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Horticulture is one of the fastest growing sectors in Uganda, exporting products worth US$100 million annually. Passion fruit (Passiflora edulis) growing and export is one of the critical contributors to this sector employing over a million farmers. However, a number of biotic and abiotic constraints have initiated widespread enterprise abandonment by farmers. Passiflora improvement efforts by conventional breeding has had limited success calling for research into alternative approaches such as genetic engineering. The study aimed at optimizing existing protocols to develop an efficient and reproducible Agrobacterium mediated transformation system to suit Uganda’s Passiflora cultivars. Agrobacterium tumefaciens strain AGL1 (OD600 of 0.5) harbouring pCAMBIA2301 containing the GUS (uidA) reporter gene was used to infect pre-cultured leaf discs. Leaf discs were then vacuum infiltrated for 1.5 min at 750 mmHg followed by a three day co-cultivation period on MS + acetosyringone (100 µM). Putatively transgenic yellow passion fruit shoots were induced on Murashige and Skoog (MS) selection media supplemented with benzylaminopurine (BAP) 8.9 µM, kanamycin (100 mgL-1mgL) and cefotaxime (500 mgL-1). Developed shoots were then transferred to elongation media (MS + 0.44 µM BAP) and later rooted on 5.37 µM naphthaleneacetic acid (NAA). Genetic transformation was monitored using GUS staining. A single independently transformed plant was confirmed by polymerase chain reaction (PCR), translating in a transformation efficiency of 0.456%. A viable in vitro transformation protocol for Uganda’s yellow passion fruit directly from leaf discs was developed using GUS reporter gene. Further investigations are required to improve the reported protocols transformation efficiency.

Key words: Passion fruit, Passiflora edulis, Passiflora improvement, genetic engineering, transformation system.

INTRODUCTION

Against the backdrop of falling and fluctuating prices of traditional export crops (coffee, tea, tobacco, cotton), the Government of Uganda embarked on promotion of the horticultural sector as an alternative source of foreign exchange (National Trade Policy, 2007). Horticulture is now one of the fastest growing sectors in Uganda exporting products worth US$100 million per year mainly to the European Union (Ministry of Agriculture, Animal Industry and Fisheries, 2019). Uganda is currently the second largest producer of fresh fruit and vegetables in sub-Saharan Africa after Nigeria (International Centre for Trade and sustainable Development, 2011).
Passion fruit (Passiflora edulis) growing and export is one of the critical contributors to the horticultural sector employing over a million small holder farmers in addition to the other players in the value chain. Uganda annually earns over US$ 200,000 from passion fruit exports (Agribusiness Development Centre., 2014; Uganda Export Promotion Board., 2016). In addition to its commercial importance, species of Passiflora also have medicinal, nutritional and ornamental value (Manders et al., 1994; Freitas et al., 2007). The demand and potential of passion fruit production is much higher compared to the current production, and this has been attributed to a number of biotic and management-related factors (Ochwo-Ssemakula et al., 2012).

Declining yields from passion fruit farming due to a number of problems ranging from pests, diseases, environmental stress, low yields, have initiated widespread enterprise abandonment by farmers. Diseases are the most significant biotic constraint with viral infections accounting for 40% yield loss and up to 40% reduction in fruit quality in some parts of the country (Ochwo-Ssemakula, 2012; Wangungu et al., 2014). Crop improvement efforts by conventional breeding techniques have had limited success since the process is relatively slow, partially hindered by sexual barriers and high ploidy levels, limited genetic variability and requires many cycles of selfing and backcrossing to eliminate deleterious genes (Varassin et al., 2001; Petri and Burgos, 2005).

These constraints have necessitated research into other alternative approaches for Passiflora improvement such as plant tissue culture and genetic engineering (Drew, 1997). However, there is lack of a reliable, efficient and reproducible transformation system that is compatible with a regeneration method for the successful transformation of Passiflora varieties in Uganda. In addition, the high heterogeneity of genus Passiflora makes genetic transformation very difficult due to a number of physiological and developmental problems. Owing to the increased genetic variability among its species, there was need to optimize existing transformation protocols to suit Uganda’s Passiflora cultivars. The study thus aimed at establishing an efficient and reproducible transformation system for Uganda’s P. edulis f. flavicarpa (yellow passion fruit) using Agrobacterium for routine utilization with an ultimate goal of improving its agronomic value.

MATERIALS AND METHODS

Study site, design and source of plant material

The study was conducted at the National Agricultural Research Laboratories (NARL) - Kawanda, an institute under the National Agriculture Research Organization (NARO). The study followed a completely randomized experimental research design. Yellow passion fruit (P. edulis f. flavicarpa) was chosen for the study since it is the most widely cultivated species of Passiflora in tropical countries owing to its tolerance to diseases, larger fruit quality and vigorous vine. High performing P. edulis (yellow) believed to be disease free were originally purchased from the Kawanda Nursery, an entity under NARL with the guidance of the Nursery manager relying on his expertise and documentation. Plants were purposively sampled according to parameters like yield, stature, growth rate, vigour and grown in an insect proof screen house with regular watering.

Immature young leaves were picked from the growing Passiflora for the study. Consent from the Ethics Committee to use the experimental materials in the study was verbal as the study was mainly a proof of concept. The constructs used did not add any known desired trait to the plants but merely a means of assaying the feasibility of transforming Uganda’s P. edulis with no possibility of releasing the genetically modified materials.

Vector and Agrobacterium cells transformation

Commercially available pCAMBIA2301 GUS transformation vector (catalogue number: 101743-186; Marker Gene Technologies, Inc; https://www.markergene.com/pcambia2301 plant-expression-vector.html) was used in the study. Binary vector pCAMBIA2301 (Figure 1) containing the GUS (uidA) reporter gene under constitutive CaMV 35S promoter, nopaline synthase (NOS) terminator, lacZα promoter and a kanamycin selection genes nptII (neomycin phosphotransferase II) for both bacteria and plant selection was used in the study.

Electrocompetent Escherichia coli cells strain JM109 were prepared using cold sterile double distilled water. Electrocompetent E. coli was then transformed with the plasmid pCAMBIA2301 using an electroporation apparatus set at 2.5 kV, 25 μF and 200 Ohms. Putatively transformed E. coli cells were then plated on Luria Bertani (LB) (10 g Bacto-tryptone, 5 g yeast extract, 10 g sodium chloride and 15 g micro-agar per litre) plates supplemented with kanamycin (100 mgL-1) and incubated overnight at 37°C. The colonies were screened for the presence of the pCAMBIA2301 using a colony polymerase chain reaction (PCR). Electrocompetent AGL1 Agrobacterium tumefaciens cells (without plasmid) were prepared using sterile double distilled water; AGL1 was then transformed with plasmid pCAMBIA2301 by electroporation. Putatively transformed A. tumefaciens were then cultured on LB agar supplemented with Kanamycin (110 mgL-1), Carbenicillin (250 mgL-1) and rifampicin (50 mgL-1). The plates were incubated at 28°C for 3 days and any colonies that developed were tested for the presence of pCAMBIA2301 using Gus specific primers.

PCR analysis to confirm transformation of E. coli JM109 and Agrobacterium tumefaciens AGL1

The transformation of the respective bacteria with the Ti plasmid was only confirmed by colony PCR using specific Gus primers. The GUS gene (~530 bp) was amplified using the following Gus specific primers (Gus Forward primer 5’- CGACGGGCTGTTGGCCATTC-3’, Gus Reverse primer 5’- TGGTGTGACCCATCGAGGAC-3’). PCR reaction mixture had a total volume of 20 μl for a 1x reaction containing 10 μl Gotaq, 0.4 μl of the forward and reverse Gus primers respectively, 1 μl DNA template (E. coli and Agrobacterium
AGL1) and sterile dH2O. The PCR cycling conditions included an initial denaturation step of 94°C for 5 min, followed by 35 cycles of 50 s at 94°C, annealing at 56°C for 1 min and 72°C for 1 min with final extension at 72°C for 7 min. PCR products were electrophoresed on a 1.35% agarose gel at 120 V, 400 mA for 40 min, stained in ethidium bromide and the DNA visualized and photographed under ultraviolet light using Genesnap software.

