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Antioxidants enhance banana embryogenic cell competence to Agrobacterium mediated transformation

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Gene transfer into the plant cell is a key step towards its successful genetic modification and its efficiency is heavily dependent on plant and bacterial cell biological status and a wide array of physical conditions. Gene transfer efficiencies in East African Highland Banana (EAHB) cell lines remains low compared to other monocotyledonous crops like rice and wheat due to factors such as high oxidative stress. The use of antioxidants is fundamental in influencing gene transfer events during *Agrobacterium*-plant cell co-cultivation. Here we report significant enhancement of gene transfer efficiency in the EAHB cultivar 'Nakinyika' (EA-AAA) by supplementing co-cultivation medium with antioxidants; ascorbic acid (AA), glutathione (GSH), tocopherol (TOC) and silver nitrate (SN). The most enhancing antioxidant by number of blue foci after histochemical assay, as a parameter of gene transfer efficiency, was ascorbic acid (174 cells) at a concentration of 40 mg/L, followed by glutathione (91 cells) and tocopherol (91 cells) both at 50 mg/L. The least enhancement was observed when ascorbic acid (39 cells), silver nitrate (41 and 31 cells) were used at concentrations of 20, 4 and 6 mg/L compared to 72 cells in controls (no anti-oxidants). Regeneration efficiency increased from 29 cells in controls to 46% for SN at 8 mg/L; 43% for GSH at 100 mg/L; 30% for TOC at 75 mg/L; and 48% for AA at 20 mg/L. Polymerase chain reaction (PCR) results using *gusA* specific primers showed that these regenerants were putative transformants and grew normally during regeneration, rooting and multiplication. GSH and TOC significantly enhanced gene transfer efficiency while AA and SN showed significant increases in shoot regeneration compared to controls. The current results show that antioxidants significantly enhance gene transfer and regeneration efficiency in recalcitrant banana cell lines and could significantly enhance the overall transformation efficiencies in cases where numerous transgenic lines are required in a short time.

Key words: Highland bananas, transgenic, browning, transformation efficiency, agrobacterium.

INTRODUCTION

Banana ranks among the 10 most important crops in the tropical and sub-tropical zones of the world (Ortiz and Swennen, 2014). Annual banana production in the world is estimated at 145 million tons of which less than 10%

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enters the commercial market suggesting that the crop is more important as food for local consumption than for export (Brown et al., 2017). In the Great Lakes region (Uganda, Rwanda, Burundi, Democratic Republic of Congo) bananas constitute between 30 - 60% of the daily per capita calorie intake with consumption rates of up to 250 kg per person per (Abele et al., 2007; Komarek, 2010).

Despite its importance, banana production is affected by a number of biotic factors such as pests and diseases and abiotic constraints like declining soil fertility (Wairegi et al., 2010; Robinson and Sauco, 2010). Breeding for host resistance using classical methods remains a tedious endeavour because of the crop's high sterility, polyploidy, linkage drag and long generation times of most of edible cultivars (Wulff et al., 2011; Namukwaya et al., 2012; Brown et al., 2017). Genetic engineering provides a promising alternative strategy for the improvement of commercial banana varieties with resistance to biotic and abiotic stresses (Ortiz and Swennen, 2014). As a pre-requisite for successful genetic manipulation, it is necessary to establish an efficient *in vitro* plant regeneration system and a method to deliver genes at a high frequency suitable for transformation (Barampuram and Zhang, 2011). Agrobacterium tumefaciens mediated transformation is the outstanding method for plant genetic transformation due to several advantages in the T-DNA transfer process, including high efficiency, low-copy number inserts, large DNA segments, low rearrangement rate and low cost (Gelvin, 2012).

