspensions of cv. 'Sukali Ndiizi, were stably transformed with a synthetic, plant-codon optimise m*Ced-9* gene. Twenty-eight independently transformed plant lines were regenerated. The lines were inoculated with Foc race 1 and observed for 13 weeks in small-plant glasshouse. Three transgenic lines showed significantly lower internal and external disease symptoms than the wild-type susceptible 'Sukali Ndiizi' banana plants used as controls. This is the first report from Africa on the generation of Fusarium wilt tolerant transgenic 'Sukali Ndiizi', a very popular but rapidly diminishing African dessert banana.**

Key words: Fusarium wilt, banana, Sukali Ndiizi, *Fusarium oxysporum* f. sp. *Cubense race 1*, programmed cell death, disease resistance.

INTRODUCTION

Fusarium wilt, was first reported in Uganda in 1952 (Leaky, 1970) and it continues to be a major threat to dessert banana production in Africa and worldwide (Ploetz and Pegg, 2000). This disease is caused by a soil-borne fungal pathogen *Fusarium oxysporum* forma specialis (f. sp.) *cubense* (Foc) that affects the root

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system hampering nutrient and water supply to the plant. Insufficient supply of nutrients eventually leads to wilting and death of plants. Based on Foc variation in virulence to specific host cultivars, four physiologically distinct 'races' have been identified. The race predominant in Uganda is race 1 (Kangire et al., 2001). East African highland bananas (AAA-EA) are resistant to (Foc race 1), but the popular dessert banana Sukali Ndiizi (AAB genome), Bogoya (Gros Michel) (AAA) and the juice bananas Kisubi (ABB) and Kayinja (ABB) are susceptible to Foc (race 1). Sukali Ndiizi is a small sweet banana characterized by having a compact bunch, short fruit fingers which are very sweet when ripe. It is a widely distributed cultivar and is gaining importance as a potential export commodity for Kenya and Uganda for both local and export markets (Tushemereirwe et al., 2001; Van Asten et al., 2008). Fusarium wilt has rapidly spread over the past decades in the region and has now become a major constraint to the production of Sukali Ndiizi causing losses up to 100% in some farms (Tushemereirwe et al., 2001).

At present, there are no cultural or chemical methods available that can effectively and sustainably control Foc (Ploetz, 2015). Fusarium chlamydospores remain viable in the soil for decades, leaving the infested fields unsuitable for growing susceptible banana cultivars. The only sustainable option for controlling this disease is through the use of resistant cultivars generated either by conventional breeding or through genetic modification. This option needs availability of resistance genes and some sources of resistance to Foc have been identified in a few wild bananas cultivars (Ploetz, 2006). Genetic engineering to enhance antifungal resistance through over-expression of genes that code for anti-fungal proteins or other diseases has emerged as a very promising strategy and has been tested in many crop plants (Collinge et al., 2010; Punja, 2001; Grover and Gowthaman, 2003). Development of Foc resistance in banana through genetic engineering may be supported by the availability of efficient and reliable banana transformation protocols (Khanna et al., 2004; Becker et al., 2000).

Different strategies have been used to identify gene products that can be expressed in transgenic tissues to counter the attack from plant pathogens. They involve destruction of pathogen structures such as chitin or glucan, neutralization of pathogen products and production of metabolites that eventually kill the pathogen, (Punja, 2001). The antimicrobial peptide (*Ace-AMP1*) gene derived from onion seeds was able to confer resistance to banana cultivar Rasthali after infection with Foc race 1 under a screen house evaluation (Mohandas et al., 2013). The neutralizing effects result from induced innate responses that involve production of defensins, phytoalexins or through signalling molecules that are initiated by R genes (Girhepuje and Shinde, 2011). Use of Programmed Cell Death (PCD) genes is one other way that has been successfully used to provide resistance against necrotrophic fungal pathogens (Gaolathe and Arunika, 2015). Human *Bcl-xL* and nematode Ced9 genes have been shown to increase tolerance of tomato to cucumber mosaic virus (Ping et al., 2004). Constitutive expression of various anti-apoptotic *Bcl-2* gene family members (including *Bcl-xL*, *Ced-9* and *Bcl-2* 3′ UTR) in transgenic tobacco plants resulted in high levels of resistance to a broad range of necrotrophic fungi (Dickman et al., 2001). This research has previously shown that when banana cell suspensions are exposed to soil pathogen, *Agrobacterium tumefaciens,* extensive cell death follows. This is accompanied by DNA laddering and fragmentation and the formation of apoptotic-like bodies (Khanna et al., 2007). These cellular responses were also inhibited in cells expressing the anti-apoptosis genes *Bcl-xL*, *Bcl-2* 3′ UTR and *Ced-9*.

A native banana MusaBAG1 and a nematode anti-apoptosis gene *Ced9* have conferred Foc race 1 resistance in transgenic banana cultivar 'Rasthali' and 'Lady Finger' respectively (Ghag et al., 2014; Paul et al., 2011). It was therefore hypothesized that a synthetic, plant codon-optimized version of *Ced9* (m*Ced9*) may also be able to confer Foc race 1 resistance to cv. Sukali Ndiizi. In this paper we demonstrate transgenic resistance in cv. Sukali Ndiizi to Foc race 1 conferred by m*Ced9* genes under glasshouse conditions.

MATERIALS AND METHODS

Binary vector pYC11 was generated by cloning a synthetic, plant-cordon optimised m*Ced-9* gene under control of the maize polyubiquitin (Ubi-1) constitutive promoter in pCambia2300 that also has the plant selection marker gene, *nptII* under the control of constitutive CaMV 35S promoter in the T-DNA region. Embryogenic cell suspensions (ECSs) of the banana cultivar 'Sukali Ndiizi (*Musa* spp. AAB group) were initiated and maintained as previously described (Namanya et al., 2004). The ECS were transformed with pYC11 construct using *Agrobacterium*-mediated transformation protocol as previously described (Khanna et al., 2004) with slight modifications. Briefly, a single confirmed colony of AGL1/pYC11 was inoculated in 10 ml of Yeast mannitol broth media containing selectable antibiotics and incubated for 3 days at 28°C. 5ml of this culture was incubated in 20 ml of LB with shaking overnight at 28°C. The bacterial culture was centrifuged at 5000 rpm for 10 min and the pellet re-suspended and induced for 3 h in bacterial re-suspension media TMA1 supplemented with 200 µM acetosyringone and shaking at 70 rpm, room temperature for 4 h. After 3 days of co-cultivation in the dark, infected ECS were washed with liquid MA2 medium supplemented with 200 µg ml^{-1} Timentin® for killing the Agrobacterium. The cells were then plated on glass filter paper and transferred to semi-solid embryo germination media supplemented with 200 µg ml^{-1}Timentin®, 100 µg ml^{-1} kanamycin and kept at 25°C in the dark. The cells on glass filters were sub-cultured on fresh media every 14 days while being observed for embryo development. Mature embryos were transferred to germination medium MA4 supplemented with 200 µg ml^{-1} Timentin and 100 µg ml^{-1} kanamycin. The germinated shoots were then transferred to MS to enable root formation. Well rooted plantlets were put on multiplication media with sub-cultures every month to get 10 clones for every line. These plants were transferred to the glass house and kept in the humid chamber for 2 weeks before being transferred to bigger pots of 200 mm diameter. The

plants were left to attain a height of 10 to 15 cm before Foc bioassays were carried out.

Molecular characterization of transgenic plants

Leaf samples were collected and total genomic DNA extracted using the modified CTAB protocol (Grover and Gowthaman, 2003). Briefly, 1 g of leaf tissue was ground in liquid nitrogen and incubated in 700 µl of CTAB extraction buffer at 65°C for 30 min. The total DNA was extracted using 700 µl of chloroform-isoamylalcohol (24:1) v/v and precipitated using an equal volume of isopropanol. After washing the pellet with 1 ml of 70% cold ethanol, the DNA was treated with RNase A, re-extracted and re-precipitated. The final precipitate was washed and re-suspended in sterile water. To detect the presence of m*ced9* and *nptII* gene sequence primer pairs. PCR was done with a reaction that contained 50 ng of plant DNA, 1.2 mM $MgCl_2$, 0.4 µM of each of the primer pairs, 1x PCR buffer, 0.24 mM dNTPs and 0.02 Unit Taq per reaction of 20 µl. The reaction mixture was subjected to an initial denaturation step of 95°C for 2 min followed by 30 cycles of 94°C for 30 s; annealing temperatures of 55°C for 30 s; 72°C for 2 min and a final extension step of 72°C for 5 min. The PCR products were run on 1% agarose. For RT-PCR analysis, RNA was extracted from 100 mg of fresh leaf tissue using an RNeasy Plant Mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions and was treated with RNase-free DNase (Promega, Madison, WI). Two-step RT-PCR was performed using RNA to cDNA EcoDry^{TM} premix protocol (Clonetech, Shiga, Japan), a pre-aliquoted, dry master mix, for reverse transcription, followed by normal PCR amplication with the appropriate gene-specific primers. The southern analysis of transgenic plants was essentially done as described by Khanna et al. (2007).