Preparation of Agrobacterium cultures for transformation of leaf discs

A single clone of AGL1 confirmed by PCR was used to prepare glycerol stocks which were stored at -80°C and routinely used for transfection of yellow passion fruit leaf discs. A week to the actual transformation, AGL1 glycerol stock was streaked onto LB agar plates supplemented with kanamycin (100 mg·L^-1), carbenicillin (250 mg·L^-1) and rifampicin (25 mg·L^-1) and grown at 28°C in a BIOCONCEPT digital incubator for 3 days. Four days prior to transformation, a two-day Agrobacterium AGL1 liquid culture was started using a single plate colony in 25 mL LB broth containing kanamycin (100 mg·L^-1), Carbenicillin (250 mg·L^-1) and rifampicin (25 mg·L^-1). Agrobacterium was grown in a MaxQ™ bench top orbital incubator shaker at 200 rpm at 28°C.

Preparation of explants for Agrobacterium mediated transformation

Immature leaves excluding buds were excised from the passion fruit plants, rinsed with detergent under running tap water and soaked for 25 min in 2% fungicide (Rovral Aquaflo™, 2mL·L^-1; Active ingredient; 500 g·L^-1 Iprodione). Leaves were surface sterilized with 70% (v/v) ethanol for two min before finally being soaked in 2.5% Sodium hypochlorite (commercial bleach) for 10 min in a laminar flow hood (Class II-Type A). At the end of each of the above steps, the leaves were rinsed thrice in sterile distilled water to clean them of the previously used solutions. Leaf discs (±1 cm^2) were sliced from sterilized leaves and precultured on Murashige and Skoog (1962) medium supplemented with 8.9 µM BAP for 1 day. Murashige and Skoog (MS) media was prepared by dissolving full strength MS basal salts and vitamins, sucrose (30 g·L^-1), appropriate BAP concentration and pH adjusted to 5.8. Gelrite (2.4 g·L^-1) was added prior to autoclaving at 121°C for 15 minutes.
All biochemicals and media constituents used in the study were tissue culture grade procured from either Sigma-Aldrich (St Louis, Missouri, USA) and Duchefa Biochemie, (RV Haarlem, Netherlands).

Agrobacterium mediated transformation of pre-cultured leaf discs and co-cultivation

On the transformation day, the overnight Agrobacterium culture was harvested by centrifugation at RCF 5,000 x g for 5 min at 4°C using a bench top Thermo Scientific Biofuge PrimoR Heraeus refrigerated centrifuge. The supernatant was discarded and the remaining bacterial cells were re-suspended in 20 mL liquid Murashige and Skoog (MS) media (without gelrite) supplemented with 8.9 μM BAP, 100 μM acetosyringone minus antibiotics for infection and co-cultivation of explants. The bacterial cell suspension was then shaken for 1.5 h at 80 rpm at room temperature on a Stuart Orbital shaker incubator to activate the Agrobacterium. Precultured leaf disc for transformation were heat shocked in plain Passion Fruit Regeneration Media (PFRM) without Agrobacterium AGL1 for 1 min at 45°C in a water bath to prevent apoptosis of injured cells. Leaf discs were then inoculated with A. tumefaciens (OD600=0.5) transformed with pCAMBIA2301 for 18-22 min with occasional shaking at 25°C in 50 mL falcon tubes. Explants suspended in Agrobacterium were vacuum infiltrated in falcon tubes for 1.5 min at 750 mmHg using a BioListic® PDS-1000/He Particle Delivery System. Leaf discs were then blotted dry on sterile blotting paper to remove excess bacteria and transferred onto non selective MS media supplemented with 8.9 μM BAP and 100 μM acetosyringone for a 3-day co-cultivation period. Cultures were incubated at 22°C in darkness with the adaxial side of the leaves in contact with media. Control leaf discs underwent the same treatment except they were not dipped in A. tumefaciens cell suspension but instead dipped in liquid PFRM supplemented with 100 μM acetosyringone minus antibiotics.

Two transformation experiments were conducted translating into two replicas, in which a total of 266 A. tumefaciens inoculated yellow passion fruit leaf discs were sub-cultured for both replicas while 56 leaf discs were used as the controls. Cultures were incubated in the dark for eight weeks at 26°C and then changed to a 16-h photoperiod (light) at 27 ± 1°C.

Selection, shoot regeneration, elongation and rooting media

After co-cultivation, inoculated discs were washed with 500 mL-1 cefotaxime and cultured on MS selection media supplemented with BAP 8.9 μM, kanamycin (100 mg L-1) and cefotaxime (500 mg L-1). Shoot regeneration from co-cultivated leaf discs was induced on MS supplemented with 8.9 μM BAP, kanamycin (100 mg L-1) for plant selection and cefotaxime (500 mg L-1) to kill bacteria. Developed shoots were subcultured onto elongation media (MS + 0.44 μM BAP, cefotaxime (300 mg L-1), kanamycin (100 mg L-1)) and cultures maintained at the 16-h photoperiod. Elongated shoots (4 cm and above) were transferred to MS media supplemented with 5.37 μM NAA, cefotaxime (300 mg L-1) and kanamycin (100 mg L-1) for root induction. Control explants were treated in the same way, except that they were neither inoculated nor co-cultivated with A. tumefaciens.

Monitoring, detection and confirmation of transformants

The histochemical assay was done to monitor the efficiency of transformation by screening for expression of β-glucuronidase (GUS gene) activity in putatively transformed yellow passion fruit explants according to protocols by Jefferson et al. (1987). Explants were assayed for expression of the gene ten days after Agrobacterium-mediated transformation following the histochemical procedure below. Explants were incubated at 37°C in a stirring buffer with final concentrations of 0.1 mM sodium phosphate, pH 7.0, 10 mM Na-EDTA, 1 mM potassium ferricyanide, 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt) and 0.1% (v/v) Triton X-100 at 37°C for 24 h. Explants were then submerged for 2 days in 90% ethanol to remove chlorophyll in order to bleach explants and enhance visualisation of the blue stain.

The integration of the transgene into the plant genome was detected and confirmed by PCR analysis using Gus specific primers. Total genomic DNA was extracted from fresh leaves of transformed and untransformed (control) plants using the cetyltrimethylammonium bromide (CTAB) method (Chaudhry et al., 1999). Further, concentration of the extracted DNA was determined using the NanoDrop 2000 (Thermo Scientific™) before dilution to 95 ng/μl to run PCR. For PCR analysis of transgenic plants, the GUS gene fragment (∼530 bp) was amplified using the above specified Gus primer set. The reaction mixture, with a total volume of 20 μl for a 1× reaction contained 10 μl Gotaq, 0.4 μl volume of the forward and reserve Gus primers, 1 μl (95 ng/20 μl reaction) DNA template and the reaction volume was made up to 20 μL using sterile dH2O. The PCR cycling conditions for GUS included an initial denaturation step of 94°C for 5 min, followed by 35 cycles of 45 s at 94°C, annealing at 56°C for 1 min, elongation at 72°C for 1 min with a final extension at 72°C for 7 min. Plasmid DNA (50 ng/20 μl) was used as a positive control and DNA (95 ng/20 μl) from a control plant as a negative control.

PCR products were electrophoresed on a 1.35% agarose gel at 120V, 400 mA for 40 min, and stained in ethidium bromide. DNA was visualized and photographed using Genesnap software (Sambrook et al., 1989). Data was analyzed using both qualitative and quantitative statistical analysis using Microsoft Excel 2007; descriptive statistical approaches using quantitative measurements was used to analyze and summarize the data in tables using measurements like averages, percentages and frequencies.

RESULTS

There was successful transformation P. edulis f. flavicarpa (yellow) passion fruit using GUS reporter gene via A. tumefaciens mediated transformation.

PCR analysis to confirm presence of pCAMBIA2301 in putatively transformed E.coli JM109 and A. tumefaciens AGL1

Amplification of the predicted size fragments of ~530 bp corresponding to the amplified internal fragments of the GUS gene for the first and second transformations in both E. coli and A. tumefaciens were positive. The approximated size fragments were observed in most of the colonies tested confirming transformation of bacteria with Ti plasmid (Figure 2). No specific amplification products were observed in case of untransformed E. coli and A. tumefaciens.

GUS histochemical assay

There were clearly visible spatial blue coloration and
spots-specks on the leaf discs stained with X-Gluc reagent whereas no blue coloration was observed in the non-transformed control explants (Figure 3). Explants with strong transient GUS expression presented bluer staining and vice versa. Positive Transient GUS staining was an indicator of successful delivery of the uidA gene via A. tumefaciens into yellow passion fruit leaf discs. GUS was an effective reporter of genetic transformation in yellow passion fruit leaf discs. Considering results from GUS staining, transformation efficiency was 70%, that is, 14 of the 20 randomly sampled putatively transformed leaf discs showed some level of transfection (chimeric blue regions).