Functional genomics studies of crop plants such as banana necessitates a cost effective testing of a huge number of candidate genes or other nucleotide sequences and require high throughput transformation systems (Ortiz and Swennen, 2014). Nevertheless, low transformation efficiency still remains a great challenge in the application of this technology in recalcitrant crops, predominantly monocotyledonous plants, similar to banana, which are not susceptible to *Agrobacterium* spp. Browning or necrosis, is a common phenomen associated with agrobacterium mediated transformation and it greatly lower the efficiency of this technique. Several studies have associated browning to the production of reactive oxgyen species (ROS), as a defense response to agrobacterium infection during transformation. This oxidative stress has been reported to inhibit growth and alter plant metabolic pathways that lead to poor plant regeneration (Dan, 2008). A number of factors including plant growth regulators and antioxidants have been reported to improve genetic transformation efficiency of plants (Dutt et al., 2011). Antioxidants include ascorbic acid that functions by to reducing explant necrosis and hence increase explants viability whereas glutathione and tocopherol reduce hyperhydricity and ROS (Dan, 2008). Ascorbic acid and Silver nitrate added in co-cultivation medium was found to increase sugarcane leaf explant viability from 10 to 90% (Gustavo et al., 1998). Similarly, incorporation of 100 mg/l glutathione and 50 mg/l tocopherol in the co-cultivation medium increased the agrobacterium mediated transformation efficiency of peanut from 3.9 to 14.6% and 10.3%, respectively (Zheng et al., 2005). Similar studies have reported positive results associated with the use of several anti-oxidants in both transformation efficiency and shoot regeneration (Dhekney et al., 2009; Dutt et al., 2011).

In this study, we hypothesised that the use of ascorbic acid, silver nitrate, glutathione and tocopherol as anti-oxidants to enhance transformation and regeneration of banana cultivar 'Nakinyika'.

MATERIALS AND METHODS

General materials

All laboratory reagents used in this study were obtained from scientific companies including Sigma (USA) and Fisher scientific (USA). Agarose was supplied by Roche Diagnostics (USA), while bacterial agar was purchased from Oxoid (Aus). All DNA molecular weight markers, reagents for Polymerase chain reaction (PCR) and restriction enzymes were purchased from Roche Diagnostics (Aus), New England Biolabs (USA), Invitrogen (Aus) or Promega (Aus). All primers used were purchase from Gene Works (Aus). Nucleic acid purification kits were purchased from Roche Diagnostics (Aus), or Promega (Aus).

Plant material

Embryogenic cell suspensions (ECS) of banana cultivar 'Nakinyika' were used. ECS were sub - cultured in cell suspension culture medium (MA2). A settled cell volume of 1 ml of ESC was sub-cultured in 50 ml of liquid MA2 and cultured at reduced density for 5 days to increase cell competence. Five days after sub culturing, ECS were collected in 50 mL falcon tubes and used as starting material for transformation experiments.

Binary vector and Agrobacterium strains

Agrobacterium tumefaciens super virulent strain EHA 105 was used in this study. The pCAMBIA2301 vector (containing a reporter g*usA* gene regulated by the CaMV35S promoter and the kanamycin selection marker gene) was used (Figure 1). The vector was transformed into *Agrobacterium* using chemical treatment (Chuanchuen et al., 2002) and maintained on YEB medium (0.1% yeast extract, 0.5% beef extract, 0.5% peptone, 0.5% sucrose and 0.04% $MgSO_4$) supplemented with kanamycin (100 mg/L). The bacterium was cultured at 28°C with continuous shaking at 150 rpm until the optical density of OD_{600nm} was attained. The bacterial cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C and re-suspended in 25 ml of TMA1 medium supplemented with 300 mM acetosyringone and varying concentrations of the four antioxidant treatments (Table 1). Prior to the co-cultivation, the bacterial suspension was activated at 25°C for 3 h with shaking at 70 rpm until an O. D_{600nm} of 0.6 was reached.

