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In vitro propagation of the new disease resistant *Coffea arabica* variety, Batian

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Batian is a true breeding commercial coffee variety that was released in Kenya in 2010. It is resistant to coffee berry disease and coffee leaf rust which are the main coffee diseases in Kenya. Coupled with early ripening, good beverage quality and high yields, demand for planting material has surpassed supply. Conventional propagation methods do not provide enough planting materials, hence the need to develop alternative methods. The objective of this study was to develop an effective *in vitro* protocol for propagating the coffee variety, Batian. Leaf explants were harvested and cultured on Murashige and Skoog (MS) media supplemented with different concentrations of cytokinins benzyl amino purine (BAP) and thidiazuron (TDZ) separately, 100 mg/l myo-inositol 3% sucrose and gelled with 0.3% gelrite. The results show differences among cytokinins levels in induction of somatic embryos. BAP at 13.3 µM gave the highest mean of embryos per explants, 6.06 ± 1.18 and highest percentage of embryogenic cultures of 58.33%. Development of somatic embryos was achieved on hormone free MS media with highest mean length of 0.32 ± 0.03 mm. Indole butyric acid at 9.8 µM was best for induction of a well-developed root system with a mean length of 1.22 ± 0.09 mm. This protocol opens new prospects for massive propagation of Batian in nine months.

Key words: Batian, somatic embryo, *Coffea arabica.*

INTRODUCTION

Kenyan coffee is of highly rated beverage quality in the world and contributes significantly to the country's economic growth. One of the constraints to coffee production in Kenya is infection by diseases. Coffee berry disease (CBD) that is caused by *Colletotrichum kahawae* and coffee leaf rust (CLR), caused by *Hemileia vastatrix* are economically important coffee diseases in Kenya (Gichuru et al., 2012). Coffee breeding programmes in the country have recently resulted into the release of the variety, Batian in 2010. This new variety is resistant to both CBD and CLR (Gichimu and Omondi, 2010). The release of Batian coincided with favourable coffee prices

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that resulted in overwhelming demand for planting materials. As a rapid multiplication method for planting materials, tissue culture offers a feasible alternative to supplement the conventional propagation methods to meet the high demand for planting materials.

Somatic embryogenesis allows for the initiation and development of embryos from somatic tissues without the involvement of sexual fusion. It is a process by which somatic cells undergo bipolar development to give rise to genetically identical whole plants in nine months. There are two types of somatic embryogenesis; direct somatic embryogenesis (DSE), where embryos originate directly from the explants and, indirect somatic embryogenesis (ISE) where embryos are derived from an embryogenic dedifferentiated callus (Carneiro, 1999). The time taken to regenerate coffee plantlets using DSE is normally shorter (nine months) as compared to the time taken for ISE (12-13 months). However, the later one is more prolific and yields much more embryos per explants. In *Coffea* sp., somatic embryogenesis was first reported by Staristky (1970) and since then, a number of protocols for somatic embryogenesis have been developed for various genotypes of coffee (Yasuda et al., 1985; Hatanaka et al., 1991, 1995; Sandra et al., 2000; Giridhar et al., 2004). This paper documents the development of an effective *in vitro* protocol for propagating the coffee variety, Batian.

MATERIALS AND METHODS

Study site and sampling

The study was carried out at the Plant Tissue Culture laboratory of the Coffee Research Foundation situated at Ruiru (Altitude 1620 m above sea level 1°06'S; 36°45'E), Kenya. Third pair of leaves were excised from mother plants grown in the greenhouse at Ruiru and placed in a beaker containing tap water and taken to the laboratory for cleaning. They were washed with tap water containing a few drops of detergent teepol© and rinsed five times. The explants were transferred to the lamina flow cabinet, immersed in 70% (v/v) ethanol for 30 s and rinsed twice with sterile distilled water. This was followed by surface sterilization using 25% of a commercial bleach (JIK) © which contains 3.85% sodium hypochlorite for 25 min.

Media preparation

Direct induction of somatic embryos was carried out using Murashige and Skoog (MS) basal salts supplemented with 30 mg/l cysteine, 100 mg/l Inositol and 2% (w/v) sucrose. This medium was supplemented with various plant growth regulators (PGR). Cytokinins were evaluated separately; benzyl amino purine (BAP) at 4.4, 8.8, 13.3, 17.7, 22.0 µM and thidiazuron (TDZ) at 4.5, 9.0, 13.6, 18.2, 22.7 µM. BAP at 2.2, 4.4, 8.8, 13.3, 17.7, 22.0 µM was used for development media. On the other hand, half strength MS media supplemented with auxins indole butyric acid (IBA) at 4.9 9.8 19.7 µM and naphthalene acetic acid (NAA) at 2.5, 5.3 10.7 µM, and 100 mg/l inositol and with 2% sucrose was used for rooting. The pH was adjusted to 5.8 using 1 N NaOH or 0.1 M HCL before agar was added and media heated to dissolve the agar and dispensed in 10 ml aliquots into culture bottles. The media was autoclaved at 1.06 kg cm^{-2} and 121°C for 15 min. Inoculation was carried out in a sterile laminar airflow hood. The leaf explants were cut into sections of $1cm^2$ and cultured in MS media to produce somatic embryos. The somatic embryos were excised and transferred to MS media containing cytokinin (BAP) for development, followed by culturing on MS media with auxins IBA and NAA singly, for root initiation.