Regeneration of putatively transformed yellow passion fruit on MS supplemented with BAP 8.9 μM

There was unsuccessful regeneration of putatively transformed plantlets for the first transformation experiment; however two sprouts were produced after approximately 16 weeks (4 months) which later died. Out of the 103 inoculated explants that survived infection, no shoot was produced translating into a regeneration efficiency of 0% for putatively transformed plantlets (Table 1). For the second transformation, there was successful regeneration of putatively transformed plantlets; four shoots were successfully induced on selection media resulting in the development of two rooted plantlets within a period of 4-5 months (Figure 4). Out of the 116 inoculated explants that survived infection, the two regenerated shoots translated into 1.72% ($\frac{2}{116} \times 100$) regeneration efficiency (Table 1).

Generally, most of the explants succumbed to kanamycin selection pressure, with the antibiotic lethal and sensitive to the untransformed tissue or partially transformed leaf discs killing them off within 3 months (Figure 4). Leaf discs started drying after 6 weeks on media; they then became brittle followed by large scale necrosis of most of the leaf disc surface within 12 weeks (3 months). Necrosis was probably due to chimerism where one leaf disc was composed of both putatively transformed and untransformed sections. Patches of the leaf disc tissue that were untransformed were selected against the kanamycin while those that were putatively transformed were tolerant to the selective agent. Chimerism was already evidenced by the GUS staining where the blue coloration was not uniformly distributed across the leaf disc surface implying that some cells of the leaf had taken up the plasmid while others had not been transformed (Figure 3).
Figure 3. Transient GUS expression of 10 day old leaf discs; A: Positive transient GUS expression for 1st transformation; B: Positive GUS staining for the 2nd transformation.

Table 1. Putatively transformed yellow passion fruit leaf discs Shoot induction statistics on MS supplemented with BAP 8.9 μM for both transformation experiments.

<table>
<thead>
<tr>
<th>Media</th>
<th>Observations of shoot induction process and progress</th>
<th>Plantlet regeneration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFRM + antibiotics (n =161)</td>
<td>Experimental setup</td>
<td>No. of infected explants</td>
</tr>
<tr>
<td>Replica 1</td>
<td>Inoculated</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>5</td>
</tr>
<tr>
<td>Replica 2</td>
<td>Inoculated</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>4</td>
</tr>
</tbody>
</table>

Plantlet Regeneration % = \[\frac{\text{Regenerated plantlets}}{\text{(Number of explants inoculated-Infected explants)}} \times 100\]. For each Replica, a total of 161 explants were used (133 explants inoculated with Agrobacterium and 28 explants as controls), infected explants were those that succumbed to Agrobacterium overgrowth or fungal contamination, Necrotic explants were those that succumbed to kanamycin selection.

PCR analysis of putatively transformed yellow passion fruit plants to confirm integration of pCAMBIA2301

Integration of T-DNA from pCAMBIA2301 was confirmed using PCR in only one of the two plantlets that survived on selection pressure. Amplification of the expected size fragment (~530 bp) using PCR was observed in only one of the two putatively transformed yellow passion fruit plantlets confirming integration of the GUS gene (Figure 5). The other plant did not show DNA amplification of the expected band size suggesting that it was an escape (Figure 5). No amplified product was observed in case of the non-transgenic control plant (negative control).

Considering only the successfully regenerated transgenic plantlet, overall transformation efficiency (Genetic transformation efficiency % = \[\frac{\text{Number of plantlets regenerated}}{\text{Number of explants inoculated- infected explants}} \times 100\]) for both transformation experiments was 0.456% \(\left(\frac{1}{219}\times100\right)\). Transformation efficiency was higher in the second transformation experiment at 0.970% \(\left(\frac{2}{205}\times100\right)\) compared to 0% in the first transformation experiment.

DISCUSSION

Introduction of target genes into the genome of elite varieties and regeneration of transgenic plants with high
efficiency are the most desirable strategies for production of transgenic plants. The study successfully transformed the commercially important yellow passion fruit variety from leaf discs via *Agrobacterium* mediated transformation resulting in the production of one transgenic plantlet. This was attributed to the availability of a plant regeneration system and to the fact that explants were susceptible to infection by *A. tumefaciens*

The study showed that *Agrobacterium* mediated genetic transformation of yellow passion fruit is feasible, however the genetic transformation efficiency was very low at 0.456%. Other researchers who have carried out similar studies on the same variety have also reported very low genetic transformation efficiency percentages with Trevisan et al. (2006) reporting genetic transformation efficiencies of 0.11 and 0.21% for two Brazilian yellow passion fruit cultivars IAC-275 and IAC-277 respectively. Monteiro et al. (2011) also reported low genetic transformation efficiencies of 0.67 and 0.19% respectively for the same abovementioned Brazilian yellow passion fruit transgenic lines in a similar study. A related study on *P. alata* reported a marginally higher transformation efficiency of 0.89% (Correa et al., 2015). The present study revealed slightly higher transformation efficiency compared to previously reported studies probably due to the lower numbers of leaf explants used in the study.

The observed low genetic transformation efficiency could be attributed to a number of possible factors. Studies have reported that transformation efficiency depends upon many factors such as regeneration potential, cultivar, physiological nature of explant, age of explant, pretreatment prior to inoculation, *Agrobacterium* strain and its density plus antibiotic concentrations used. Studies by Davis et al. (1991) and Madhulatha et al., (2007) reported that co-cultivation time, different growth regulators and their concentrations, transformation conditions all play an important role in the success of transformations.

The recorded low genetic transformation efficiency by the study was majorly due to limitations in plantlet regeneration; there was observed difficulties in promoting shoot elongation and plantlet development. This was further amplified by the low number of initial explants used. Plant regeneration is an integral part of most plant transformation strategies, and can often prove to be the most challenging aspect of a plant transformation protocol. Successful transformation of any plant depends

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**Figure 4.** Putatively transformed yellow passion fruit plants; **A**: 4 week old putative transgenic buds; **B**: 4½ months old putative transgenic sprouts of yellow passion fruit on selection media; **C**: 28 week (7 month) old putatively transformed yellow passion fruit plantlet; **D**: 14 week (3½ month) old browned leaf discs on selective media.
on the development of a quick and efficient regeneration system. An efficient regeneration protocol which is compatible to various transformation techniques is a prerequisite for successful application of genetic engineering (Manders et al., 1994). Trevisan et al., (2006) and Monteiro et al. (2011) to a considerable extent attributed their low genetic transformation efficiencies to limitations in shoot induction and plantlet development. Studies have reported that the basic requirement for a successful gene transfer system for producing transgenic plants is the availability of a target tissue made up of a large number of regenerable cells that are accessible to the gene transfer treatment (Birch, 1997; Cho et al., 2004). These studies continued to emphasize that these cells must retain the capacity to regenerate even after being subjected to varied explant preparation and selection treatments. It was obvious from the study that superior regeneration potential is important for successful passion fruit transformation.

The study observed adverse deleterious effects of kanamycin on morphogenesis and development of transformed leaf discs with large scale browning and necrosis most probably due to the high concentration of kanamycin (100 mgL\(^{-1}\)) used. These findings were supported by Holford et al. (1992) and Lin et al. (1995) who noted that some antibiotics have a detrimental effect on plant tissue cultures. Since only a limited number of explant cells are usually transformed after inoculation/co-culture with \textit{A. tumefaciens}, this leads to chimeric tissue consisting of transformed and untransformed cells. The observed large scale necrosis was probably due to chimerism where the portions of the leaf disc tissue that were untransformed were selected against by the kanamycin creating a cascade effect resulting in the elimination of the entire leaf disc. Developing a selection procedure for non genetically uniform organisms favouring the growth of transformed cells over untransformed cells is extremely critical and difficult (Hanke et al., 2007).

Selection agents like kanamycin significantly decrease the relative density of viable cells by eliminating untransformed cells resulting in severe growth inhibition of the surviving transgenic cells. This was collaborated by Winkler and Quoirin (2002) who reported in a similar study that no yellow passion fruit leaf explant survived on media supplemented with kanamycin (100 mgL\(^{-1}\)) with explants becoming chlorotic and later necrotic at the end of thirty days. Cefotaxime was also used in media to kill and prevent bacterial growth yet studies have reported on its negative effect on shoot induction and development (Okay and Pedersen, 1988; Ling et al., 1998). There was an escape which regenerated on selection media during the study. The regeneration of non transformed plants according to Ghorbel et al. (2000) could be due to inefficient selection where surrounding transformed cells offer protection to non transformed cells from the selection pressure.