Transformation, selection and regeneration

Banana cells were transformed with *Agrobacterium* strain EHA

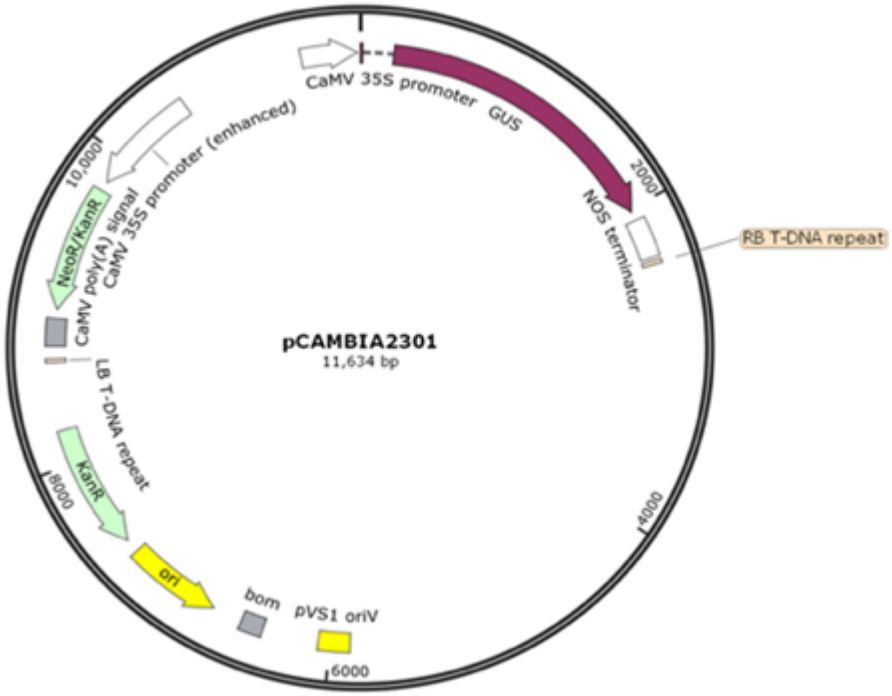

Figure 1. Schematic presentation of T-DNA of binary vector pCAMIBIA2301 used for transformation. It contains aminoglycoside phosphotransferase gene for plant cell selection and the *gusA* gene has a 5' extension with a catalase intron to ensure expression in plants but not bacteria.

Table 1. Media compositions: The different media types used at banana ECS culture, Agrobacterium-mediated transformation and regeneration of plants stages.

Medium type	Medium composition
ECS sub-culture	MA2 (Cell suspension culture medium); Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962) supplemented with 100 mg/L glutamine, 100 mg/L malt extract,1 mg/L2,4-Dichlorophenoxyaceticacid (2,4-D), 45 g/L sucrose, pH 5.3
Infection	TMA1(liquid) - (MS Macronutrients, MS Micronutrients, MS vitamins, 1 mg/L biotin, 100 mg/L malt extract, 100 mg/L glutamine, 230 mg/L proline, 5 g/L polyvinylpyrrolidone-10 (PVP-10), 200 mg/L cysteine,1 mg/L indoleacetic acid (IAA), 1 mg/L naphthaleneacetic acid(NAA),4 mg/L 2,4-D, 85.5 g/L sucrose, pH 5.3) supplemented with 200 mM acetosyringone and different varying concentrations of four antioxidants
Co-cultivation/resting	TMA1 (solid) - (MS Macronutrients, MS Micronutrients, MS vitamins, 1 mg/L biotin, 100 mg/L malt extract, 100 mg/L glutamine, 230 mg/L proline,5 g/L polyvinylpyrrolidone-10 (PVP-10), 200 mg/L cysteine,1 mg/L indoleacetic acid (IAA), 1 mg/L naphthaleneacetic acid(NAA),4 mg/L 2,4-D, 85.5 g/L sucrose, pH 6) supplemented with 300 mM cetosyringone and different varying concentrations of four antioxidants
Selection	MA3; RD1; MA4; Schenk and Hildebrandt (SH)Macronutrients, SH Micronutrients (Schenk and Hildebrandt, 1972), MS vitamins, 100 mg/L glutamine, 100 mg/L malt extract, 1 mg/L biotin, 230 mg/L proline, 45 g/L sucrose, 10 g/L lactose, 0.05 mg/L zeatin,0.1 mg/L kinetin, 0.2 mg/L NAA, 3 g/L gelrite, pH 5.7; supplemented with timentin (200 mg/L) and kanamycin (50 mg/L) with varying concentrations of different antioxidants
Regeneration	(MS salts and vitamins, 100 mg/L myo-inositol, 5 mg/L BAP, 30 g/L sucrose, 3 g/L gel rite, pH 5.8)
Rooting	MS salts and vitamins, 10 mg/L ascorbic acid, 100 mg/L myo-inositol, 1 mg/L indole-3-butyric acid (IBA), 30 g/L sucrose, 3 g/L gelrite, pH 5.8

harbouring pCAMBIA2301 through co-cultivation of the cells the *Agrobacterium* as described by Khanna et al. (2004) and Magambo et al. (2016). Five days after the co-cultivation in the dark at 22°C, the *Agrobacterium*-infected cells were washed and transferred onto selective embryo formation medium (MA3; Table 1), that was supplemented with timentin (200 mg/L) and kanamycin (50 mg/L) to kill off the bacterium. The different antioxidants were added to the selective media at varying concentrations and the culture were

Table 2. Different Antioxidants and their varying concentrations used in the study.