Experimental design, data collection and analysis

The experiments were laid out in completely randomized design (CRD). For embryo induction each treatment was repeated three times with 48 explants per repetition. For development of somatic embryos, each treatment was repeated three times with 30 explants per repetition. For root induction, each treatment was repeated three times with 20 explants per repetition. Data on number of somatic embryos per explants, percentage embryogenic cultures, length of developing embryos as well as length of roots were recorded. All the data were subjected to one-way analysis of variance (ANOVA) and the significant differences between treatment means were assessed by Duncan's multiple test range (DMRT). The results are expressed as a mean ± SE. Percent (%) embryogenic cultures was calculated as total number of explants with embryos/total number of cultured explants x 100, while percentage root induction was calculated as total number of plantlets with roots/total number of cultured plantlets x 100.

RESULTS

Somatic embryos were observed from the cut edges of the leaves 30 days after culture. Several stages of somatic embryogenesis were observed in the same culture (Figure 1). These somatic embryos later developed to the cotelydonary stage after 90 days. The MS media supplemented with BAP 8.8 µM gave the highest number (6.06 ± 1.18) of embryos per explant as well as the highest percentage of embryogenic cultures (58.33%). There were no embryos regenerated when the concentration of BAP was increased from 8.8 to 22.0 µM (Table 1).

MS media supplemented with TDZ 9.0 µM gave the highest mean number (2.06 ± 0.63) of embryos per explant and the highest percentage of embryogenic cultures (33.33%). There was no response when the concentration of TDZ was increased from 9.0 to 22.7 µM (Table 2).

On development media, the somatic embryos increased in size and synthesized photosynthetic pigments (Figure 2A). These pigments facilitate photosynthetic activity, extra storage reserves like lipids, triglycerides, proteins and other hydrates which favor *in vitro* germination (Nasim et al., 2010). Somatic embryos were considered to have germinated by development of the shoot (epicotyl) and elongated with the presence of a radicle structure which is a precursor to root induction (Figure 2B). In this study, embryos cultured on embryo elongation media responded by forming shoots only

Figure 1. Formation of somatic embryos at cut edges of the leaf discs.

Table 1. Effect of BAP on regeneration of somatic embryos in Batian variety.

Concentration (µM)	Embryogenic cultures (%)	Mean no. of embryos per explant
4.4	16.67	1.93 ± 0.86^{b}
8.8	14.58	1.75 ± 0.79^{b}
13.3	58.33	6.06 ± 1.18^{a}
17.7	25.00	1.11 ± 0.37^{b}
22.0	0.00	0.0 ± 0.0^{b}
P value		<.0001

n= 48. Values represent means ± SE. Means within a column followed by different letters are significantly different at P = 0.05.

Table 2. Effect of TDZ on regeneration of somatic embryos in Batian variety.

Concentration (µM)	Embryogenic cultures (%)	Mean no. of embryos per explant
4.5	0.00	0.0 ± 0.0^{b}
9.0	33.33	2.06 ± 0.63^{a}
13.6	18.75	1.00 ± 0.45^{ab}
18.2	16.67	0.82 ± 0.44^{b}
22.7	0.00	0.0 ± 0.0^{b}
P value		<0.0001

n= 48. Values represent means± SE. Means within a column followed by different letters are significantly different at P = 0.05.