Since the explants that were used for the study were picked from the field and thus had to undergo surface

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**Figure 5.** PCR amplification of a ~530 fragment product of the \textit{GUS} gene using Gus primers for Putatively transformed yellow passion fruit plants on a 1.35% agarose gel; \textbf{L-O’GeneRuler}\textsuperscript{TM} 1kb Plus DNA Ladder; \textbf{PC}-Positive Control using plasmid pCAMBIA2301 DNA; \textbf{NC} Negative Control using DNA from an untransformed control plant; \textbf{Lanes 1-2}-DNA samples extracted from putatively transformed passion fruit plants.
sterilization, the harsh sterilization disinfection procedure could have had a negative impact on the regeneration potential of the leaf discs. Oyebanji et al. (2009) stated that it is important to recognize that the sterilization period may differ according to the host species. He continued to argue that the optimal period of disinfection should be determined when working with a new species to eliminate the negative effects of over sterilization which normally results in damage or even death to the leaf tissue.

The element of the appropriate age, physiological condition and genotype of the leaf explants also arose. Leaf explants were picked from fully grown passion fruit plants and not seedlings as recommended by most studies which could have hindered leaf disc transformation and regeneration. Trevisan et al., (2006), in his transformation study collected young leaves from 50 to 75-day old seedlings. Studies have cautioned against the use of older explants since the age of the plantlets is critical for transformation efficiency favouring the use of young healthy green well expanded leaves from the two to three week old in vitro plantlets for genetic transformation (Chabaud et al., 1988; Trevisan et al., 2006).

On the other hand, the efficiency of A. tumefaciens transformation considering the transient expression of β-glucuronidase activity was approximately 70%; this percentage was extremely high compared to the ultimate whole plant transformation efficiency of 0.456%. This showed that transgenic yellow passion fruit regeneration from successfully transformed leaf discs was the limiting factor as discussed above. The observed high transformation efficiency evidenced by GUS staining could be attributed to a number of procedures used in the development of the reported transformation protocol that included a pre-culturing step, vacuum infiltration, optimal cocultivation period and addition of acetosyringone. A number of studies have reported that addition of exogenous acetosyringone increases Agrobacterium T-DNA transfer into host cells (Sheikholeslam and Weeks, 1987; Adachi et al., 2005).

Agrobacterium-mediated transformation assisted by vacuum infiltration was first reported in Arabidopsis by Bechtold et al. (1993) and has since been used in other plants such as soybeans, wheat, rice etc. Vacuum infiltration generates a negative atmospheric pressure that causes the air spaces between the cells in the plant tissue to decrease allowing the infiltration of the infective transformation vector to relocate into the plant tissue. Co-cultivation of explants with A. tumefaciens for three days allowed bacterial cells to infect and interact with explant cells effecting gene transfer into explant cells. It is essential to establish optimum co-culture conditions of explants and the Agrobacterium to increase frequency of transformation and avoid bacterial overgrowth due to prolonged co-cultivation period (Villemont et al., 1997; Suzuki et al., 2001).

Preculturing explants for one to three days prior to inoculation and co-cultivation with Agrobacterium has been shown to improve genetic transformation frequencies in many plants. Preculturing of explants had a positive effect on the induction of actively dividing competent cells for transformation (Villemont et al., 1997). Suma et al. (2008) reported that preculture of young buds of ginger on callus induction medium for three days increased the transformation frequency almost eight fold compared to explants that did not undergo preculture.

Conclusion
In this study, a viable transformation protocol for Uganda’s yellow passion fruit directly from leaf discs was developed using the GUS reporter gene. Further investigations are needed to decipher the effect of a number of variables in order to improve the protocols transformation efficiency. The method reported here provides new opportunities for the crop improvement of Uganda’s passion fruit with agronomically useful traits.

ABBREVIATIONS
2,4-D-2,4-dichlorophenoxyacetic acid; GA3-Gibberelic acid; GUS-β-glucuronidase; MS, Murashige and Skoog medium; NAA, naphthaleneacetic acid; PCR, Polymerase chain reaction; T-DNA-Transfer DNA; PFRM, Passion Fruit Regeneration Media; X-Gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexyl ammonium salt; BAP, Benzylaminopurine.

CONFLICT OF INTERESTS
The authors have not declared any conflict of interests.

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Full Length Research Paper

Dehairing of animal hides and skins by alkaline proteases of Aspergillus oryzae for efficient processing to leather products in Tanzania

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The leather industry is one among the most vital sectors for economic development contributing to high earnings. However, tanning of hides and skins is constrained by primitive technology and the use of hazardous chemicals that contribute to environmental pollution. This study was conducted to evaluate the efficacy of alkaline protease on dehairing animal hides and skins under different conditions. Alkaline protease was extracted from Aspergillus oryzae MG429773 cultured by solid state fermentation with a medium containing rice husks supplemented with 1% mineral solution incubated at 30 to 35°C for 7 days at pHs ranging from 7.5 to 9.5. The filtrate was used as a crude enzyme solution and also partially purified for a dehairing bioassay. Four pieces of cattle hide and goat skin (10 cm × 10 cm) were soaked or sprayed with enzyme solutions for a maximum of 5 days. Percentages of depilation of the hides and skins were recorded at 6 and 12 h intervals. Enzyme produced by A. oryzae showed the highest dehairing activity from 15 to 20\% concentration of partially purified enzyme whereby 100\% of goat and cattle hairs were removed within 24 and 48 h, respectively. For 5 and 10\% enzyme concentration, 93.75 and 68.75\% of hide and skin depilated in 72 h, whereas for the control 0.0 to 6.5\% depilation was achieved in 72 h in hide and skins, respectively. Application of the A. oryzae protease by spray method was superior over a dipping method that released hair in effluent and caused skin decay after 5 days of exposure. This study indicates that enzyme extracted from cheaply available resources is efficient for dehairing by a spray method could be a potential technology for application by local tanners to improve the leather industry in Tanzania.

Key words: Alkaline protease, enzymatic dehairing, hides and skins, leather industry, fungal biotechnology.

INTRODUCTION

The leather industry is an important segment of the evolving industry in developing countries including Tanzania. The leather industry contributes to growth of the domestic production and adds to foreign currency reserves (China and Ndaro, 2015; Humphrey, 2003). Several countries including China, India and Ethiopia

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improve their economies through export of leather products such as coaches, clothes and shoes although the later faced a number of challenges (Mahmud, 2000 (Fan and Scott, 2003; Amiti and Freund, 2010). Tanzania is endowed with large number of cattle and goats, but animal by-products are underutilized for economic purposes. Cattle hides, sheep and goat skins are poorly handled and exploited due to the low level of processing technologies (China and Ndaro, 2015; Eifert et al., 2005; Murphy, 2002; Mahmud, 2000) including application of hazardous chemical such as sodium sulphide and chromium that are toxic and cause pollution of water bodies and are hazardous for all kinds of organisms. All these processes release waste and render leather as one of the most polluting industries due to high amount of chemical and solid wastes especially hair and chrome released into environment (China and Ndaro, 2015; Jian et al., 2011; Ozgunay et al., 2007). Leather processing involves multiple processes such as soaking, liming, deliming, tanning bating and degreasening and use of a considerable amount of chemicals and water to produce leather (Dixit et al., 2015; Thanikaivelan et al., 2004). Soaking hides or skin to soften and remove hair is the most important step in leather processing requiring proper performance to facilitate the proceeding steps (Thanikaivelan et al., 2004). Such processes are dehairing, liming, with use of alkaline sodium sulphide to complete hair removal which in turn accumulate toxic sulphide and chrome released into environment (Sawant and Nagendran, 2014). Use of these hazardous chemicals releases toxic substance to environment. This has raised concern among environmental activists and has hindered growth of the tanning industry in Tanzania (Kumar et al., 2008; Perkins, 1983). Enzymatic processing of leather is gaining popularity worldwide due to safety and quality of leather products (Fathima et al., 2010; Thanikaivelan et al., 2004). Enzyme biotechnology is an environmentally friendly alternative for some processes such as deliming, bating and degreasening and enzyme treatment can be used for removal of unwanted protein materials with the exception of the pickling process which is an acidic treatment of the hides/skins to increase shelf life of leather (Saran et al., 2013; Thanikaivelan et al., 2002). Tanning is the process using complex chemicals such as chromium VI (CrVI) to change the skins and hides into colourful and flexible materials. Biotechnological tanning reduces the effects of chromium in effluents (Jenitta et al., 2013; Suresh et al., 2001). Neutralization, dyeing and degreasening are the final processes to improve the structure, texture and appearance of leather. But the common dye used is an azo dye compound that persists in sewage and might harm the environment. The application of alternative technologies such as enzymic dehairing would minimize application of toxic chemicals and their effects on humans and other organisms. Microbial processing is a good alternative to simplify leather processing by reducing processing steps into a few important ones and making the products more safe, attractive and durable (Dayanandan et al., 2003). Several proteases including keratinase from filamentous fungi have been reported as effective in leather for soaking, dehairing and degreasing (Choudhary et al., 2004; Gupta and Ramnani, 2006). Aspergillus species is one of the most studied genera for production of industrial enzymes (Chellapandi, 2010) whereby Aspergillus oryzae has been reported as effective fungus for removal of chromium from tanning effluent (Nouri et al., 2005). In this study, local isolates of A. oryzae (MG429773) from previous study (Zekeya et al., 2019) were used for production of alkaline protease using rice husks as medium. Use of rice husk supplemented with nitrogen source (Pedri et al., 2015) is reported to add value to over 4.1 million tons of rice husks produced annually in Tanzania with scarce utilization (Saied et al., 2014). Furthermore, it replaces use of food stuff like sugar cane, corn wheat in solid state fermentation, which is threatening food security (Pensupa et al., 2013). Then crude enzyme was evaluated for its efficacy for dehairing of cattle hides and goat skins. The overall aim is to reduce economic and environmental costs encountered with conventional tanning in Tanzania.