Antioxidants name	Concentrations (mg)		
Ascorbic acid	20	40	60
Glutathione	50	100	150
Tocophel	25	50	75
Silver nitrate	4	6	8

incubated for 3 months with fortnightly transfer onto fresh medium. Putatively transformed embryos were then transferred to semi-solid RD1 medium (Table 1) amended with kanamycin (50 mg/L). Embryos germinating on this medium were transferred to medium MA4 (Table 1). The shoots developed on MA4 were transferred to proliferation medium (Table 1) and after 2 weeks, individual shoots were transferred to rooting medium. The regenerated plants were further used for molecular analysis.

Antioxidants

Antioxidants have been reported in the control of tissue browning and necrosis in plant tissue culture. Previous studies indicated that, tissue browning/ necrosis leading to poor plant regeneration in vitro and successful use of antioxidants is a key in solving the problems in the tissue culture (Tang et al., 2004; Naser et al., 2019). Therefore, using several antioxidants in many plant species such as AA, GSH, TOC and SN (Table 2) and other antioxidant namely, Cysteine, Dithiothreitol (DTT) and Polyvinylpyrrolidene (PVPP) have yielded tremendous results both at transformation and regeneration stages (Mei et al., 2011). The four antioxidants used in this study, each was varied in three different concentrations (Table 2) and each concentration was replicated three times.

Histochemical GUS assays

GUS assays were conducted on infected banana cell suspension after 5 days of co-cultivation with the *Agrobacterium* following the method of (Jefferson et al., 1987). Three samples from each concentration of antioxidant were randomly selected and stained. The frequency of transient GUS expression was determined as the percentage of the number of infected cells that exhibited the blue colour. After the regeneration process, the transformation frequency was determined as the percentage of the number of shoots regenerated against the total number of embryos cultured on selection media (Figure 4).

PCR analysis

Genomic DNA was isolated from 11 randomly selected putative transformed plantlets by means of the CTAB protocol (Gawel and Jarret, 1991; Stewart and Via, 1993) and used PCR analysis. PCR was performed using specific primers for the *gusA* gene, comprising the forward primer 5'TTTAACTATGCCGGGATCCATCGC-3'and reverse primer 5'CCAGTCGAGCATCTCTTCAGCGTA-3'. The PCR master mixes (20 µl) contained 0.5 ng of gDNA or 200 ng of plasmid DNA template, 10 µl of GoTaq Green master mix (Promega) and 5 pmol of each primer. The PCR parameters were as follows, the initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and a final extension of 72°C for 5 min. PCR amplicons were separated on agarose gel and stained in ethidium bromide before visualization under UV light.

Data collection and analysis

Data on stained and regenerated shoots was collected by counting shoots per medium type and presented as images and graphs. The means presented were for three replicates. Analysis of variance (ANOVA) was conducted using XLSTAT software version 9.0 (Addin soft, SARL, 2010). The means were separated using Tukey's HSD test at $P<0.05$ significance threshold of 0.05 and a post-hoc was applied thereafter.

RESULTS

Transformation, selection and regeneration of transgenic banana

Transient GUS expression assays showed that Cultivar "Nakinyika" ECS were successfully transformed. There were more GUS expressions in transformed embryogenic cells that had antioxidants added in the co-cultivation media compared to the controls where antioxidants were not added (Figure 2A and B). Different concentrations of ascorbic acid, glutathione, silver nitrate and tocopherol added to MA3 media resulted into variable numbers of GUS stained cells (Figure 3). Transformation efficiency results indicate that, cultivar 'Nakinyika' ECS were successfully transformed based on GUS expression assays (Figure 2: A (no anti-oxidant) and B (anti-oxidant). In addition, B which had antioxidant added showed more stained cells than A indicating, there was an improvement in the transformation as a result of antioxidants.