Fungal cultures and inoculum preparation

F. oxysporum f. sp. *cubense* race 1 (VCG 0124) isolate was obtained from plant pathology laboratories at NARO, Uganda. The fungus was inoculated on full strength potato dextrose agar (PDA) supplemented with 200 µg ml^{-1} ampicillin and incubated for 7 days at 27°C. Millet grain (*Echinochloa esculenta*) was rinsed in tap, soaked overnight and rinsed with distilled water before autoclaving at 121°C for 1 h. Five potato dextrose agar stabs of 1 mm^3 were taken from a uniformly growing fungal culture using a sterile blade and inoculated into 250 g of cooled sterile millet grain. The cultures were incubated at room temperature (23 to 25°C) and mixed daily for 10 days to ensure even distribution of the growing fungus.

For quantifying different spore concentrations, Foc race 1 was grown on full strength PDA and incubated for 7 days at 27°C. Mycelium of grown Foc race 1 was first harvested by scraping using a sterile blade and placed in a falcon tube with 20 ml of sterile water. The contents were mixed vigorously and filtered through a sterile nylon mesh to separate the mycelium from the spores. The spores in the filtrate were washed twice in 20 ml of water, centrifuged at 1000 rpm and suspended in 1 ml of sterile water. The conidia concentration in the suspension was determined with a haemocytometer and concentration adjusted to 2×10^4 spores ml^{-1} and 2×10^6 spores ml^{-1}. For millet inoculation, 50 ml of each spore concentration was added to 250 g of sterile millet and 5 agar stabs were also used for this experiment.

Small-plant bioassay

Twenty-eight lines of tissue-cultured transgenic plants (5 to 10 clones per line) of the 'Sukali Ndiizi cultivar and five wild-type tissue-cultured banana plants of 'Sukali Ndiizi were acclimatized and transplanted into 100-mm diameter pots in sterile soil for 8 weeks in a glasshouse at 27°C under natural light conditions.

Selected transgenic banana lines and wild-type control plants were potted into 200 mm pots with sterile soil and inoculated with five mycelia agar stabs of Foc race 1 in a glasshouse trial. Plants were maintained with regular watering and fertiliser regime in a greenhouse with an average temperature between 25 and 27°C.

Assessment of disease symptoms

Twelve weeks after inoculation, external and internal symptoms of Fusarium wilt infection were assessed using a modified version of the method described by Mak et al. (2001). External symptoms of the Fusarium wilt were assessed by scoring each plant for the intensity of the three main disease symptoms (Table 2). This was based on a modified version of the method described by Mak et al. (2001). Yellowing and wilting were assessed using a 5-point scoring scale where 1 = healthy, no sign of symptoms, 2 = slight symptoms, mainly on lower leaves, 3 = advanced symptoms (~50%), 4 = extensive symptoms (~90%) and 5 = entire plant affected (dead plant). Splitting of the stem was assessed using a 3-point scoring scale where 1 = no sign of splitting, 2 = slight splitting at the base of the plant and 3 = extensive splitting. These point scale values determined the Leaf symptom index (LSI) of the cultivar or line.

For assessing the internal symptoms, plants were removed from the pots and the pseudostem was removed, leaving behind the corm and the root region. The plants were washed to remove soil from the roots then split longitudinally through the corm. Scores for corm infection intensity were made and an 8-point scale was used to compare the level of discolouration and to get the corm discolouration index (RDI). The LSI and RDI were used to obtain the disease severity index (DSI) that was used to determine susceptibility or resistance levels of the cultivar.

Statistical analysis

For pathogenicity test experiments, the Disease Severity Index of each cultivar and transgenic line was computed from the LSI and the RDI according to Mak et al. (2001).

$$DSI = \frac{\Sigma(\text{No.of scale x No.of plantlets in the scale})}{\Sigma\ (\text{No of treated plantlets})}$$

Plants with DSI scale of 1 for both LSI and RDI were classified as resistant. Plants with DSI scale between 1.1 and 2 for LSI and between 1.1 and 3 for RDI were classified as tolerant. Susceptible plants had DSI scale between 2.1 and 3 for LSI and between 3.1 and 5 for RDI. Highly susceptible plants had DSI scale higher than 3 for LSI and higher than 5 for RDI.

The data generated for the transgenic lines was analyzed using DSI values and ANOVA using the Genstat software (14th edition). Significance was determined using Dunnett's test ($p < 0.05$).

RESULTS

Transformation and regeneration of transgenic plants

Embryogenic cell suspensions of 'Sukali Ndiizi' transformed with binary vector pYC11 were selected on kanamycin and the effectiveness of selection was evident within the first three weeks as the untransformed cells started turning brown and dying. Transformed cells

Figure 1. Selection and regeneration of cells transformed with m*Ced9.* (A) Cells on selection media (MA3). (B) Developed embryos on selection media (MA3). (C) Mature embryos on selection media (RD1) (D) Developed shoots on multiplication medium.

developed into small white embryos which increased in size and matured over a period of two months. Matured embryos started germinating within 3 weeks when transferred to the germination media. A total of 84 embryos germinated and 69 shoots regenerated into plants (Figure 1).

Molecular characterisation

Total genomic DNA was extracted from the 69 putative transgenic plants. In case of the lines that were positively transformed, primers specific for *nptII* amplified the expected product size of 620 bp and primers specific for m*Ced9* produced a band of the expected product size of 291 bp (Figure 2). A total of 42 lines tested were positive for both *nptII* and m*Ced9* transgenes.

From these 42 lines, total RNA was extracted from leaf tissues and a two-step RT-PCR was performed using primers specific for m*Ced9* and all lines produced a band of the expected product size of 291 bp (Figure 3).

Nine transgenic lines 4, 12, 20, 21, 27, 55, 72, 83 and 96 were selected for southern analysis to determine the copy number of the transgene (Figure 4). The selected lines were a representation of those that were susceptible (4, 12, 21) and those that were tolerant line (27, 55, 72, 83, 96) after screening with Foc 1. Copy number ranged from 1 to more than 3. Line 20 had two copies while line 55 and 83 had one. There was no direct correlation observed between transgene copy number and level of tolerance to Fusarium infection.

Glasshouse trials

Pathogenicity of Foc race 1 isolate

It has previously been confirmed that East African highland banana cultivars (e.g. Nakinyika) are resistant to Foc race 1 while ‘Sukali Ndiizi’ is susceptible under field conditions (Kangire et al., 2001). When 10 tissue cultured plants each from these two cultivars were infected, the Foc race 1 isolate used in this study was able to infect both cultivars. Sukali Ndiizi was severely infected even at 2 x 10^4 spores ml^{-1}spore concentrations while Nakinyika showed only mild symptoms even at the concentration of

Figure 2. Representative PCR of transgenic Sukali Ndiizi lines transformed with pYC11. (A and B) Amplification of *nptII*. (C and D) Amplification of m*Ced9*. Lanes 4 to 37 and 39 to 99 are test plants; + is plasmid DNA control, - is non-transformed control plant, W: water control, M: Hyper ladder I molecular marker for A and B gels, Hyper ladder II molecular marker for C and D gels.

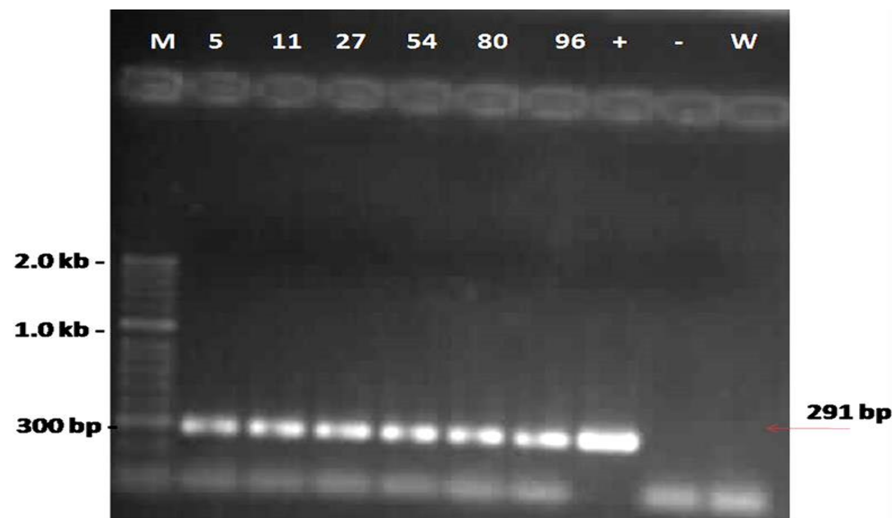

Figure 3. RT-PCR of selected transgenic Sukali Ndiizi lines transformed with pYC11. Lanes 5 to 96 are test plants +: Plasmid DNA, - : Non-transformed control plant, W: Water control, Hyper ladder II molecular marker.