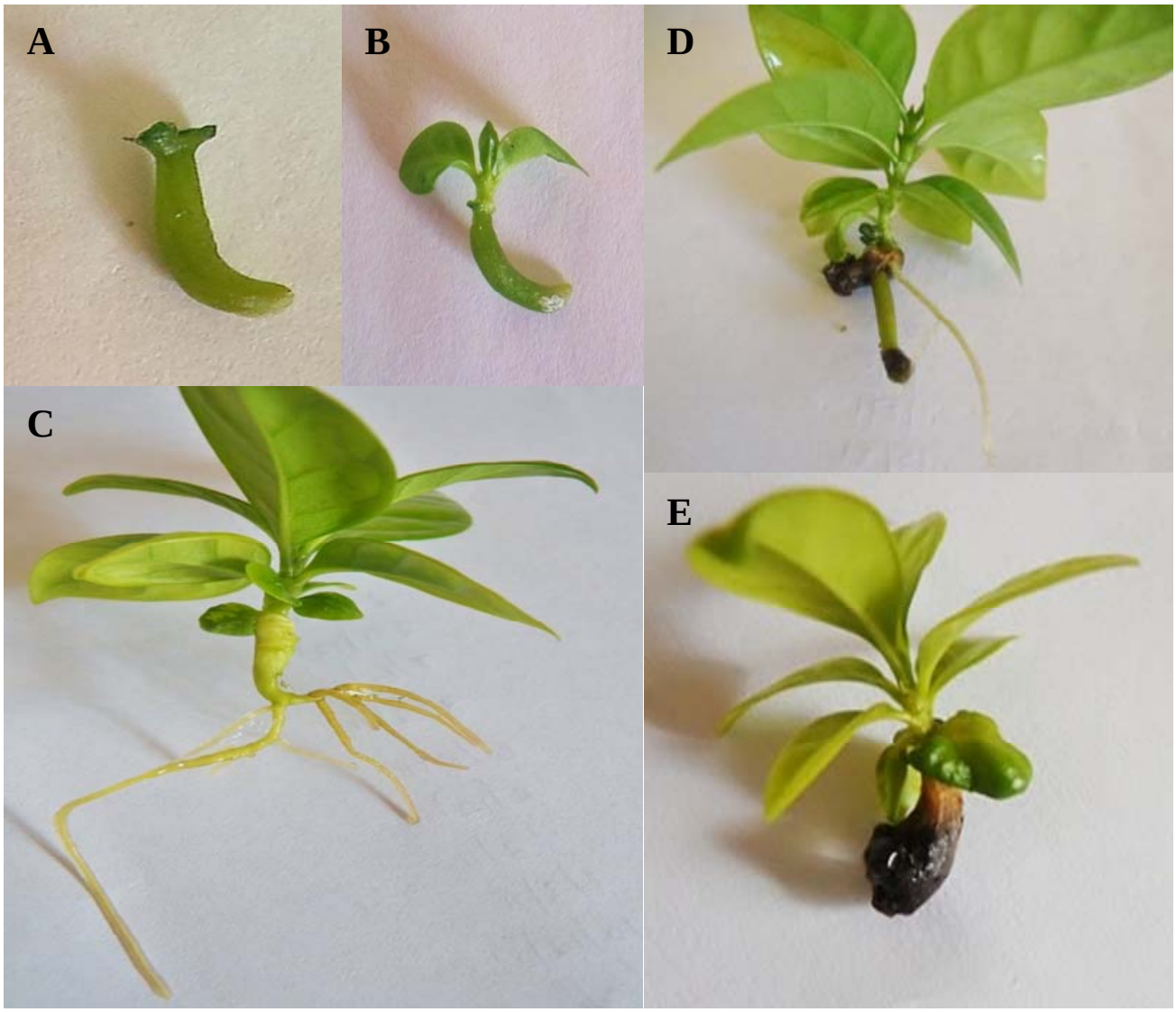

Figure 2. The different regeneration stages of somatic embryos. **A,** Maturing somatic embryo after 14 days in germination media. **B,** Matured somatic embryo ready for root induction. **C,** Rooting of plantlet on NAA 4.9 µM 8 weeks after culture in rooting media. **D,** Inhibited root elongation on NAA 10.7 µM. **E.** Desiccation of plantlet due to high levels of auxins (IBA 19.7 µM).

hence the need for a rooting step. The effects of BAP on elongation of somatic embryos showed no significant difference. However, control had the highest mean length of 0.27 ± 0.05 mm while BAP at 4.4 µM had the lowest mean absolute length (Table 3).

MS media supplemented with NAA 2.5 µM gave the highest mean root length of 1.02 ± 0.14 mm and highest percentage (66.67%) of root induction (Table 4). Low levels of auxins promoted root elongation (Figure 2C). Increasing the concentration of NAA from 5.3 to 10.7 µM resulted in inhibition of root elongation (Figure 2D).

The embryos cultured on media supplemented with 9.8 µM IBA gave the highest mean root length, 1.22 ± 0.09 mm. Increasing IBA from 9.8 to 19.7 µM resulted in reduction in the percentage root induction as well as the mean root length. This was similar to the trend observed for somatic embryo cultured on medium supplemented with NAA where an increase in concentration resulted in low percentages of root induction (Figure 2E). On the other hand, 9.8 µM IBA gave the highest mean length and the highest percentage (68.62 %) of root induction (Table 5).

DISCUSSION

In vitro growth is highly dependent on the interaction between naturally occurring endogenous substances and

Table 3. Effect of BAP on development of somatic embryo of Batian.