Results indicate that antioxidant increased transformation efficiency over the controls as presented in Figure 3. The highest number of stained cells (174 cells) was realized with Ascorbic acid at a concentration of 40 mg/L, followed by Glutathione (91 cells.) at 50 mg/L and Tocopherol (91 cells) at 50 mg/L. The least staining was observed when Ascorbic acid (39 cells), Silver nitrate (41 cells and 31 cells) were used at concentrations of 20, 4 and 6 mg/L, respectively (Figure 3). Therefore, all anti-oxidants can be useful for regeneration mostly the concentrations that gave better response than the control. Comparison of the various anti-oxidant concentrations at both transformation efficiency and regeneration showed

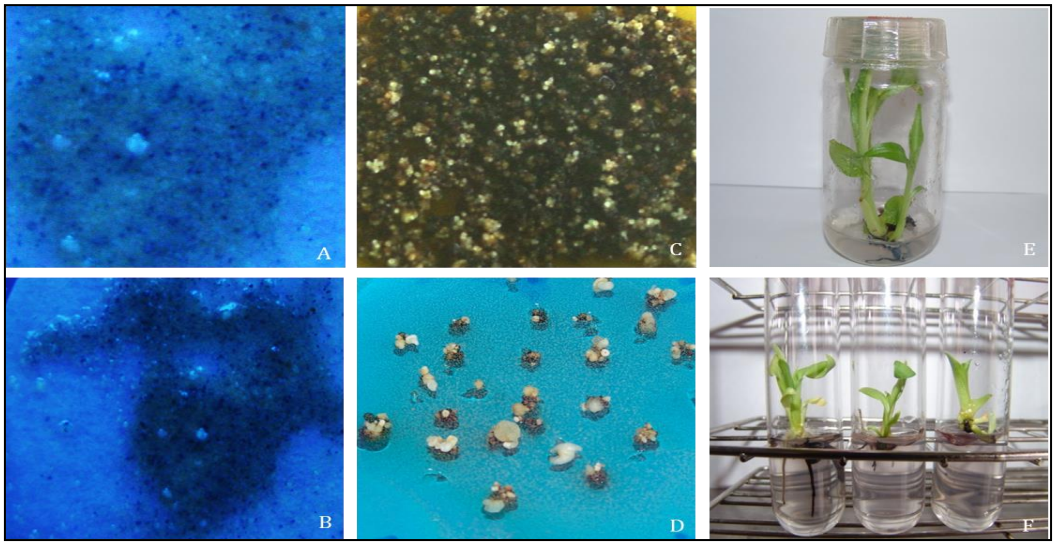

Figure 2. Schematic presentation of A: histochemical GUS assays with no anti-oxidant, B: histochemical GUS assay with anti-oxidant, blue foci represent the transformed ECS of cultivar 'Nakinyika', C: cells on selection medium (MA3), D: embryos germinating on selective medium (RD1) following Agrobacterium-mediate transformation, E: putatively transgenic shots on selective medium, F: transgenic.