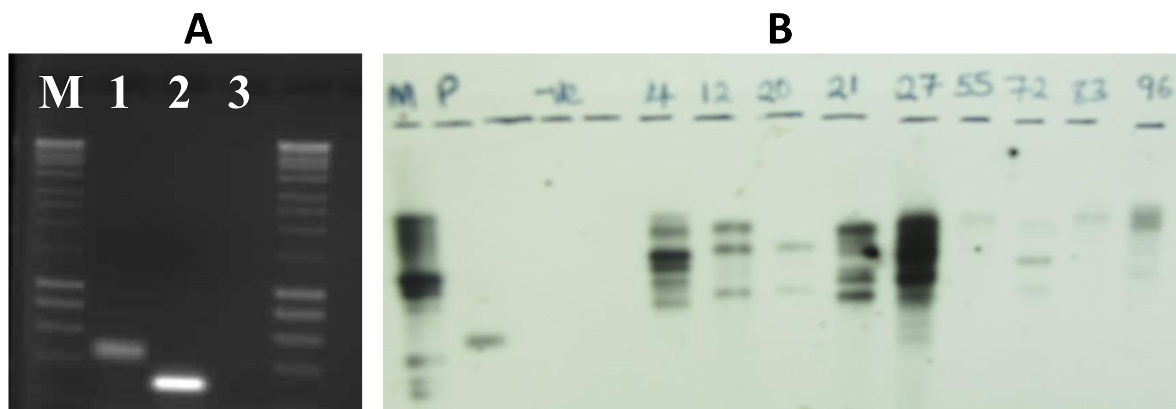

Figure 4. Probe labeling and southern blot analysis of selected transgenic lines. A: DIG label PCR probe using plasmid m*Ced-9*. M) Molecular weight marker. 1. Labeled m*Ced9* 2. Un-labeled m*Ced9*. 3. Water control. B: Southern analysis of transgenic lines: M) DIG labeled marker. P) Plasmid pYC11. –ve) is un-transformed Sukali Ndiizi plant. 4, 12, 20, 21, 27, 55, 72, 83, 96 are transgenic lines.

Table 1. Disease severity index (DSI) of control plants infected with different inoculum concentrations.

Cultivar	Symptom	Treatment 1 ($2x10^4$ spores ml^{-1})	Treatment 2 ($2x10^6$ spores ml^{-1})	Treatment 3 (5 agar stabs)
Sukali Ndiizi	Yellowing	2.9 *Susceptible*	3.9 *Highly susceptible*	3.1 *Highly susceptible*
	Wilting	1.4 *Tolerant*	2.2 *Tolerant*	2.5 *Tolerant*
	Corm discolouration	5.2 *Highly susceptible*	6.4 *Highly susceptible*	5.4 *Highly susceptible*
Nakinyika	Yellowing	2.0 *Tolerant*	1.0 *Resistant*	1.4 *Tolerant*
	Wilting	1.4 *Tolerant*	1.2 *Tolerant*	1.6 *Tolerant*
	Corm discolouration	1.0 *Resistant*	1.4 *Tolerant*	1.0 *Resistant*

Scale for the leaf index (yellowing and wilting): Resistant = 1, Tolerant 1.1- 2.0, Susceptible 2.1-3.0 and highly susceptible 3.1-4.0. Scale for corm discolouration: Resistant = 1, Tolerant 1.1- 3.0, Susceptible 3.1-5.0 and highly susceptible 5.1-8.0.

2 x 10^6 spores ml^{-1} (Table 1). Table 1 shows the computed disease severity index (DSI) values for the yellowing, wilting and corm discolouration, 8 weeks after infection. Corm discolouration was found to be the best indicator for assessing disease susceptibility and tolerance since wilting and yellowing was giving variable responses (Figure 5). All deductions that were later made were based on corm discolouration responses. Nakinyika was confirmed resistant and Sukali Ndiizi susceptible therefore the Foc race 1 isolate used in this study was considered pathogenic to Sukali Ndiizi and was subsequently used in assessing the transgenics.

Determination of Foc race 1 inoculum for consistent infection

To evaluate the best inoculum (spores or mycelia), thirty plants each of Sukali Ndiizi and Nakinyika were infected with 250 g of millet pre-infected with 2 different concentrations of fungus spore suspensions (50 ml of fungal suspension containing 2 x 10^4 and 2 x 10^6 spores ml^{-1}) or with 5 mycelium-covered agar stabs. Infected plants were observed for eight weeks. Sukali Ndiizi leaves showed yellowing two weeks after infection as compared to Nakinyika which showed milder yellowing but only after 4 weeks. Sukali Ndiizi plants infected with millet with 2 x 10^6 spores ml^{-1} and 5 agar stabs treatments were comparable for yellowing after 2 weeks and for wilting after 4 weeks. For the wilting and corm discolouration symptoms, all the treatments showed the same level of responses in Sukali Ndiizi after 8 weeks. However DSI values obtained for Sukali Ndiizi from millet treatment with 2 x 10^4 spores ml^{-1} were low (Table 1) in all cases compared with the other two treatments, indicating that the results with that inoculum were not the best indicators in the small pot assay. Furthermore, in treatment using 2 x 10^6 spores ml^{-1}, Nakinyika a cultivar known to be resistant to Foc race 1 showed some infection, indicating that the inoculum concentration was much higher than

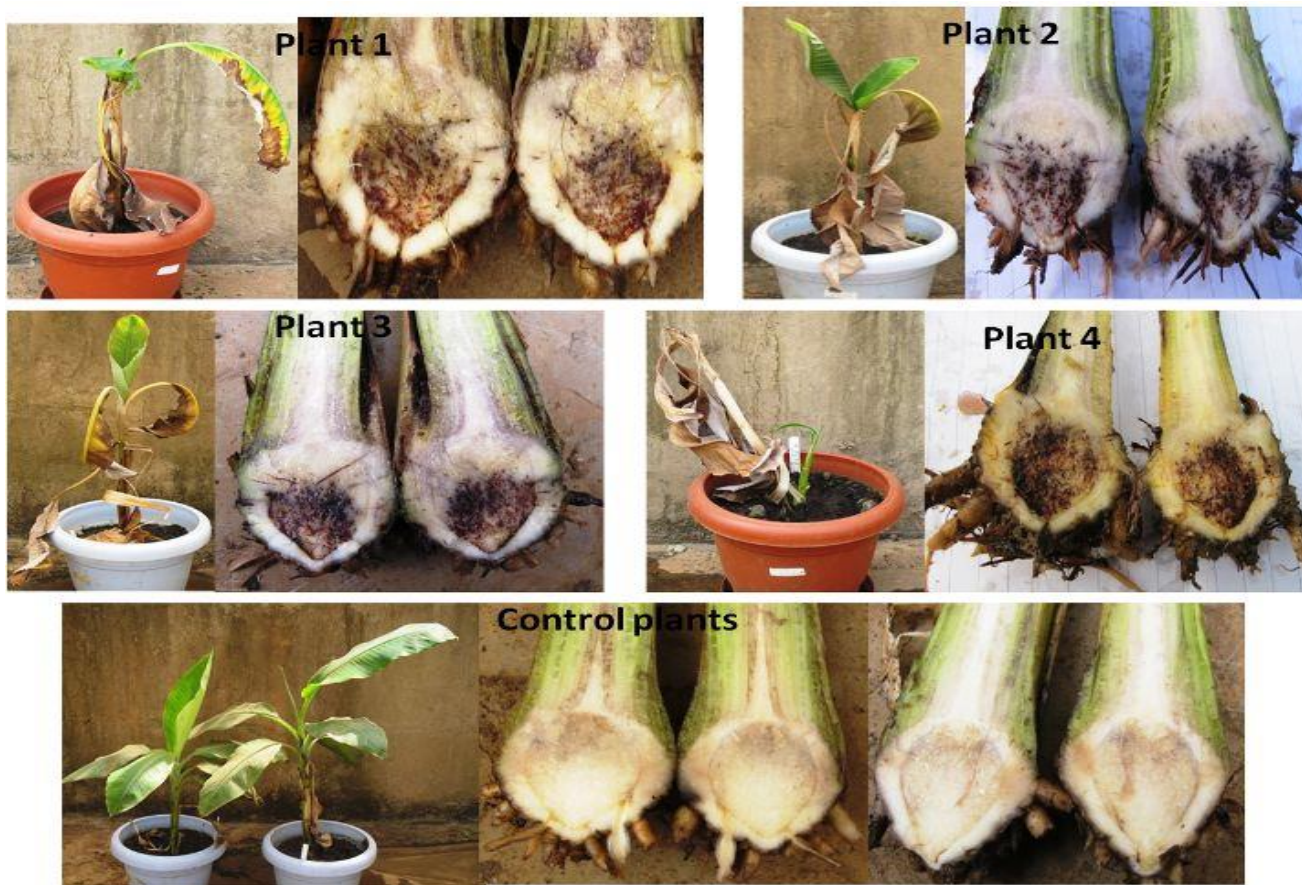

Figure 5. Representative picture showing internal and external symptoms of plants. Tissue culture derived plants at 8 weeks after infection. Plants 1, 2, 3 and 4 are Sukali Ndiizi cultivar and control plants are Nakinyika cultivar.

the one to which plants are normally exposed in the field (Table 1). Therefore, instead of spores, millet infected with five mycelium-covered agar stabs was selected as a method of inoculation for all subsequent infection experiments.