MATERIALS AND METHODS

The fungus was collected from Nelson Mandela African Institution of Science and Technology laboratory. Rice husks were supplied from Moshi rice milling and the brown sugar (sucrose) purchased at local shops in Arusha. The mineral solution included sodium chloride (NaCl), ammonium sulphate ((NH₄)₂SO₄ and manganese (II) sulphate (MnSO₄.H₂O) and agar supplied by Sigma Aldrich, Chemie Gmbh, Germany whereas Ammonium sulphate ((NH₄)₂SO₄ from LOBA and Trichloroacetic acid AR (TCCA) from LOBA Chemie Pvt, Mumbai, India. Yeast extract from SIGMA ALDRICH, Louis, USA and Tris Hydrochloric acid (C₃H₇NO₃HCl) and Casein was supplied by Duchefa Biochemie, Haarlem, The Netherlands. Growth media, potato dextrose agar (PDA), potato dextrose broth (PDB), tween 80 and tween 20 were supplied by HiMEDIA Laboratories Pvt, Mumbai India and sunflower oil from Singda Sunflower (local).

Collection and isolation of fungal isolate

Two fungal isolates were supplied by Nelson Mandela African Institution of Science and Technology and sub-cultured in-potato dextrose broth (PDB) followed by serial dilution in a 96 well microplate preloaded with antibiotic (Ciprofloxacin) to prevent bacterial growth in the well. When a pure colony grew in a well it was isolated using a needle and incubated on potato dextrose agar (PDA).

Enzyme production and fermentation conditions

Enzymes from A. oryzae; MG429773 and MG429774 were produced in solid state fermentation on 10 g of broken rice husk mixed with 1% w/v of casein, 5% sugar, 1% yeast extract all together moistened with 20 mL of mineral salt solution (0.15%) containing (0.05 g NH₄NO₃, 0.025 g NaCl, 0.05 g (NH₄)₂SO₄) dissolved in 1000 ml of distilled water at a pH adjusted to 8.5. The
mixture was sterilized by autoclaving at 121°C for 15 min and inoculated with 1 mL of 1x10^7 conidia accessed by a haemocytometer and incubated at 35°C for 7 days.

Protease hydrolysis assay

The proteolytic activity of the crude enzyme solution was determined by using casein as a substrate. 450 mL of 1% (w/v) of casein was mixed in 50 mL of Tris-HCl buffer at 8.5 pH followed by addition of 50 mL of the crude enzyme extract and incubated in water bath at 40°C for 20 min. After 20 min, enzyme reaction was determined by addition of 500 mL of Trichloroacetic acid and incubated at 35°C for 12 h. After 12 h, enzyme activity was visualized as clear zones around the petri dishes due to hydrolysis of media (PDA containing casein) in the after addition of indicator solution.

Enzyme isolation

Ten milliliters of 0.1% triton X-100 was added to 2 g of fermented rice husk mixed and homogenized by using magnetic stirrer 100 rpm for 1 h. Solids were removed by using Whatman no. 1 filter paper followed by centrifugation of solution at 8000 rpm for 15 min. The resulting supernatant was used as the crude enzyme for hair removal bioassay.

Partial purification of enzyme

The most effective enzyme solution from isolate A. oryzae, MG429773 from the cell free supernatant portion of the culture was precipitated with ammonium sulphate to a saturation point of 60 to 80%. The salt was added slowly in small quantities under constant stirring by a magnetic stirrer to equilibrate the salt for maintaining equal concentration throughout the culture filtrate. After 1 h, the solids were collected by centrifugation at 10,000 g for 15 min at 4°C and the solids of different fractions was suspended (re-dissolved) separately in Tris-HCl buffer (pH 8.5). The re-suspended pellets were dialyzed against Tris-HCl buffer (pH-8.5) by placing them into a visking dialysis membrane. Salt was removed by dialysis with constant stirring for 12 h and periodic changeover of buffer 7 times. The dialyzed fraction was used as the enzyme for bioassay and samples with high activity were lyophilized and stored as solid pellets at -4°C in freezer.

Skin and hide dehairing bioassay

The dehairing bioassay was conducted on pieces of cattle hide with an area of 10 cm x 10 cm with a weight of 13.81±0.64 g and 0.41±0.2 cm thick whereas goat skin measured (10 cm x10 cm) area with weight of 4.82±0.22 g and 0.21±0.02 cm thick. Hide and skin samples were separately placed into beakers and soaked in distilled water overnight and left to dry at room temperature (29.5±2.4°C) for 2 h and then soaked into 2, 5, 10, 15 and 20% of crude enzyme solutions of A. oryzae MG429773 with a control of 0.1% triton X-100. The fungal isolate was grown in larger volumes testing the effects of different parameters including substrate (rice, wheat, maize grain), nitrogen source, carbon source, pH effect, surfactant (triton x-100, tween 80, tween 20 and vegetable oil), temperature and partially purified.

To evaluate the effect of dipping against spray method on dehairing activity 2, 5, 10, 15, and 20% of partial purified enzyme and control (0.1% triton X-100) was applied. Degree of hair removal was calculated by subtracting dehaired portion over total skin/hide portion. The experiment was replicated four times and data was recorded after every 12 h.

Data analysis

Data on enzymic dehairing of cattle hide and goat skin was determined by assessing effect of fungal isolates in different conditions. Substrate, carbon source, nitrogen source, temperature, pH and surfactant were the factors for analysis of degree of hair removal. In each treatment, pieces of hides and skins were subdivided into ten fractions each measuring 1 cm. Portion of hair removed over total area of skin exposed to treatment times 100% was calculated. Scores were graded as: 0=skin or hide portions without depliation, 1-3/10=25% portions of skin or hide depilated (partial dehairing), 5-6/10=50% portions of skin or hide depilated (moderate dehairing), 7-9/10=75% portions of skin or hide depilated (incomplete dehairing), and 10/10 portions of skin or hide depilated=100% (complete dehairing).

RESULTS

The results obtained showed that dehairing activity of enzyme was time and concentration dependent. 15% of crude enzyme exhibited high activity of depliation compared to 10%, 5%, and for the control no hair was removed after 48 h (Figure 1). Dehairing activity was fast on goat skin compared to cattle hide after 48 h (Figure 2).

The effect of substrate and carbon source on enzyme activity was different between treatment where rice husks showed high activity of up 100% dehairing compared to cotton seeds, wheat grain and PDA where the last showed only 56.25% dehairing after 48 h (Figure 3).

The effect of the nitrogen source on enzyme activity varied between substrates with the use of collagen resulting in the highest enzymic dehairing activity compared to casein, yeast extract, ammonium nitrate and ammonium sulphate (Figure 4).

pH also affected enzyme activity were alkaline pH in the range of 8.5 to 9.5 favoured enzyme activity, and low enzyme activity was observed in acidic and neutral conditions (Figure 5). The effect of temperature on enzyme production was high at temperature range of 35 to 40°C whereas enzyme activity slowed down at temperature below 30°C and above 40°C (Figure 6). Results also showed that surfactant application enhanced enzyme activity in which triton x-100 showed high enzymic dehairing than tween 80, tween 20 and sunflower oil (Figure 7).