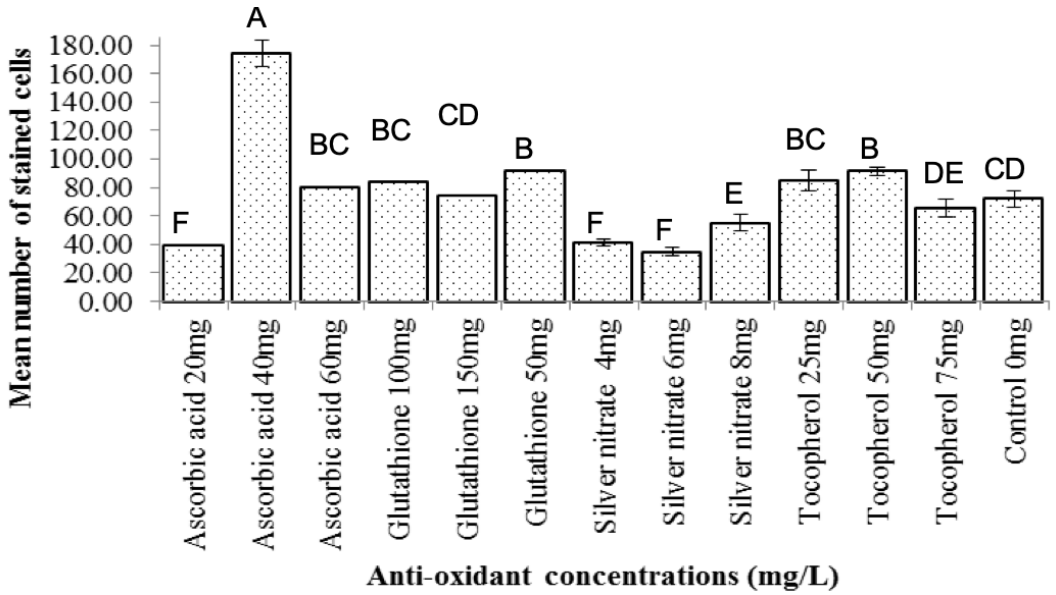

Figure 3. Effects of anti-oxidants on transformation efficiency of banana cultivar 'Nakinyika'. Means with the same letters were not significantly different from each other at p≥0.05%. Results are presented as mean ± standard error. Means are of three replicates.

that the three anti-oxidants (Ascorbic acid, Glutathione and Tocopherol) performed well at co-cultivation level basing on the number of stained cells, than the control of no anti-oxidant except Silver nitrate. At regeneration level, all the four anti-oxidants at the varied concentrations gave better results than the control, based on from the number of shoots obtained. The exception was Glutathione at 50 mg/L whose effect did not differ from the control. However, at both transformation and regeneration levels, ascorbic acid was better than the rest of the antioxidants, followed by Glutathione (Figures 3 and 4). From the data, all antioxidants gave better

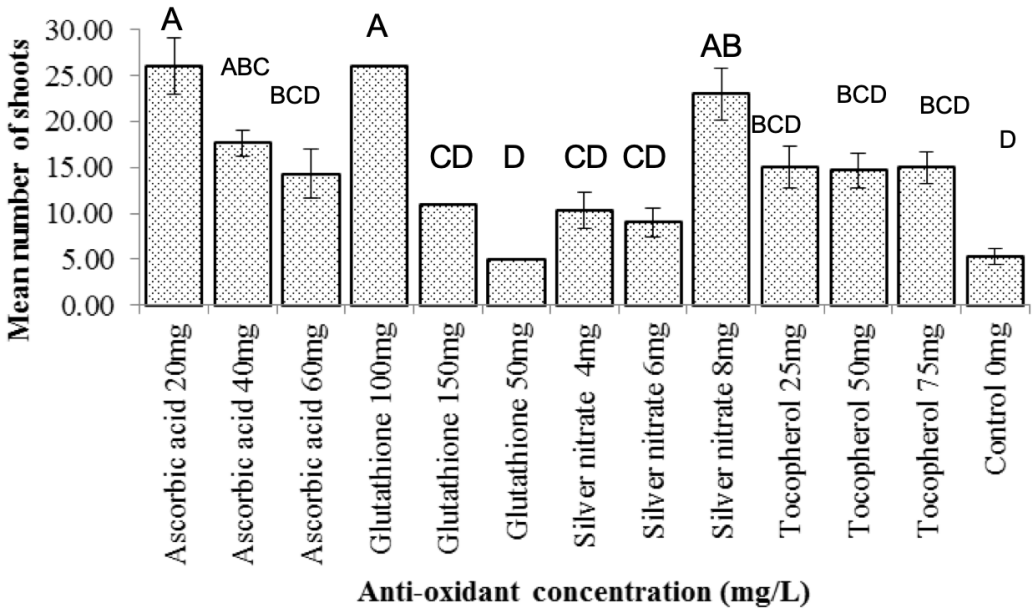

Figure 4. Effects of anti-oxidants on shoot regeneration of banana cultivar 'Nakinyika'Means with the same letters were not significantly different from each other at p≥0.05%. Results are presented as mean ± standard error. Means are of three replicates.