Evaluating the reaction of transgenics to Foc race 1 infection

Twenty-eight transgenic lines of Sukali Ndiizi with 5 to 10 replicates each were inoculated with 250 g of millet infected with Foc race 1 using fivemycelium-coated agar stabs. Scores for external symptoms were recorded after 6, 8 weeks and 13 weeks. External symptoms were clearly visible six weeks after infection and intensified with increase in the duration of exposure to the fungus. By thirteen weeks, most lines showed variability in the level of susceptibility to Foc race 1 infection for all the symptoms, that is, yellowing ($p < 0.001$), wilting ($p < 0.001$) and corm discolouration ($p < 0.001$). When all the mean symptom scores were subjected to Dunnett's test, six lines were found to be significantly different from the infected control plants based on the corm discolouration ($p < 0.001$). Line 27 had the lowest mean symptom score for both external and internal symptoms and the corm was almost symptom free (Table 2).

Based on the mean symptom score for corm discolouration, the transgenic lines were placed into three categories of disease severity (Figure 6). Lines 27, 72 and 83 were classified as tolerant, 12, 37, 55 and 96 as susceptible while the rest as highly susceptible to Foc race 1 infection. Within each transgenic line, the clones that showed any degree of infection were counted. Four lines had 2 or less clones infected, two lines had 3 clones while the rest had more than5 clones with corm discoloration symptoms.

DISCUSSION

Generating Fusarium resistant lines

Options for the control of Fusarium wilt are limited and there is lack of commercially suitable resistant cultivars. Hence, the introduction of resistance genes into banana

Table 2. Disease severity index (DSI) of transgenic lines 13 weeks after Foc race 1 infection.

Transgenic line numbers	Number of clones	Mean symptom scores		
		Yellowing	Wilting	Corm discoloration
4	8	4.5	4.6	7.5
5	7	3	3.1	6.3
6	6	4.2	4	6
8	10	3.8	3.9	6.5
9	6	3	3.2	5.5
11	10	3.6	3.9	6.6
12	6	2	**2.5***	**3.1***
14	7	2.7	2.7	5.7
21	7	3.9	3.9	7.1
27	7	**1.5***	**1.8***	**2.3***
28	8	4.5	4.8	7.6
30	10	3.4	3.5	6.5
31	8	2.5	3.1	5.4
33	6	2.8	3.3	6.2
37	5	**1.2***	**2.0***	4.4
39	8	3.8	3.6	6.5
46	5	3.3	3.7	6.3
48	7	4	4.1	7.1
53	8	3.8	3.9	6.6
55	7	2.3	2.4	**3.7***
61	7	3.4	3.7	6
65	8	4.4	4.9	6.1
66	9	3.6	3.8	6.4
72	7	**1.9***	**2.1***	**2.4***
80	7	4.9	4.9	6
83	9	**1.8***	**2.1***	**2.4***
96	6	2	2.5	**3.5***
99	7	2.6	2.7	6.3
Infected control	5	4.6	4.8	7.6

*Denotes lines that are significantly different from the non-transgenic control plants. Differences were considered significant at $P < 0.05$.

plants via biotechnological means is one of the ways for developing resistant banana cultivars. Previously, recalcitrance to transformation hindered the attempts towards introducing novel traits in banana (Ganapathi et al., 2001). However, the transformation and regeneration frequencies of several banana cultivars have been improved over the years (Khanna et al., 2004; Arinaitwe, 2008). The results obtained in this study further demonstrate the capacity of our laboratory to successfully transform banana cultivars using *A. tumefaciens* and male flower derived embryogenic cell suspensions. The *Agrobacterium* mediated method has been used to develop traits like resistance to banana bacterial wilt disease (Leena et al., 2010) and stimulation of the banana cell cycle (Talengera et al., 2012).

Genetic engineering to enhance antifungal resistance through over-expression of various transgenes has emerged as a very promising strategy and has been tested in many crop plants. Strategies like expressing R-genes, pathogenesis-related (PR)/antimicrobial genes, detoxification of pathogen virulence factors, increasing structural barriers, and the modification of defence-signalling pathways have been used to produce transgenic plants (Grover and Gowthaman, 2003). The use of RNAi technology has also shown survival of transgenics after infection which is attributed to the down regulation of *F. oxysporum* genes (Hu et al., 2015). There are however, no current commercially available transgenic plant species with increased resistance towards fungal pathogens (Wally and Punja, 2010). There are an increasing number of examples of plant-pathogen interaction strategies that result in control of cell death. We have previously reported in Australia that nematode anti-apoptosis gene *Ced9* can be expressed in ‘Lady

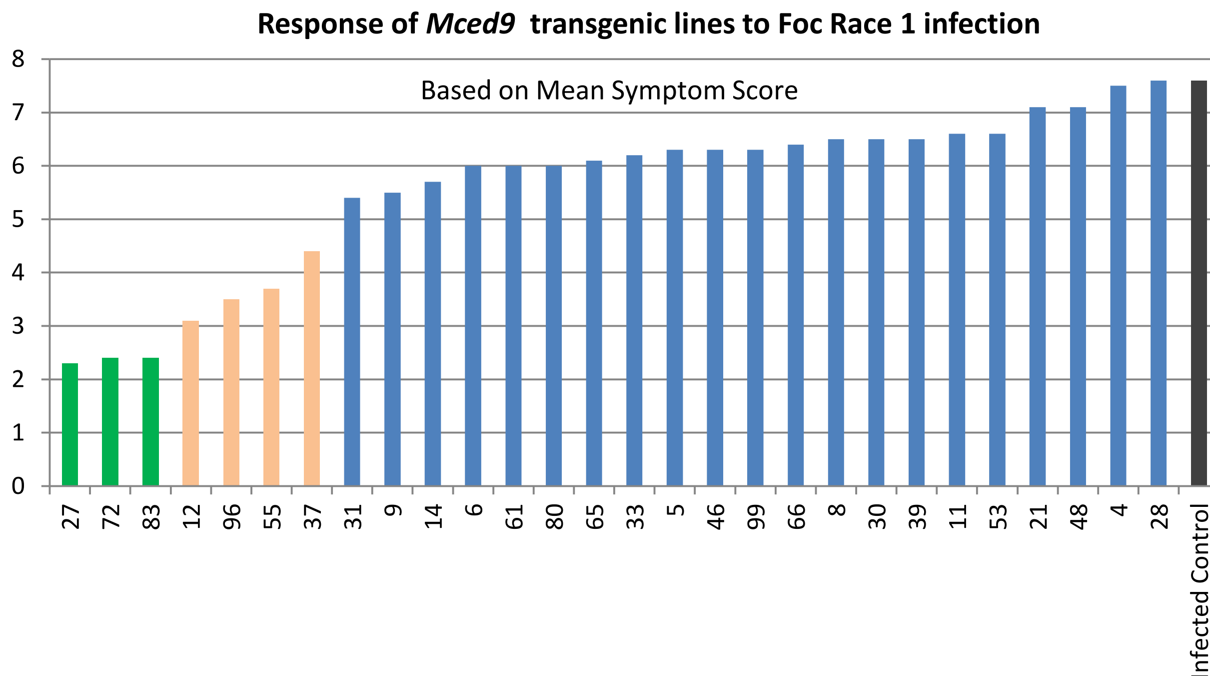

Figure 6. Levels of disease severity shown by the m*Ced9* transgenic lines after Foc race 1 infection. The disease severity index of transgenic lines showing the three disease levels: Tolerant, susceptible (12, 37, 55, 96) and highly susceptible (4, 5, 6, 8, 9, 11, 14, 21, 28, 30, 33, 39, 46, 48, 53, 61, 65, 66, 80, 99).

Finger' (AAB) banana to generate resistance to Foc race1 (Paul et al., 2011). A plant codon optimized version of *Ced9* (m*Ced9*) was therefore expected to confer Foc race 1 resistance to 'Sukali Ndiizi', an AAB banana cultivar from East Africa and the results obtained further confirmed the potential for cell death inhibition in plant protection against pathogens.

In this study, transformation of Sukali Ndiizi with m*Ced9* was successfully achieved and a large number of independent transgenic lines were obtained and characterised. Generally, for stable transgene expression, lines with a single copy are preferred and several studies have shown that transgenics generated using *Agrobacterium*-mediated transformation system have lower transgene insertions with high frequencies of single transgene copy. In this study, the transgenic lines generated had copy number ranging from single to multiple and there was not found any direct correlation between copy number and Foc tolerance. The three best lines had variable copy transgene number.

Small plant bioassay and Foc inoculations

Fusarium infects the banana plant though the roots and the fungal hyphae adhere to the host root surface before fungal infection commences. The pathogen moves through the root parenchyma tissue until it reaches the vascular tissue and blocks the xylem vessels (Mai and Abawi, 1987) causing a reddish-brown discoloration of the corm and pseudostem. Wilting symptoms observed are the combined result of fungal spores and mycelium blocking the xylem, toxin production, and host defence responses such as tyloses, gums and gels (Beckman and Roberts, 1995). Leaves of infected banana plants eventually become bright yellow, before they wilt and collapse around the pseudostem.

The two-month-old plantlets (10-15 cm tall) that were used to screen for Foc tolerance were found to be suitable and the pathogenicity of the inoculum used was confirmed using known resistant and susceptible cultivars. As expected, East African Highland banana cultivar 'Nakinyika' showed high tolerance to Fusarium wilt, while 'Sukali Ndiizi' was susceptible. All inoculated, susceptible plants showed both foliage and corm symptoms within the 13 weeks of trial, whereas the non-inoculated plants remained symptom-free.