The dehairing method for enzymic depilation of cattle hide and goat skin was more effective using the spray method which induced dehairing within a short time as compared with the dipping method depending on concentration of enzyme and time (Figure 8). An increase in incubation time improved the dehairing activity and a high percentage of hair removal was observed after exposure for 48 h indicating that enzyme activity was time dependent (Figure 9). Enzymic dehairing also varied for type of animal hide or skin and the method used. For goat skin depilation was faster than for cattle hide and the spray
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Figure 1. Percentage of dehaired cattle hide after exposure to various concentrations of crude protease at maximum of 96 h.

Figure 2. Percentage of dehaired goat skin after exposure to various concentrations of crude protease at maximum of 96 h.

method produced good quality of skins and hides (Figure 10).

DISCUSSION

Enzymic dehairing of hides and skins have been reported as effective and safe for both human and environment (Choudhary et al., 2004). Recently isolated fungus from Tanzania was revealed to depilate goat and cattle hair within 48 h with quick activity in goat skin compared to cattle hide. The enzyme activity was time and dose dependent in which more activity using higher enzyme concentration that lower doses even when exposed for a long time for cattle hide and less significant for the goat skin.

The fermentation conditions are essential to obtain high enzyme activity. Various conditions were investigated for their influence on enzyme activity. The solid substrate is a key factor for enzyme production and maximum enzyme activity was obtained for rice husk as compared to wheat grain, cotton seed flower and PDA indicating that rice was superior source of carbon for fermentation of A. oryzae enzyme, cheap and easy in downstream
Figure 3. Effect of carbon source on dehairing hide by crude alkaline protease over time.

Figure 4. Effect of Nitrogen source on dehairing hide by crude alkaline protease over time.

Figure 5. Effect of pH on dehairing of cattle hide by crude alkaline protease of A. oryzae.
process compared to special media including PDA (Sandhya et al., 2005). Good performance of rice husks entails value addition to this abundantly available agricultural waste to replace use of cotton seed and wheat grain, which are food, thereby ensuring food security while protecting the environment.

Nitrogen is another important fermentation medium ingredient and the best source of nitrogen was casein yielding a high amount of enzyme compared to ammonium sulphate, ammonium nitrate and yeast extract when used solely. Earlier studies reported that organic sources of nitrogen greatly improve the production of enzymes (Dong et al., 2005). This might be the reason for good performance observed with collagen and casein.

Temperature influences enzyme activity and also fungal enzymes have minimum, maximum and optimal temperature for specific activity. This study showed that the optimum temperature for production of alkaline protease from A. oryzae is 35°C grown for 5 days under static conditions in an incubator followed by 3 days on an incubator shaker. At temperatures below 30°C and above 40°C, enzyme activity declined, and more incubation time was required to reach optimal activity of the enzyme due to sensitivity and specificity of microbes and enzyme on temperature.

It is well established that enzymes are most active in a pH range of 5 to 10, varying from one type to another. Protease enzymes are active at pH rage of 7.5 to 8.5. A
change in pH can protonate or deprotonate a side group (carboxyl and amino termini), thereby changing its chemical features and conformation, resulting into loss of interaction with an adjacent subunit, hence decrease in substrate affinity. Alteration of pH can further lead to protein un-folding, thereby completely deactivating the enzyme. Higher protease activity was observed at alkaline pH at the range of 8.5 to 9.5 than in neutral and acidic pH. This shows that alkalinity favor the dehairing activity of A. oryzae protease similar to in chemical dehairing where lime and sodium sulphide exhibit dehairing activity at alkaline pH above 8.5.

Surfactants are important for emulsification and of hydrophobic molecules in water and play role as
dispersant and wetting agent. Triton X-100 was the best surfactant in this study. It enhanced extraction and emulsification of enzyme during incubation and in water solution (Rastogi et al., 2008). Triton X-100 has advantage over tween 80 and tween 20 as it is heat tolerant and tolerant on wide range of parameter when included in culture media (Chen et al., 2005). Enzymic dehairing was effective and faster for goat skin compared to cattle hide probably due to its thicker layer with more proteinous material than for goat skin. The pieces with similar area had different weight and volume as described earlier. The spray method exhibited higher dehairing activity for goat skin compared to dipping method especially in cattle hide. The thickness of hide prevented quick penetration of enzyme into animal hide. The skin depilation by spray method was the best as no hairs remained in solution whereas in dipping method high amount of hair was retained in solution which might lead to environmental pollution. This study suggests that, enzymic dehairing by spray method could save dual purposes; efficient depilation and dry/firm hair could be processed for making other products such as painting brushes than being waste in dipping method.

**Conclusion**

Alkaline protease produced by *A. oryzae* (MG429773) was effective in dehairing goat skin and cattle hides when rice husk was used as the solid substrate and source of carbon and casein as source of nitrogen at temperature range of 35 to 40°C and pH range of 8.5 to 9.5. In addition, 0.1% of triton X-100 was found to be good surfactant during enzyme extraction. This study found that an alkaline protease has potential activity for dehairing hides and skins. However, further experiments including purification and trials by local tanners should be conducted to evaluate cost and environmental benefits in comparison to conventional for validating its application in processing leather products in Tanzania.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.

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Exploring the nutritional efficiency of genotypes of *Coffea arabica* L. from different parental lineages in contrasting environments for N availability


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The different responses of improved genotypes to alterations of the availability of nutrients indicate that it is possible to modulate the nutritional efficiency by exploring the interaction between the intrinsic response of a genotype and the level of nutrient supply. The objective of this research was to assess the response of genotypes of *Coffea arabica* L., from different parental lineages, to contrasting environments for N supply, using parameters of growth and nutritional efficiency indexes to explore a possible increase in the nutritional efficiency. The experiment followed a 3×3 factorial scheme, in a completely randomized design, with 3 improved genotypes (Acauã, Katipó and Topázio) and 3 environments with different levels of N availability in the soil (50, 100 and 200% of the recommended supply). The growth and nutritional efficiency of *C. arabica* is influenced by the effects of the intrinsic differences among genotypes and can be modulated by changes in the environment, based on the response to different supplies of N in the soil. Genotypes from different parental linages are able to present highly contrasting responses to the fertilization with N, indicating a high variability to be explored. Among the studied genotypes, Topázio presents higher accumulation of biomass and high nutritional efficiency for absorbing, translocating and using N in environments with low fertility; while Acauã presents higher efficiency in environments with higher N supply.

**Key words:** Coffee, genotypes, mineral nutrition, variability.

**INTRODUCTION**

The coffee industry stands out for its great importance to Brazilian socioeconomic development, being one of the main Brazilian exports. The species *Coffea arabica* L. currently covers an area of 1.74 million ha, corresponding to 80% of the total area of coffee plantations in Brazil, which may be responsible for a production of over 36

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million bags of coffee in the current year (Conab-Companhia Nacional de Abastecimento, 2019). There is a constant need for new technologies aimed to increase the coffee productivity in a more sustainable manner, such as the use of newer and improved cultivars, new pruning practices and rational fertilization programs (Prezotti and Bragança, 2013; Martins et al., 2015; Rodrigues et al., 2017).

The ongoing breeding programs in Brazil have developed and recommended several new cultivars of *C. arabica* along the past decades. These cultivars present higher crop yield, often associated to other agronomic advantages, such as higher beverage quality, resistance to plant diseases and tolerance to environmental stresses (Oliveira and Pereira, 2008). Therefore, a considerable number of improved genotypes are available for commercial plantations, also representing a valuable genetic source for the improvement of other agronomic traits still unexplored by the current breeding programs.

Beside the use of improved cultivars, the fertilization management is another important research object, since the fertilization is responsible for a high proportion of the production costs of coffee plantations, and the highly productive genotypes tend to also have high nutritional demands, especially during the stages of flowering and fruit formation (Fonseca et al., 2015; Sakiyama et al., 2015). Among the nutrients, N must be highlighted in the fertilization programs for coffee, since it is highly required in the metabolism, allowing the vegetative growth and photosynthetic process, and is highly mobile in the soil; therefore, this nutrient is commonly subjected to losses by lixiviation or volatilization in Brazilian edaphoclimatic conditions (Malavolta, 1993; Sakiyama et al., 2015; Taiz et al., 2017).