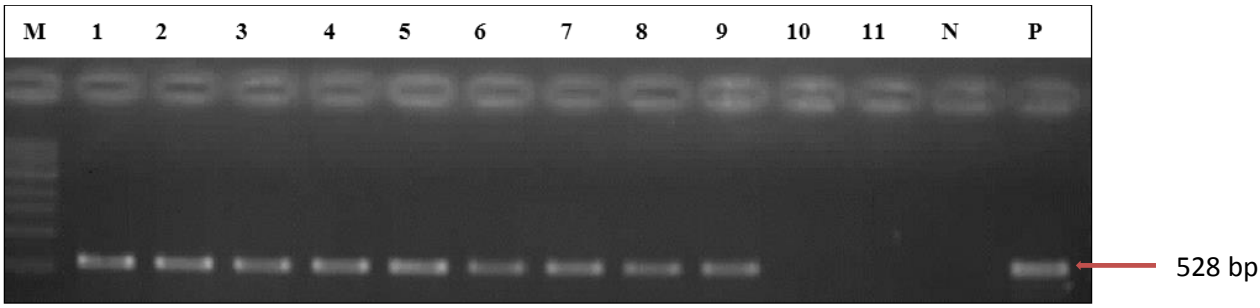

Figure 5. PCR amplification using Gusgene primers Lane M: DNA maker. Lanes 1-11: transgenic plants, lane N: Nontransgenic control P: Plasmid DNA.

responses at shoot regeneration other than at co-cultivation, the best antoxidants being ascorbic Acid at 20 mg, glutathione at 100 mg and silver nitrate at a rate of 8 mg/L (Figure 4).

PCR analysis

PCR analysis using gene specific primers gave the expected 528 bp fragment for coding region of *gusA* gene. Out of eleven putatively transformed plants that were randomly selected for confirmation, 9 lines were PCR positive while two lines did not show any amplification (Figure 5). In comparision, there was no amplfication observed in the non-transformed control.

There were no bands seen from some samples that were anaylsed for the Gus gene.

DISCUSSION

In contrast, browning and necrosis of transformed cells and difficulty to regenerate transgenic plants from the transformed cells are common phenomena in *Agrobacterium*-mediated transformation process in many plant species (Hiei et al., 2014). The problems of browning and necrosis affecting transformation' and occurrence of shoot escapes severely reduce transformation efficiency. *Agrobacterium*-mediated transformation is an injury process and therefore reactive oxygen species (ROS) such as superoxide radical (O-2), hydrogen peroxide (H_2O_2), the hydroxyl radical (OH), and

the peroxyl radical (RO2) are generated causing tissue browning and necrosis (Sharma et al.,2012). Additionally, dead cells release toxins that kill other cells in their proximity, resulting into low regeneration (Lindsey and Gallois, 1990). Therefore, an effective antioxidant is needed to protect the infected cells and improve their survival and successful transformation. Antioxidants act through inihibition of polyphenol oxidase enzymes that catalyse oxidation phenolics into highly reactive quinones that cause browning in injured tissues (Khosroushahi et al., 2011; Sharma et al., 2012; Selvarajan et al., 2018). Previous studies have reported use of various antioxidants in other plant species fore xample, it was observed that, applying 400 mg/1 cysteine in the coculture medium increased both the frequency of transient a-glucuronidase (GUS) expression in target cells of corm by 56% more than the control which yielded only 17% (Frame et al., 2002; Selvarajan et al., 2018). In addition, Toldi et al. (2002) demonstrated that, reduction in hyperhydricity of leaf explants, increase in leaf explant viability, as well as the frequency of transformation from 13% (without glutathione) to 45% in Agrobacterium mediated transformation of a desiccation-tolerant plant and *Craterostigma plantagineum*, was due to the addition of glutathione in the selection medium. Furthermore, previous research reported that, when ascorbic acid, sodium selenite, DL-a-tocopherol, and glutathione were added in a cocultivation medium, there was reduction on ROS production, antioxidant activity, and stable transformation efficiency during peanut Agrobacterium-mediated transformation (Zheng et al., 2005). They found that glutathione, tocopherol, and selenite not only eliminated the formation of H^sub 2^O^sub 2^ produced in wound tissue during preparation of leaf expiants and their cocultivation with A. tumefaciens, but also decreased malondialdehyde (MDA) formation and enhanced the activities of the antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). Furthermore, it was reported that, when cysteine was added incocultivation medium at a rate of 400 mg/L, it increased the Agrobacterium infection in the cotyledonary node of soybean (Olhoft et al., 2001). They further reported that, a combination of cysteine and DTT in the co- cultivation medium significantly increased the transformation efficiency (Olhoft et al., 2003).