Concentration (µM)	Mean shoot length (mm)
Control	0.27 ± 0.05[a]
2.2	0.20 ± 0.05[a]
4.4	0.15 ± 0.05[a]
8.8	0.23 ± 0.05[a]
17.7	0.19 ± 0.05[a]
P value	<0.0001

n= 30. Values represent means± SE. Means within a column followed by different letters are significantly different at P = 0.05.

Table 4. Effects of different NAA concentrations on rooting of Batian.

Concentration (µM)	Mean root length (mm)	Root induction (%)
2.5	1.02 ± 0.14[a]	66.67
5.3	0.72 ± 0.18[ab]	53.84
10.7	0.53 ± 0.11[b]	50
P value	<0.0001	

n = 20. Values represent means± SE. Means within a column followed by different letters are significantly different at P = 0.05.

Table 5. Effects of different IBA concentrations on rooting of Batian.

Concentration (µM)	Mean root length (mm)	Root induction (%)
4.9	1.05 ± 0.09[ab]	52.38
9.8	1.22 ± 0.09[a]	68.62
19.7	0.85 ± 0.09[b]	36.11
P value	<0.0001	

Values represent means± SE. Means within a column followed by different letters are significantly different at *P* = 0.05.

the analogous synthetic growth regulators added to the medium (George, 1993). During the current study, somatic embryos were formed only at the cut edges. These results are in agreement with those of Hatanaka et al*.* (1991) who reported that somatic embryos were formed only at the cut edges which were in contact with the media containing growth regulators. They suggested that the cut surface (wounding) might have been sites for rapid uptake of minerals and PGR which resulted in the high percentage of embryogenic cultures. These results are also in agreement with that of Gatica et al. (2007) and Catie and Yasuda et al. (1985) who reported that culture media supplemented with low levels of BAP stimulated development of somatic embryos. During the present study, increasing the concentration of BAP resulted in the decrease of number of somatic embryos. This is contrary to the results of Yasuda et al. (1985) who observed that media supplemented with 4 to 6 mg/l BAP were more efficient in induction of somatic embryos of F1 hybrid *Coffea arabica*. These differences could be explained by the influence of genotype and other precise conditions that may not have been reproduced in the two studies.

TDZ is chiefly used as cotton defoliant (Giridhar et al., 2004) but it also acts as a growth regulator in tissue culture systems. In peanut and geranium, the use of TDZ has effectively replaced the requirement of auxins and/or cytokinins in induction of somatic embryogenesis (Gill and Saxena, 1993). In this study, results show that TDZ at 9.0 µM produced the highest (33%) frequency of embryogenic cultures. However, these results are contrary to those of Kahia (1999) who reported 100% embryogenic cultures when coffee leaf explants of Ruiru 11 an F1 hybrid were cultured on modified MS media supplemented with 1 µM TDZ, and Gill and Saxena (1993)

who reported 100% induction of somatic on *Nicotiana tabacum* L. leaf explants using TDZ. In this study, the use of TDZ in formation of somatic embryos was achieved with low concentrations. These is in agreement with the study of Giridhar et al. (2004) who reported that low levels of TDZ stimulated direct somatic embryogenesis in *Coffea*.

The time taken to regenerate coffee plantlets using DSE is normally shorter (nine months) as compared to the time taken for ISE which is 12-13 months (Ducos et al., 2010; Etienne et al., 2010). It is a two-step procedure thus similar to high frequency somatic embryogenesis (HFSE). On the other hand, the later one is more prolific and yields much more embryos per explants. Due to the long period, the cultures stay in a PGR supplemented media, the chances of somaclonal variation in ISE are much higher. The protocol developed in this study ensures production of true to type plantlets.

Auxins stimulate root initiation by activating quiescent pericycle cells to initiate division and then expansion which facilitate lateral root emergence, although response varies with concentration (Fukaki and Tasaka, 2009). Taiz and Zegler, (2003) reported that auxins are required for root induction; though, root growth is inhibited at higher auxin concentration. Kollmeier et al. (2000) stated that high auxin concentration inhibits root elongation. This was also reported by Riov and Yang (1989) who observed that auxins intensify the rate of ethylene biosynthesis. Subsequently, it is conceivable that high concentration of NAA and IBA induced ethylene biosynthesis which is inhibitory to root elongation. IBA proved to be better than NAA in induction of rooting system across all evaluated concentrations.

In general, the effectiveness of each PGR on the various regeneration stages of the selected *C. arabica* varieties was influenced by the concentration. During the current study, a reproducible somatic embryogenesis protocol for regenerating Batian in nine months was developed. The protocol involves culturing the leaf disc explants on half strength MS media supplemented with BAP 13.3 µM. The somatic embryos elongated on hormone free full strength MS media. The somatic embryos were rooted on half strength MS media supplemented with 19.7 µM IBA.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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