The nutritional efficiency may vary according to the species and even among genotypes of the same species. In the agronomic context, the nutritional efficiency is related to the ability of a given genotype to absorb, distribute and use the nutrient in order to grow and yield in relation to the given supply of nutrients (Baligar and Fageria, 1997). Parameters of nutritional efficiency have been used to discriminate genotypes of different plant species worldwide (Muurinen et al., 2006; Rozane et al., 2007; Beche et al., 2014).

For *C. arabica*, parameters of nutritional efficiency have been successfully used to identify differences among coffee genotypes in Brazil (Martins et al., 2015; Rodrigues et al., 2015). These studies also report different responses of improved genotypes to alterations in the availability of nutrients. This indicates that it is possible to modulate the nutritional efficiency by exploring the interaction between the intrinsic response of a genotype and the level of nutrient supply in order to enhance the fertilization programs. The objective of this research was to assess the response of genotypes of *C. arabica*, from different parental lineages, to contrasting environments for N supply, using parameters of growth and nutritional efficiency indexes to explore a possible increase in the nutritional efficiency.

**MATERIALS AND METHODS**

**Local and soil conditions**

The experiment was executed in protected environment, in a greenhouse, located in the experimental area of the Center of Agrarian Sciences and Engineering of the Federal University of Espirito Santo (CCEAE-UFES), in the municipality of Alegre, Espirito Santo State, Southeast Region of Brazil. The site is located at 20°45' S latitude and 41°33' W longitude, and present average altitude of 277.41 m over sea level. Homogeneous samples of a red-yellow Oxisol were collected at a depth of 10 to 40 cm, discarding the first 10 cm in order to reduce the effect of the presence of organic matter, more present on surface layers. The soil was analyzed to determine its physical and chemical characterization (Table 1). After characterization, the soil was dried in shade and homogenized in a 2.0 mm mesh sieve before being separated into 10 dm³ samples, standardized by weighing in precision scale, using the soil density. The samples were transferred to sealed plastic pots, capable of holding 12 dm³ of total internal volume.

**Experimental design**

The experiment studied the interaction between the effects of the genotypic differences and environmental modifications regarding the availability of nutrients in the soil, following a 3×3 factorial scheme. The factors corresponded to three coffee genotypes cultivated under conditions of three levels of N availability in the soil: 50, 100 and 200% of the recommended supply. The trial was arranged in a completely randomized design (CRD) with three repetitions and one plant per experimental pot (14 dm³ of capacity).

**Selection of genotypes**

Three genotypes of *C. arabica* L. were selected based on their recommendation as cultivars of above-average crop yield for the region of this study and by their contrasting parental origins and ripening cycles. The selected genotypes were Acauã, Katipo and Topazio, which present desirable agronomic traits (Table 2), such as potential to good beverage quality or some level of resistance to common stresses. The seedlings were acquired in certified nurseries, selected to compose a homogeneous group regarding size, leafiness, phytosanitary and nutritional aspects. At the stage of three fully developed pairs of leaves, the seedlings were transplanted to the prepared plastic pots.

**Cultivation practices**

According to the results of the physical and chemical characterization of the soil, its fertility was correct and the nutritional availability of all nutrients, excepted N, was raised to the levels considered adequate to protected environments, based on the recommendations of Novais et al. (1991). The fertilizations with phosphorus and potassium were performed in a single application previously to transplanting of the seedlings, using KH₂PO₄ salt diluted in water and mixed in the entire soil samples. The irrigation was performed daily, in order to maintain adequate levels of soil moisture for the development of the plants. Phytosanitary management was performed according to its eventual need, applying only manual control strategies to evade chemical interventions. All the practices were planned in accordance with the
Table 1. Physical and chemical characteristics of the soil used as substrate.

<table>
<thead>
<tr>
<th>Characterization</th>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sand proportion (g kg⁻¹)</td>
<td>47.0</td>
</tr>
<tr>
<td></td>
<td>Silt proportion (g kg⁻¹)</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>Clay proportion (g kg⁻¹)</td>
<td>47.0</td>
</tr>
<tr>
<td>Density</td>
<td>Soil density (kg dm⁻³)</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>Particle density (kg dm⁻³)</td>
<td>2.81</td>
</tr>
<tr>
<td></td>
<td>Porosity (m³)</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>6.37</td>
</tr>
<tr>
<td></td>
<td>P (mg dm⁻³)</td>
<td>5.65</td>
</tr>
<tr>
<td></td>
<td>K (mg dm⁻³)</td>
<td>83.00</td>
</tr>
<tr>
<td>Fertility</td>
<td>Ca (cmol, dm⁻³)</td>
<td>3.73</td>
</tr>
<tr>
<td></td>
<td>Mg (cmol, dm⁻³)</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>Al (cmol, dm⁻³)</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>H+Al (cmol, dm⁻³)</td>
<td>3.63</td>
</tr>
<tr>
<td></td>
<td>Sum of bases (cmol, dm⁻³)</td>
<td>4.83</td>
</tr>
<tr>
<td></td>
<td>Potential cation-exchange capacity (cmol, dm⁻³)</td>
<td>8.46</td>
</tr>
<tr>
<td></td>
<td>Effective cation-exchange capacity (cmol, dm⁻³)</td>
<td>4.83</td>
</tr>
<tr>
<td></td>
<td>Base saturation (%)</td>
<td>57.1</td>
</tr>
</tbody>
</table>


Table 2. Characteristics of the studied genotypes.

<table>
<thead>
<tr>
<th>Progenitor</th>
<th>Genotype</th>
<th>Acuã</th>
<th>Katipó 245-3-7</th>
<th>Topázie MG1190</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sachimor IAC 1668</td>
<td>High</td>
<td>High (early years)</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>Caturra Vermelho</td>
<td>Late</td>
<td>Early</td>
<td>Intermediate</td>
</tr>
<tr>
<td></td>
<td>Mundo Novo IAC 388-17</td>
<td>Short</td>
<td>Short</td>
<td>Short to average</td>
</tr>
<tr>
<td></td>
<td>Híbrido Timor</td>
<td>High</td>
<td>Average</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>Mundo Novo 515</td>
<td>Average</td>
<td>Red</td>
<td>Yellow</td>
</tr>
<tr>
<td></td>
<td>Beverage quality</td>
<td>Good</td>
<td>Good</td>
<td>Good</td>
</tr>
</tbody>
</table>

Source: Ferrão et al. (2005); Queiroz et al. (2007); Carvalho (2008); Sakiyama et al. (2015).

current recommendations for Arabica coffee cultivation (Reis and Cunha, 2010).

Alteration of N supply

The N fertilizations started 30 days after transplanting the seedling to the prepared pots, and were performed in four proportional parcels, separated by 30-days intervals between them. The different levels of N supply were applied to each designed plot following the levels of 50, 100 and 200% of the recommended levels (Novais et al., 1991), which resulted respectively in the addition of 1.11, 2.22 and 4.44 g of CO(NH)₂₂ salt per pot. The urea salt was diluted in distilled water and applied over the soil surface, distant 10 cm around the plant collar.

Evaluation of growth and nutritional efficiency

After 180 days of cultivation under conditions of different N supplies, the plants were cut; separating leaves, stems and roots; separated in paper bags, which were then dried in laboratory drying oven, with forced air circulation at 65°C (STF SP-102/2000 CIR), until their mass achieve constant weight. After drying, the plant organs were weighed on analytical balance (AUW-220D; precision: 0.00001 g) to obtain the measures of roots dry matter (RDM), above-ground dry matter (ADM), as the sum of leaves and stems) and total dry matter (TDM, as sum of roots and above-ground dry matter).

The dried tissues were triturated in Wiley-type mill (Cienlab EC-430, 8 blades, 1725 rpm, using a 0.42 mm mesh stainless steel sieve) to obtain a homogeneous powder. Triplicate samples of 0.5 g of the powder were transferred to Taylor tubes (25 mm × 200 mm)
and submitted to sulfuric digestion (H$_2$SO$_4$), in order to quantify the organic N content of roots (RNC), above-ground (ANC) and total (TNC) (Silva, 1999). The determined values of dry matter and N content were used to estimate the following indexes of nutritional efficiency: N absorption efficiency (NAE), based on the total nutrient content and the root dry matter (Swiader et al., 1994); N translocation efficiency (NTE), based on the above-ground and total nutrient content (Li et al., 1991); and N use efficiency (NUE), based on the total dry matter and total nutrient content (Siddiqi and Glass, 1981).