Different infection methods have previously been used for screening banana plants with Fusarium wilt. These include immersion of roots in spore suspensions of known concentrations or use of a substrate like Fumonisin. The present study used millet grain which favours spore multiplication and survival of the fungus (Smith et al., 2008). Although even resistant plants can get infected if very high fungal inoculum load is used and they can show some foliar symptoms, however, corm damage occurs only in susceptible lines. Infection using different forms of Foc race1 inoculum with Sukali Ndiizi and

Nakinyika proved that better consistency is obtained when mycelium-covered agar stabs are used to infect millet grain as compared to the spore suspensions. In other studies with other cultivars, infections have been achieved with spore suspensions (Pei et al., 2005; Mahdavi et al., 2012; Wei-ming et al., 2011). However, there are differences in the proportion of different spore types (macroconidia, microconidia and chlamydospores) that different fungal isolates produce even when they belong to the same race (Groenewald et al., 2006). The proportion of spore types present in the infection culture affects the virulence of the fungus. The agar stabs predominantly contain mycelium which could be a reason for better infection since the mycelium is responsible for nutrient absorption, quick colonisation and hence quick multiplication of the fungus.

The internal and external glass house symptoms in this study showed 4 to 8 weeks after infection as opposed to field conditions where symptoms start becoming visible only after 5 to 6 months of infection. Field conditions have the limitations of being costly due to the need for space, lot of man power and the requirement for strict quarantine. The field environment, usually has factors affecting disease expression such as inoculum concentration, temperature and other variables that are difficult to control they are highly effective and reliable method for screening resistant lines.

An alternative method is to first screen a large number of lines using young plantlets in the glasshouse, under controlled conditions and using measured inoculum and pathogenicity tested races of Foc.

Assessing Fusarium wilt tolerance

Data analysis, which included yellowing, wilting and corm discolouration, showed variable Foc disease responses among the tested transgenic lines. The evaluated transgenic lines were categorized as tolerant, susceptible or highly susceptible, according to how they responded to the fungal infection (Table 1) based on scoring ranges by Mak et al. (2001). Three lines showed tolerance and four lines showed low susceptibility whereas the rest of the lines were almost as susceptible as the wild type controls. When the transgenic plants were compared statistically with the infected non-transgenic plants, lines 12, 27 55, 72, 83 and 96 were significantly better than the control plants ($p < 0.001$). Similar observations have been previously reported in tobacco expressing *Ced9* with the level of resistance displayed ranging from highly tolerant to completely resistant after *S. sclerotiorum* infection (Dickman et al., 2001).

For pathogenicity studies, the number of infected clones of a given line can also be considered indicative of the level of susceptibility. Lines with less than 3 infected clones are better indicators of resistance than those with a large number of clones showing infection. Four lines had only 1 or 2 clones that showed severe corm damage after Foc race 1 infection. Of these lines, two (line 27 and 72) were only mildly affected by Foc race 1 infection. Since all the infected transgenic lines were PCR checked, the variable response levels among these transgenic lines could be due to the different integration patterns that could have occurred for each line. Variation in expression and stability which is reported to be caused by environmental effects, promoter methylation, inter-loci interactions and gene silencing can also be responsible for the differences observed (Marenkova et al., 2012). The lines generated as part of this study were evaluated for a single growth cycle and the performance of these genes in ratoon crops was not assessed. For assessing long term transgene expression stability, these lines should be evaluated in the field for at least up to the 10th ratoon crop.

*Transgene choice-*m*Ced9*

Genes that target the pathogen itself or neutralise the pathogen derived metabolites are being exploited in many crops for providing resistance to Foc in susceptible cultivars. Two lines of a susceptible banana cultivar transformed with a plant ferrodoxin like protein (*flp*) gene resulted in 14.2 and 20.8% disease severities compared to the wild type which had 41.6% after Foc 4 infection (Mei et al., 2011). Similarly, transgenic Pisang Nangka cultivar with the rice thaumatin-like protein (*tlp*) had disease incidence of 29.4% compared to the control plant which had 89.1% after 4 weeks of infection (Mahdavi et al., 2012). Although such genes like the thaumatin-like protein have shown lower disease severities compared to the control plants, the necrotrophic nature of *F. oxysporum* demands for a better resistance strategy that would prevent the fungus from killing the plant cells even after infection. Such a strategy will provide more sustainable Fusarium wilt resistance. Anti-apoptosis genes that are able to prevent cell death after infection have shown promising protection in tomatoes, tobacco and banana when challenged with various pathogens (Dickman et al., 2001; Ping et al., 2004; Paul et al., 2011). In the present study, the effect of Foc race 1 infection on transgenic Sukali Ndiizi banana containing the plant-codon optimised synthetic version of a nematode anti-apoptosis gene *Ced9* was evaluated. Even though *Ced9* was previously used for 'proof-of-concept' that it can confer Foc race 1 tolerance to bananas, we have also been aware of the possible regulatory problems related to using a nematode gene for product development. Consequently, we designed a synthetic m*Ced9* (modified *Ced9*) gene with the same amino-acid sequence as *Ced-9* but with altered DNA sequence using plant-preferred codons.

Although many animal anti-apoptosis genes have been shown to prevent cell death and confer biotic and abiotic

stress tolerance to plants (Dickman et al., 2001; Paul et al., 2011), the mode of action of anti-apoptosis genes in plants is not clearly known. Bcl-2 3'UTR has previously been shown to protect plant cells from biotic and abiotic stress (Dickman et al., 2001) and some studies have indicated that microRNAs, e.g. miR-7 regulate the expression of anti-apoptosis gene Bcl-2 through direct 3'UTR interactions (Xiong et al., 2011), in this study, we started with the hypothesis that in case of *Ced9*, it is probably the anti-apoptosis protein and not the DNA/RNA sequences that are involved in generating fungal resistance in the transgenics. This hypothesis was confirmed in this study because 'Sukali Ndiizi' m*Ced9* transgenics showed Foc race1 tolerance similar to *Ced9* transgenics of 'Lady Finger'.

Animal derived anti-apoptosis genes have shown promising protection in plants when challenged with various pathogens. The human *Bcl-2*, nematode *Ced9* and baculovirus *op-iap* were all able to control S. *sclerotiorum* in transgenic tobacco (Dickman et al., 2001), while the human *Bcl-xL* and nematode *Ced9* genes increased tolerance of tomato to cucumber mosaic virus (Ping et al., 2004). Such findings suggest that the products of these anti-apoptosis genes interact with the natural homologues present in plants. Furthermore, some plant Pathogen Related (*PR*) genes and *R* genes in plants have been found to be closely related to mammalian *Apaf*[1] and the nematode *ced4* which genes are known to be regulators of programmed cell death. Recently, it has also been found that the products of these genes (*Apaf*[1], *Ced4* and plant *R* gene) also share amino-terminal effector domains which could further show homology (Christina and Arunika, 2012). Some *Bcl-2* associated anthanogenes (BAG) have been found (Juqiang et al., 2003) and in *Arabidopsis*, eight BAG genes have been identified and the AtBAG6 gene is associated with reduced disease development in *B. cinerea* (Kabbage and Dickman, 2008). In a recent study where native banana genes MusaDAD1, MusaBAG1 and MusaBI1 were used, only MusaBAG1 showed high levels of resistance to Foc 1 (Ghag et al., 2014). It is therefore possible that plant protection via cell death inhibition in naturally resistant plants occurs through BAG genes which interfere with the products of R genes and pathogenesis related proteins to eventually prevent death of cells.

Conclusions

The results obtained in this study further demonstrate the capacity to successfully transform 'Sukali Ndiizi' cultivar and other elite cultivars using *A. tumefaciens* and male flower derived embryogenic cell suspensions. Modified *C. elegans* m*Ced9* gene driven by maize ubiquitin promoter expressed in the transgenics generated could provide significant protection to at least three transgenic Sukali Ndiizi lines against Fusarium wilt. These lines can now be multiplied and evaluated in a disease screening trial in the field with reference cultivars for resistance and tolerance included.

Although this study used a plant codon optimised synthetic gene of nematode origin successfully, using genes of plant origin like the BAG genes would be preferable for purposes of public acceptance (Enoch et al., 2011; Ghag et al., 2014). Identification and isolation of other sequence homologues of the genes involved in programmed cell death can be exploited for generating Fusarium wilt resistant banana cisgenics.

Conflict of Interests

The authors have not declared any conflict of interests.

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Abbreviations

CTAB, Cetyltrimethylammonium bromide; **PCR,** polymerase chain reaction; **RT-PCR,** reverse transcription - polymerase chain reactions; **dNTPs,** deoxynucleoside 5'-triphosphates; **DNA,** deoxyribonucleic acid; **cDNA,** complementary DNA; **PDA,** potato dextrose agar; **LSI,** leaf symptom index; **RDI,** corm discolouration index; **DSI,** disease severity index; **ANOVA,** analysis of variance.