Similarly in the present study, Ascorbic acid at 40 mg/L was the most effective antioxidant in transformation. Above this concentration, the antioxidant effects were not different from the other tested antioxidants. Ascorbic acid, Glutathione and Tocopherol were effective at about 50 mg/L at cell level during transformation. The observed ineffectiveness below and above this concentration is likely to be due to insufficient levels and toxicity, respectively. In contrast, the high concentrations were generally not deterrent at the tissue and plant level during regeneration and subsequent culturing.

The effect of silver nitrate on transformation success was not different from the control. Low transformation levels of silver nitrate could be partly linked to its antibacterial effects (Pandian et al., 2010). This toxicity might have reduced the agrobacterium population affecting the transformation process. Overall, the effect of the antioxidants at transformation did not match with the shoot regeneration trend. Cell injury and related oxidation was more limiting at the cell transformation stage. Due to the low cell surface exposure at the subsequent culture procedures of transfers and regeneration there was little oxidation which could be handled even at a lower ascorbic acid level of 10 mg/L. Contrarily to its effect at transformation, silver nitrate incoporated at 8 mg/L resulted into higher shoots recovery. This propmotive effect could be attributed to improvement in callus growth and embryogenesis through inhibition of ethylene as reported in wheat (Cristea et al., 2012).

Ascorbic acid is a common anti-oxidant in banana micro propagation media using the meristem culture where it is used at 10 to 20 mg/L in less and highly browning prone cultivars, respectively. It is suggestive that Ascorbic acid should be used at 40 mg/L during transformation and subsequently reduced to 20 ml/L at the plant regeneration stage. There was a significant anti-oxidant dependency in *Agrobacterium*-mediated banana transformation. Hiei et al. (2014) demonstrated that the increase in transformation frequency was primarily due to the increase in transgene integration efficiency rather than in tissue regeneration efficiency. Silver nitrate at a rate of 8mg, ascorbic acid at 20 mg and glutathione at 100 mg should be useful at cell-bacterium co-cultivation. Similarly, silver nitrate, ascorbic acid and glutathione can be adapted for shoot regeneration. For the PCR, the results confirmed that the GUS gene was successfully incorporated into ‘Nakinyika’ cultivar due to its presence observed in the 9 randomly selected lines. These results correlate well with the assessment of the transformation efficiency after co-cultivation when cells were stained which confirmed higher transformation in relation to the transgene presence. Similarly, the negative results observed in the two samples could be due to the presence of contaminants within the DNA samples or these could have been escapes. Due to cultivar differences in phenolic contents, these antioxidants should be evaluated on other banana genotypes as well.

Conclusion

The results of this study show that, antioxidants significantly enhance gene transfer and regeneration efficiency in recalcitrant banana cell lines and could significantly enhance the overall transformation efficiencies and generation of numerous transgenic lines. This study has established an efficient procedure for the *Agrobacterium*-mediated transformation of banana. Principally, we have developed an optimized co-

cultivation and regeneration conditions through supplementation with the antioxidants ascorbic acid, silver nitrate and glutathione for transgenic banana plants of the 'Nakinyika' cultivar. Our results provide an opportunity for further reproduction of transgenic banana plant cultivars.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ROS, Reactive Oxgyen Species; **RO2,** Peroxyl radical; **OH,** Hydroxyl radical; **H_2O_2,** Hydrgen peroxide; **O-2,** Super oxide radical; **DNA,** Deoxyribo Nucleic Acid; **PCR,** Polymerase chain reaction; **GSH,**Glutathione; **TOC,** Tocopherol; **AA,** Ascorbic acid; **SN,** Silver nitrate; **ECS,** Embryogenic cell suspension; **CTAB,**Centyltrimethyl ammonium bromide; **Mg,** Milligram; **SH,** Schenk and Hildebrandt; **RD1,** Embryo development media; **mL,** Millilitre; **GUS,** ß-glucuronidase; **G,**Gram; **ANOVA,** Analysis of Variance; **NAA,** Napthalene acetic acid; **IAA,** Indole acetic acid.

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