Data analysis

The collected data was subjected to analysis of variance (p ≤ 0.05), using the F-test to identify the existence of significant interactions among the factors and the unfolding of levels for each factor was performed according to the statistical need, using the Tukey’s test (p ≤ 0.05). The analyses were done using the statistical software Sisvar (Ferreira, 2011).

RESULTS AND DISCUSSION

The response of the genotypes to the cultivation in environments with different N supplies showed the existence of significant interaction between the effects of both genotypic and environmental effects over the determination of the growth and nutritional efficiency of the plants.

Similar responses were observed among the genotypes for the root growth, although with different magnitudes (Figure 1a). The high availability of N in the environment with 200% of the recommendation did not cause an increase in root development (RDM). The abundant supply of the nutrient may have made it promptly available for the root system, not requiring a larger growth for the plant to support its nutritional demand. The promotion of the root development in conditions of low supply, as observed for Topázio (Figure 1b), may be explained by the nutritional deficiency inducing the root growth in order to explore larger volumes of soil and acquire the nutrient to sustain the metabolism and the growth of the aerial organs (Neto et al., 2016).

The genotypes also presented different responses for the accumulation of dry matter on above-ground organs (Figure 1c, d) and in total biomass production (Figure 1e f). Acauã presented the lowest means for both ADM and TDM when cultivated with only 50% of the N supply (Figure 1d and f). This genotype achieved similar means to others when cultivated in environments with N supply under the recommended level; however, it never achieved the same above-ground and total biomass accumulation than Topázio in any of the scenarios (Figure 1c and e). The genotype Topázio was described by Carvalho et al. (2012) as being well adapted to different environments, presenting vigorous growth and high yield under different conditions. This genotype presented high accumulations of biomass regardless of the differences among the three studied environments. It is reported that genotypes of C. arabica, subjected to cultivation in modified environments for nutrient availability in the soil, can present high genetic variability for growth (Rodrigues et al., 2017), which could justify the different patterns of biomass allocation observed among the three genotypes in response to the N supply. Martins et al. (2015) explains that even older parental genotypes used in the breeding of the species, such as Mundo Novo and Catuial, present different responses regarding their nutritional efficiencies, which can be modulated by modifications in the nutritional availability of the environment in which they are cultivated.

Different responses in terms of concentrating the absorbed N along the different organs were also observed among the genotypes (Figure 2a, c and e). The genotypes Acauã and Katipô showed gains in the accumulation of N in the all vegetative organs as the N availability increased in the soil (Figure b, d and f). The genotype Topázio, however, accumulated higher amounts of N on the aerial organs regardless of the fertilization (Figure 2d) and in the roots under condition of low supply of N (Figure 2b), resulting in higher N content in the plant in the environment with 50% of the N supply, probably due to a robust activity of its root system.

The different responses among the genotypes for root N content reaffirm this particular pattern, as the concentration of this nutrient for the genotypes Acauã and Katipô, at the level corresponding to 100 and 200% of N supply, surpassed Topázio, which in turn was superior to all others at the level of 50% of N supply (Figure 2a). For the concentration in above-ground organs, the genotype Topázio presented higher means for the environments with up to 100% of the N supply; however, to the environment with 200% of the N supply, the genotypes achieved similar concentration of this nutrient (Figure 2c).

There is a noticeable inversion of patterns among genotypes for the total N content of the plants. While Topázio exceeded the N concentration of all other genotypes at the level of 50% of the nutrient supply, this genotype had similar concentration than Acauã at 100% and was surpassed by all genotypes when cultivated in the environment with 200% of N supply. The genotype Acauã present gains in N content, accumulating more of this nutrient as the availability in the soil increases, presenting the higher TNC at the environment with 200% of the N supply (Figure 2e). The ability of the coffee genotypes to absorb and utilize nutrients is linked to intrinsic characteristics of the genotype (Martins et al., 2013a; Rodrigues et al., 2015), which may explain the differentiated response of the genotypes regarding the uptake and accumulation of N in the plant tissues (Table 2). As well as the different nutritional efficiencies that each genotype presented in the modified environments (Figure 3).
For N absorption efficiency, gains were observed for the genotypes Acauã and Katipó in the nutrient uptake as more of it was supplied in the fertilization, while the genotype Topázio kept similar efficiency regardless of the availability of N in the soil (Figure 3b). Swiader et al., (1994) relate the absorption efficiency to the ability of a genotype to acquire the nutrient from the soil in relation to its available root system, which reaffirms the vigorous growth and activity of the roots of Topázio as an important factor to maintain its nutritional efficiency in different soil conditions. Between the genotypes Acauã and Katipó, the gains in N absorption efficiency with the increase on its availability is shaper for Katipó, which ended up being the genotype with the highest efficiency at the environment with 200% of N supply (Figure 3a).

Variability for the nutritional efficiency among genotypes of other species of coffee have been reported in several other researches (Amaral et al., 2011; Martins et al., 2013a, b; 2016; Colodetti et al., 2015; Tomaz et al., 2011), including differences for the efficiency in absorb
and utilize N (Colodetti et al., 2014; Machado et al., 2016). Fageria (1998) relates the different efficiencies observed in among genotypes of one species with the ability of one specific genotype to adapt its root system to stress conditions, which supports the patterns of efficiency observed for Topázieio. Analyzing the efficiency of N translocation, it is possible to observe differentiated responses among the genotypes for each environment (Figure 3c). Overall, it is possible to observe a relative superiority of the genotype Topázieio and lower efficiency of the genotype Katipó, in all three scenarios of N availability.

The change of N supply had no effect over the translocation efficiency for the genotype Katipó (Figure 3d). However, Acauã presented the higher efficiency when cultivated with the recommended supply (100%) and Topázieio presented higher efficiency for the environments with the recommended supply or above.
The translocation efficiency, according to Li et al. (1991) relates to the amount of nutrient contained in the aerial organs of a plant with the amount of nutrients in the plant as a whole. Thus, the NTE is directly related to the ability of the plant to carry the absorbed nutrients from root system to its aerial systems to sustain the metabolism of the photosynthetically active tissues (Martins et al., 2015). Higher capacity to translocate N can be related to the productivity of a plant, since higher N contents in aerial organs tend to sustain a greater chloroplast synthesis, increasing the overall photosynthetic yield of this plant (Faquin, 2005; Epstein and Bloom, 2006; Taiz et al., 2017).

A considerable variability was observed among genotypes for the N use efficiency (Figure 3e). In the environments with N supply of 100% and bellow, the genotype Topázio is highlighted by its higher efficiency and capacity to convert biomass. For the environment with high supply (200%), the genotype Acuã surpass the efficiency of all others. This fact indicates that Topázio
may be efficient to utilize the available nutrient and convert it to produce biomass even in environments with low availability, while Acauã probably presents higher nutritional demands and becomes more efficient in soils of higher fertility.

The N use efficiency presented two distinct patterns among the studied genotypes. For the genotype Topápio, highest use efficiency occurred at the 50% of N supply, decreasing as the supply increased in the soil. In contrast, the genotypes Acauã and Katipó presented higher efficiencies as the levels of N available in the soil increased (Figure 3f). The use efficiency is related to the amount of biomass which the plant is capable of producing by the amount of nutrient available in the tissues (Siddiqi and Glass, 1981). Therefore, it can be inferred that a genotype which is able to produce more biomass requiring a smaller amount of nutrient tends to be more efficient to use of this nutrient, which is a highly desirable agronomic trait (Tomaz et al., 2008).

Considering the overall different behavior of the genotypes, it becomes possible to explore their efficiency traits in a more rational way. For breeding programs, the contrasting patterns make it possible to use these genotypes in efforts to improve the nutritional efficiency both for environments with intense use of fertilization, exploring Acauã; or soils with low natural fertility, exploring the traits of Topápio. For nutritional management, the response of each genotype makes it possible to funnel efforts to supply the plantation with adequate amounts of fertilization in order to explore the efficiency to reduce costs.

Conclusion

The growth and nutritional efficiency of C. arabica is influenced by the effects of the intrinsic differences among genotypes and can be modulated by changes in the environment, based on the response to different supplies of N in the soil. Genotypes from different parental lineages are able to present highly contrasting responses to the fertilization with N, indicating a high variability to be explored among genotypes. Among the studied genotypes, Topápio presents higher accumulation of biomass and high nutritional efficiency for absorbing, translocating and using N in environments with low fertility; while Acauã presents higher efficiency in environments with higher supplies of N.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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