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Full Length Research Paper

Evaluation of potential bio-control agents on root-knot nematode *Meloidogyne incognita* and wilt causing fungus *Fusarium oxysporum* f.sp. *conglutinans in vitro*

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Indigenous strains of *Trichoderma viride* (ITCC No. 6889), *Pseudomonas fluorescens* (ITCC No. B0034) and *Purpureocillium lilacinum* (ITCC No.6887) were isolated from undisturbed forest eco-system of Southern India. These three bio-mediators were evaluated for their antagonism towards root knot nematode, *Meloidogyne incognita* and *Fusarium oxysporum* f.sp. *conglutinans in vitro*. Cell free culture filtrate of these strains significantly inhibited the egg hatching and caused juvenile (J_2) mortality of *M. incognita* at 25, 50, 75 and 100% concentrations. Maximum inhibition in egg hatching and juvenile mortality were recorded in *P. lilacinum* as 94.21 and 91.28%, respectively after 120 h. It was followed by *T. viride* and *P. fluorescens* which recorded 92.72 and 91.46% and 89.12 and 90.14% inhibition in egg hatching and juvenile mortality, respectively after 120 h. Antagonism of *T. viride* on *F. oxysporum* was recorded maximum on the 5th day as 45.82%. Similarly, the antagonism on the 5th day for both the bio-agents of *P. lilacinum* and *P. fluorescens* were recorded as 45.26 and 44.19%, respectively.

Key words: Biocontrol agents, culture filtrate, *Fusarium oxysporum*, *Meloidogyne incognita*.

INTRODUCTION

Root-Knot nematodes are causing a notable damage to a wide range of vegetable crops causing significant yield loss in tropical and sub-tropical agriculture (Sikora and Fernandez, 2005). The symptoms of nematode disease are manifested by the formation of root galls accompanied by stunted growth, chlorosis and loss of viability of the plant (Babu et al., 1999). *Fusarium* wilt is soil borne fungal pathogen which can sustain many years in the soil without a host (Ignjatov et al., 2012). *F. oxysporum* has a worldwide distribution and causes severe root rot or vascular wilt in ample range of plant families (Enya et al., 2008; Lievens et al., 2008; Michielse and Rep, 2009). This fungal pathogen infects the seed and early stages of seedling growth, causing seed decay and damping-off (Punja et al., 2004).

Trichoderma viride is an effective bio-control agent

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against numerous soil borne plant pathogens and can easily colonize plant rhizosphere and helps in the plant growth promotion (Verma et al., 2007; Savazzini et al., 2009; John et al., 2010). *T. viride* proved to be effective against root knot nematode, *Meloidogyne* species and reduced its damage on several crops (Meyer et al., 2001; Abd et al., 2007). Various reports on nematophagous fungus *Purpureocillium lilacinum* prove its efficacy as effective bio-control agent on *Meloidogyne* spp. on various crops (Jatala, 1986; Rao and Reddy, 1994; Rao et al., 1999; Khan and Williams, 1998; Mohd et al., 2009; Brand et al., 2010). It has more rate of occurrence in tropical and subtropical areas (Morgan-Jones et al., 1984; Chen et al., 1996).

Pseudomonas fluorescens is a Plant Growth Promoting Rhizobacteria (PGPR) effective against soil borne pathogens including root-knot nematodes (Perveen et al., 1998; Siddiqui et al., 1999; Rao et al., 2002; Rao, 2007; Otsu et al., 2004). The concept of PGPR has been documented with the isolation of many bacterial strains which exhibit the desirable characteristics of root colonization, disease suppression, plant growth stimulation and biocontrol (Molla et al., 2001; Beneduzi et al., 2008).

Process of DNA extraction from fungal cultures eliminates many unknown interfering substances which allow to identify species specific organisms using ribosomal DNA by PCR (Don et al., 2000; Bryan et al., 1995). Currently, many methods are available for the isolation of fungal genomic DNA (Plaza et al., 2004; Melo et al., 2006). *Pseudomonas* species were isolated from soil eco-system that was naturally suppressive to many plant diseases like *Fusarium* wilt and black rot of tobacco (Thomashow et al., 1990; Raaijmakers and Weller, 1998; De Boer et al., 1999; William et al., 1991).

Culture filtrates of bio-control agents are reported to be antagonistic to a wide range of plant parasitic nematodes in *in vitro* (Reibinger, 1995; Hallaman and Sikora, 1994; Meyer et al., 2004; Vu, 2005; Abd et al., 2007). In the current investigation, efforts were made to isolate the bio-agents and evaluate the effect of three bio-agents, namely, *T. viride*, *P. fluorescens* and *P. lilacinum* against *M. incognita* and *Fusarium oxysporum* f.sp. *conglutinans* in *in vitro*.

MATERIALS AND METHODS

Isolation of bio-agents, collection of *M. incognita* egg mass and *F. oxysporum*

Cultures of *T. viride* (ITCC No.6889), *P. fluorescens* (ITCC No. B0034) and *P. lilacinum* (ITCC No. 6887) were maintained on nutrient agar (NA) (Himedia chemicals, India) for bacterial cultures and Potato dextrose agar (PDA) (HIMEDIA chemicals, India) for fungi by cryopreservation method (Sudheer, 2010). The isolates were sub-cultured and used for further study. Root knot nematode culture was obtained from infected cauliflower plants grown in farmers field (Doddaballapur, Bengaluru rural district, Karnataka, India). Identification of *M. incognita* was confirmed by perineal cuticular pattern (PCP) under stereo microscope and used for further studies. *F. oxysporum* f. sp. *conglutinans* was isolated from fusarium infected cauliflower plants. Preparation of inoculum was made by culturing the *F. oxysporum* isolate in PDA amended with streptomycin sulphate for 7 days at 25 ± 2°C.

DNA isolation from *T. viride*, *P. lilacinum* and *P. fluorescens* and PCR

Genomic DNA was isolated from cultures of *T. viride* and *P. lilacinus* as per the protocol of Raeder and Broda (1985). Bacterial genomic DNA of *P. fluorescens* was isolated using Nucleospin tissue extraction kit (Macherey-Nagal, Germany). Polymerization Chain Reaction (PCR) of 25 µl PCR mixture was prepared for molecular identification of *T. viride* and *P. lilacinum* with the following protocol. Final PCR volume contained 19.7 µl of PCR grade water, 0.5 µl of each forward and reverse primers (0.2 pmol; Bio-serve Pvt. Ltd, India), 0.5 µl of dNTPs (10 mM; Fermentas Inc, Canada), 2.5 µl (10X) of Taq buffer (Bangalore Genei Pvt.Ltd, India), and 0.3 µl of Taq DNA polymerase (Bangalore Genei Pvt.Ltd, India) and 1 µl of template DNA (25 ng/µl). PCR amplifications were carried out in Eppendorf master cycler gradient (vapo.protect, Germany). Amplification reactions were performed in master cycler with heated lid. The primer pairs ITS F (TCCGTAGGTGAACCTGCGG) and ITSR (TCCTCCGCTTA-TTGATATGC) were used for the amplification of region including the ITS 1, 5.8 S and ITS 2 (Hurtado et al., 2008). The initial denaturation for 5 min at 94°C was followed by 35 cycles of 45 s at 94°C, 35 s at 43°C, 40 s at 72°C and a final extension of 10 min at 72°C.

16s rDNA amplification of *P. fluorescens* was carried out using universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) (Weisburg et al., 1991) in thermo cycler (Eppendorf vapo.protect, Germany) using the aforementioned components which followed for final PCR mixture of 25 µl except 16s primers and DNA template (25 ng/µl). The PCR cycle of initial denaturation was done at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 45 s, primer annealing at 51°C for 35 s and elongation at 72°C for 40 s. Final extended elongation was done for 10 min at 72°C. The obtained PCR products were gel electro-phoresis on 1.5% agarose gel (Figures 1 and 2).

The PCR products were sequenced at Bio-serve, Hyderabad, Telangana state, India. All the sequenced PCR products were confirmed using NCBI mega blast for its species identity of ITS region and 16s region. The molecular identified strains were submitted at NCBI.

Effect of culture filtrate of *P. fluorescens* on egg hatching of *M. incognita*

A single colony from pure culture of *P. fluorescens* taken from 24 h old culture plates was inoculated into 50 ml of sterilized King's B broth (HIMEDIA chemicals, India) in 100 ml Erlenmeyer flasks. These flasks were incubated in a shaker incubator at 150 rpm speed and 37°C for 24 h. The bacterial growth after 24 h was tested for their luminosity under transilluminator at 250 to 260 nm. *P. fluorescens* culture filtrate was obtained by centrifugation (Eppendorf refrigerated centrifuge 5415) at 10,000 rpm for 15 min at 4°C. The supernatant culture filtrate was collected and passed through syringe filter of 0.22 µm (Millipore PVDF Durapore 13 mm diameter). Consequently, collected culture filtrate was tested for the absence of any viable cell and used to study the effect on egg hatching and juvenile mortality of *M. incognita*.

P. fluorescens culture filtrate was made into four concentrations of 100, 75, 50 and 25% by adding sterile distilled water. Three millilters of each concentrations of culture filtrate was transferred to sterile Petri-dishes of 5 cm diameter. *M. incognita* egg masses

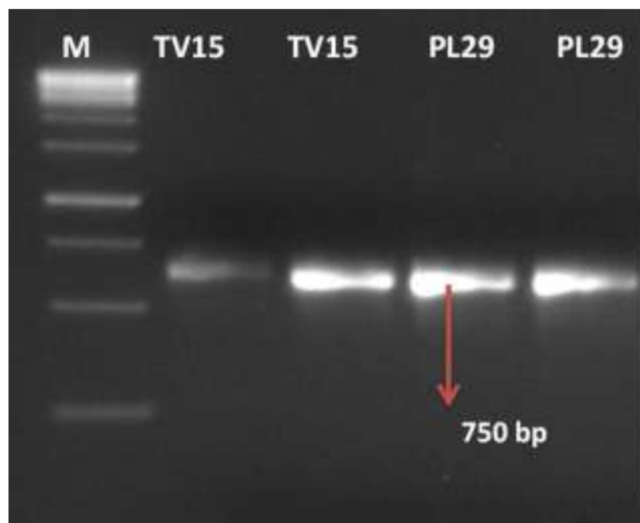

Figure 1. PCR amplified ITS region of *T. viride* and *P. lilacinum*.

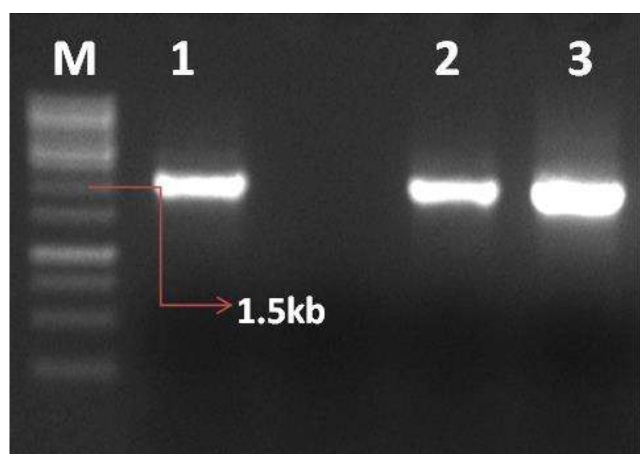

Figure 2. PCR amplified 16s region of *P. fluorescens* (1 to 3).

were collected and surface sterilized using 0.1% sodium hypochlorite for 30 s and rinse the treated egg masses with sterile distilled water. Each petri dish was placed with five egg masses containing culture filtrate of each concentration and incubated at room temperature. *M. incognita* egg masses placed in King' B and distilled water served as control. The number of juveniles (J_2) hatched in all the four concentrations were recorded at 24, 48, 72, 96 and 120 h of exposure.

The percentage suppression in hatching of juveniles (J_2) was calculated using the following formula:

Percentage of hatching suppression = [1- (Ht/Hc)] ×100

Where, Ht is the number of juveniles hatched in treatment and Hc is the number of juveniles hatched in control.

Effect of culture filtrate of *P. fluorescens* on mortality of *M. incognita* juveniles

Culture filtrate was made into four concentrations of 100, 75, 50 and 25% by adding sterile distilled water. Three millilters culture filtrate of each concentration was transferred to sterile Petri-dishes of 5 cm diameter. Freshly hatched 100 *M. incognita* juveniles (J_2) were placed in each Petri dish and incubated at room temperature (25 to 30°C). Petri dishes containing sterile water and autoclaved King's B broth placed with juveniles (J_2) served as control and treatment.. Total number of dead nematodes were counted after 24, 48, 72, 96 and 120 h of exposure and percentage mortality of juveniles was calculated.

Effect of culture filtrate of *T. viride* and *P. lilacinum* on egg hatching of *M. incognita*

Freshly sub-cultured *T. viride* and *P. lilacinum* of 5 mm disc were inoculated in 100 ml sterilized potato dextrose broth – PDB (HIMEDIA chemicals, India) in 250 ml Erlenmeyer flask. The flasks were incubated at 27 ± 1°C for 8 days. From each culture, 50 ml of broth containing 2.8×10^6 spores (CFU/ml) was centrifuged at 13000 rpm at 4°C for 20 min (Eppendorf refrigerated centrifuge 5415). The obtained pellet was discarded and supernatant was collected which passed through 0.45 µm syringe filter (Millipore PVDF Durapore 13 mm diameter). The culture filtrate thus obtained was tested for the absence of any fungal spores by plating it on PDA. *T. viride* and *P. lilacinum* culture filtrate was made into four concentrations of 100, 75, 50 and 25% by adding sterile distilled water. They were tested for hatching and J_2 mortality as per the aforementioned procedure for *P. fluorescens*.

Effect of *T. viride* and *P. lilacinum* antagonists against *F. oxysporum* f.sp. *conglutinans* in *in vitro*

Five day old cultures of *T. viride* (TV-15), *P. lilacinum* (PL-29) and *F. oxysporum* discs (5 mm diameter) were grown on PDA and punched in the periphery in 90 mm petri plates. Each culture was inoculated separately on PDA plates as control. Cultures of *T. viride* with *F. oxysporum* and *P. lilacinum* with *F. oxysporum* were inoculated separately on PDA plates at 20 mm distance from periphery of the petriplate as per dual culture method. Each treatment was replicated thrice and incubated at 27±2°C for antagonistic study. Observations were recorded on growth of each fungal culture for 5 days. The percentage of inhibition of *F. oxysporum* was calculated (Vincent, 1927).

PI =C-T/C×100

Where, PI is the percentage inhibition over control, C is the control of *F. oxysporum* without *T. viride* (mm), and T is the growth of *F. oxysporum* with *T. viride* (mm).

Effect *of P. fluorescens* antagonists against *F. oxysporum* f.sp. *conglutinans* in *in vitro*

Both the cultures of 1 day old *P. fluorescens* and 5 days old *F. oxysporum* were inoculated on Nutrient Agar (NA) and PDA plates (90 mm), respectively. As per dual culture method, *P. fluorescens* was inoculated by spread plate method on PDA plate. *F. oxysporum* disc (5 mm) was inoculated in the centre of PDA plate. Half streak of *P. fluorescens* and 5 mm *F. oxysporum* disc inoculated 20 mm distance from periphery of the plate in the same PDA plates were also maintained for antagonistic study. The same procedure for *T. viride* and *P. lilacinum* antagonistic study was followed.

Table 1. Effects of the cell-free culture filtrate of *T. viride* on egg hatching and mortality of *M. incognita* in *in vitro.*

Concentration (h)	% Suppression in hatching (T)					% J_2 Mortality (T)				
	24 h	48 h	72 h	96 h	120 h	24 h	48 h	72 h	96 h	120 h
25%	51.25 (45.71)	52.41 (46.37)	53.28 (46.88)	54.72 (47.71)	55.18 (47.97)	56 (48.45)	65.13 (53.81)	67.41 (55.20)	68.23 (55.71)	69.72 (56.62)
50%	62.21 (52.09)	63.54 (52.87)	64.11 (53.20)	64.98 (53.74)	65.72 (54.16)	68.51 (55.95)	70.28 (56.96)	71.37 (57.66)	73.25 (58.86)	75.12 (60.09)
75%	69.18 (56.29)	70.35 (57.00)	71.83 (57.94)	72.64 (58.48)	73.84 (59.24)	76.23 (60.84)	78.52 (62.52)	79.25 (62.97)	81 (64.23)	82.51 (65.34)
100%	81.24 (64.34)	82.16 (66.37)	83.08 (65.98)	84.31 (66.84)	92.72 (74.83)	83.15 (65.80)	85.48 (67.64)	86.41 (68.58)	88.27 (70.12)	91.46 (73.05)
Media alone (PDB)	11.02 (19.29)	12.27 (20.30)	13.48 (21.51)	14.13 (22.00)	15.09 (22.80)	10.46 (18.79)	11.35 (19.63)	12.51 (20.66)	13.18 (21.26)	14.64 (22.42)
Control (Distilled water)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)

	CD (0.01)	SED	CD (0.01)	SED
C	2.24	0.85	2.19	0.83
T	2.05	0.78	2.00	0.76
CxT	5.02	1.91	4.91	1.87

Figures in parentheses are arc sine transformed values.

RESULTS

Molecular identification of bio-agents using gene specific primers

Ribosomal DNA internal transcribed spacers region was amplified using fungal genus specific ITS-1 and ITS-4 primers (Figure 1). Bacterial strain was amplified using 16s primers (Figure 2). Molecular identification of these PCR amplified strains were confirmed using NCBI mega blast. *T. viride* and *P. lilacinum* matching to National Centre for Biotechnology Information (NCBI) were 94 and 96%, respectively and *P. fluorescens* was 97%. These strains were submitted at NCBI with the following accession numbers *T. viride* - KP271026, *P. lilacinum*-KP271028 and *P. fluorescens* - KP27102.

Effect of culture filtrates of three bio-agents on *M. incognita*

Results of studies on culture filtrates showed that the efficacy of the isolates of *T. viride*, *P. lilacinum* and *P. fluorescens* on hatching of *M. incognita* eggs and J_2 mortality increased with the increase in concentration of the culture filtrates. These investigations clearly indicated that as the duration of exposure to culture filtrate of the bacterium and fungal cultures increased, suppression in hatching and mortality of juveniles (J_2) also increased. The maximum suppression in egg hatching was recorded in *P. lilacinum* as 94.21% and juvenile mortality as 91.28% after 120 h at 100% concentration (Table 1). It was followed by *T. viride* which recorded the inhibition in egg hatching as 92.72%, and juvenile mortality as 89.12% after 120 h (Table 2). In *P. fluorescens*, suppression in egg hatching and juvenile mortality was recorded as 91.46 and 90.14%, respectively after 120 h (Table 3).

Effect of bio-agents antagonism on *F. oxysporum*

T. viride showed antagonistic activity on *F. oxysporum* on the 5th day which was recorded as 45.82%. It was followed by *P. lilacinum* and *P. fluorescens*, for which the antagonism was recorded on the 5th day as 45.26 and 44.19% (Figure 3).

DISCUSSION

During the experimental investigations, native strains of *T. viride*, *P. lilacinum* and *P. fluorescens* were isolated from different regions of South India. The identity of these isolates were confirmed through molecular techniques and evaluated for their antinemic and antifungal activity *in vitro*. The culture filtrate studies revealed that all the bio-control agents were effective in suppressing the egg hatching of *M. incognita* and causing J_2 mortality which increased with increase in time of exposure of eggs to cell free culture filtrate as well with increase in concentration.

Deformation of juveniles was observed in most of the eggs in the present study. The results

Table 2. Effects of cell-free culture filtrate of *P. lilacinum* on egg hatching and mortality of *M. incognita* in *in vitro*.

Concentration (h)	Suppression in hatching (%)					J_2 Mortality (%)				
	24 h	48 h	72 h	96 h	120 h	24 h	48 h	72 h	96 h	120 h
25%	53.17 (46.81)	54.38 (47.51)	55.09 (47.92)	57.34 (49.26)	58.71 (50.04)	58.24 (49.74)	69.31 (56.37)	71.33 (57.63)	73.48 (59.00)	75.08 (60.05)
50%	65.08 (53.78)	66.41 (54.58)	67.26 (55.10)	68.43 (43.83)	69.12 (56.25)	72.24 (58.21)	74.04 (59.38)	74.81 (59.89)	76.15 (60.83)	77.34 (61.57)
75%	71.27 (57.59)	72.41 (58.32)	73.81 (59.23)	74.08 (59.40)	75.38 (60.27)	78.13 (62.28)	79.14 (63.12)	80.24 (65.01)	82.40 (65.23)	84.52 (66.86)
100%	83.07 (65.78)	85.24 (67.97)	86.18 (68.25)	87.92 (69.95)	94.21 (76.16)	82.04 (64.93)	84.25 (66.64)	85.13 (67.33)	89.14 (70.83)	91.28 (72.92)
Media alone (PDB)	10.42 (18.78)	11.07 (19.38)	12.46 (20.60)	13.51 (21.49)	13.05 (21.11)	11.14 (19.19)	12.21 (20.42)	13.06 (21.15)	13.68 (21.69)	14.04 (21.99)
Control (Sterile water)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)
	CD (0.01)			**SED**			**CD (0.01)**		**SED**	
C	1.99			0.76			3.45		1.31	
T	1.82			0.69			3.15		1.20	
C×T	4.46			1.70			7.72		2.95	

Figures in parentheses are arc sine transformed values.

Table 3. Effects of the cell-free culture filtrate of *P. fluorescens* on egg hatching and mortality of *M. incognita* in *in vitro*.

Concentration (h)	% of Hatching suppression					% J_2 Mortality				
	24 h	48 h	72 h	96 h	120 h	24 h	48 h	72 h	96 h	120 h
25%	52.08 (46.18)	53.49 (46.99)	53.94 (47.26)	55.04 (47.89)	55.78 (48.31)	52.64 (46.51)	63.05 (52.64)	64.22 (53.26)	65.18 (53.84)	66.52 (54.67)
50%	61.04 (51.37)	62.13 (52.03)	63.46 (52.81)	63.91 (53.08)	64.76 (53.59)	64.04 (53.15)	65.17 (53.85)	66.54 (54.66)	67.25 (55.10)	68.84 (56.19)
75%	65.24 (53.90)	66.37 (54.65)	67.19 (55.10)	68.46 (55.86)	69.81 (56.84)	72.64 (58.49)	73.42 (58.97)	74.49 (59.67)	76.21 (60.68)	78.55 (62.51)
100%	79.28 (63.05)	81.44 (64.51)	82.06 (64.98)	83.05 (65.76)	89.12 (70.83)	80.28 (63.68)	81.27 (64.37)	83.22 (65.84)	84.76 (67.05)	90.14 (71.76)
Media alone (NB)	10.18 (18.37)	11.64 (19.91)	12.08 (20.28)	13.34 (21.39)	14.24 (22.12)	11.52 (19.80)	12.41 (20.58)	13.08 (21.08)	14.64 (22.44)	15.16 (22.89)
Control (Sterile water)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)	0 (0.52)
	CD (0.01)		**SED**			**CD (0.01)**			**SED**	
C	1.95		0.74			2.09			0.80	
T	1.78		0.68			1.91			0.73	
C×T	4.37		1.67			4.69			1.79	

Figures in parentheses are arc sine transformed values.

indicated the production of nematicidal compounds in the culture filtrates. These nematicidal compounds produced by *T. viride*, *P. fluorescens* and *P. lilacinum* seemed to play an important role in causing nematode mortality. Many soil borne nematode trapping fungi, endoparasitic fungi, parasites of nematode eggs and cysts were reported to produce toxic metabolites against nematodes (Li et al., 2007). In earlier reports,

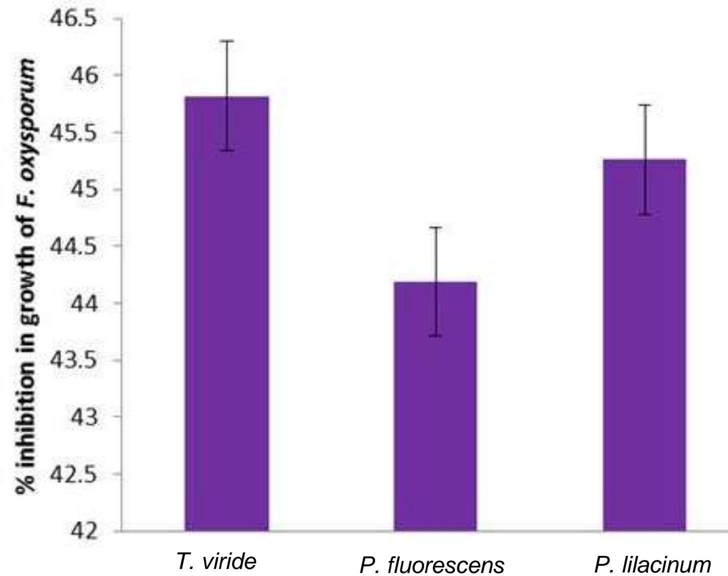

Figure 3. Effect of *T. viride*, *P. fluorescens* and *P. lilacinum* antagonism on growth inhibition of *F. oxysporum* in *in vitro.*

isolates of *P. lilacinum*, an egg parasite of root knot and cyst nematode, showed potential nematoxic activity (Shamim et al., 2012).

These observations also fall in line with experimental evidence as indicated by Regina et al. (1998) and Hanna et al. (1999) who reported that mortality of *M. incognita* increased with increase in exposure time as well as the concentration of culture filtrate. Bin et al. (2005) showed that culture filtrates of rhizobacterium are heat stable and resistant to extreme pH values, which suggested that the antibiotic rather than protein might be responsible for the nematicidal activity.

Antagonism was observed in 3 bio-control agents, namely, *T. viride, P. lilacinum* and *P. fluorescens* against *F. oxysporum in vitro*. *Trichoderma* species are widely used as biocontrol agents to reduce the disease incidence caused by plant pathogenic fungi and many soil borne pathogens (Papavizas, 1985; Sivan and Chet, 1986). Pau et al. (2012) investigated the effect of antagonism on *P. lilacinum* in *in vitro*. The culture filtrates and their antagonism proved the biocontrol efficiency of these microbes. Results on antagonism clearly indicated the effect of bio-agents in *in vitro* on *F. oxysporum*. More antagonism was recorded with *T. viride* followed by *P. lilacinum* and *P. fluorescens*. Hence, it can be exploited further in development of formulations and evaluation under field conditions.

Conclusion

The findings on the effect of culture filtrates of three bio-agents, namely, *T. viride*, *P. fluorescens* and *P. lilacinum* showed on root-knot nematode *M. incognita* in *in vitro.* The experiments also proved the antagonism of these bio-control agents on suppression of *F. oxysporum* f.sp. *conglutinans*. For the resultant data, the effect on *M. incognita* and on *F. oxysporum* was an apparent indication to control the nematode induced disease complex in *in vivo* in cauliflower (Rajinikanth et al., 2013) by application of eco-friendly bio-control agents of bio-nematicide (*P. lilacinum*), bio-fungicide (*T. viride*) and bio-bactericide (*P. fluorescens*).

Conflict of Interests

There is no conflict of interest.

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