International Journal of Development and Sustainability
ISSN: 2186-8662 – www.isdsnet.com/ijds
Volume 7 Number 11 (2018): Pages 2807-2820
ISDS Article ID: IJDS18082001

Coffea arabica Cultivar Ruiru 11 sibs exhibit significant variation in plant regeneration through direct somatic embryogenesis

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Abstract

This experiment evaluated the response of 11 selected recalcitrant coffee 'Ruiru 11' sibs to plantlet regeneration through direct somatic embryogenesis. Third leaf pair explants from field-grown mother plants were harvested, sterilized and induced for up to 12 months in half-strength Murashige and Skoog basal salts medium supplemented with 1 ml/L thidiazuron, 0.2 g/L thiamine, 0.1 g/L nicotinic acid, 0.1 g/L pyridoxine, 20 g/L sucrose, 100 mg/L myo-inositol, 100 mg/L cysteine and 3 g/L gelrite. Embryos were sequentially transferred to media for shooting for 8 weeks, rooting for 6 weeks and weaning for 4 weeks. The explants were effectively sterilized using 30% sodium hypochlorite (3.85 v/v) for 20 minutes. The sibs showed significant (P<0.05) genotypic variation, with sibs 11, 41, 71 and 121 from 'Catimor 86'and those from 'Catimor 90, 128 and 134' regenerating somatic embryos, whereas sibs 52 and 142 from 'Catimor 88' failing to do so. Sib 137 had the highest 63% embryogenic rate and 3.7 average embryos per explant. Sibs 71 and 137 embryos developed longest shoots, with 84% of sib 137 embryos developing shoots. Sibs 93 and 71 embryos developed longest roots, with 91% of sib 71 embryos developing roots. About 91% of sib 71 plantlets survived acclimatization. Sibs 137, 71 and 93 were classified as highly embryogenic, whereas sibs 52 and 142 were non-embryogenic. Sib 71 best responded to plantlet regeneration through direct somatic embryogenesis. Therefore, sib 71 plantlets should be used in future as stocks for mother plants for mass propagation of 'Ruiru 11'.

Keywords:Four to eight keywords come here. Divide the keywords by semicolon.

Cite this article as: Mayoli, R.N., Isutsa, D.K. and Nyende, A.B. (2018), "Coffea arabica Cultivar Ruiru 11 sibs exhibit significant variation in plant regeneration through direct somatic embryogenesis", *International Journal of Development and Sustainability*, Vol. 7 No. 11, pp. 2807-2820.

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1. Introduction

Somatic embryogenesis is the developmental reorganization of somatic cells toward the embryogenic pathway and the process includes initiation of embryogenic cultures, multiplication of embryogenic cultures, maturation of the somatic embryos and germination (Pullman et al., 2003; Helmersson et al., 2004). Merkle et al. (1995) postulated that with increased probability of somatic embryo gene expression matching that of zygotic embryos (Fehér et al., 2003), the higher the probability of achieving highly efficient regenerative systems. Mass propagation of plants through somatic embryogenesis is the most economically viable application of somatic embryogenesis (Merkle et al., 1990). Regeneration via somatic embryogenesis is considered pivotal for application of cell culture methods to the genetic improvement of crop plants due to their potential in agricultural production and crop improvement programmes (Elmeer, 2013).

Several endogenous and exogenous factors exert influences on the growth, development and performance of somatic plant cells in culture (Neumann et al., 2009). Among these factors, the genotype is of critical importance in eliciting the desired response to somatic cell development into plants (Neumann et al., 2009). Regeneration studies have shown that the frequency and efficiency of somatic embryogenesis dependent on the genotype, and not all cultivars are amenable to somatic embryogenesis, regeneration and/or transformation (Hankoua et al., 2005; Atehnkeng et al., 2006). Molina et al. (2002) reported that some genotypes respond well, whereas others are recalcitrant, which suggests that regeneration is genetically controlled.

Acquisition of regeneration potential is determined at several levels, and the variation readily demonstrates this in response often observed among different genotypes within the same species and their heritability (Delporte et al., 2014). This is important for selection since it allows for the estimation of the regeneration capacity of a progeny by the response of its parent. Many genotype-dependent effects are caused by the interaction between the plant genotype and the culture environment. Vasil and Vasil (1986) suggested that the differential response of genotypes may be due to differential expression, which in turn depends upon spatial and temporal distribution and their physiological and developmental stages.

Ruiru 11 is one of the arabica coffee cultivars that generally combines CBD resistance with high yields and fine quality (Van der Vossen and Walyaro, 1980; Griffiths et al., 1971; Hindorf and Omondi, 2011; Gichimu, 2013). The cultivar is a composite of 66 F1 hybrid sibs each derived from a cross between a specific female and male population (Omondi et al., 2001). Ruiru 11 progenies have variable beverage quality and yield (Kathurima, 2013; Gichimu, 2013), some of which is attributed to environmental effects. Coffee production in Kenya has been constrained by many factors, including inadequate planting materials needed to expand coffee acreage (CRF, 2011). This paper reports results of evaluating responses of 11 selected recalcitrant coffee cultivar Ruiru 11 sibs to plantlet regeneration through direct somatic embryogenesis.

2. Materials and methods

The research was done in the Kenya Agricultural and Livestock Research Organization-Coffee Research Institute laboratories and greenhouses at Ruiru in Kenya. The site is situated 1.05°S and 36.45°E at an elevation

of 1608 m above the sea level and has humic nitosol soils (Jaetzold et al., 2007). Out of the 60 sibs available, 11 were selected based on cup quality (Gichimu et al., 2012a), raw bean quality (Van der Vossen, 2009; Kathurima et al., 2010; Gichimu et al., 2012b), and yields (Gichimu, 2013). The selected sibs were: R11-11, R11-41, R11-52, R11-71, R11-91, R11-93, R11-100, R11-121, R11-131, R11-137 and R11-142 (Table 1). Ruiru 11 sib 121 was used in development of sterilization procedure for field-grown 'Ruiru 11' explants.

Third leaf pair explants were excised from field-grown mother plants, washed under running tap water followed by water containing Teepol detergent and finally sterile distilled water. The subsequent sterilization steps took place in a laminar flow cabinet where the explants were dipped quickly for 30 seconds in 70% alcohol and rinsed 2-3 times in sterilized distilled water. The explants were further sterilized using 20%, 25% or 30% sodium hypochlorite (3.85 v/v) for 20, 25 or 30 minutes, followed by rinsing 4 times in sterilized distilled water. The sterilized explants were trimmed to remove sterilizing agent-affected parts, entire mid-ribs and leaf tips. The sterilized explants were cut into 1 cm^2 leaf discs and cultured.

The explants were induced in half-strength Murashige and Skoog (MS) (1962) basal salts medium, augmented with 1 ml/L thidiazuron, 0.2 g/L thiamine, 0.1 g/L nicotinic acid, 0.1 g/L pyridoxine, 20 g/L sucrose, 100 mg/L myo-inositol, 100 mg/L cysteine and 3 g/L gelrite. The medium was adjusted to pH 5.7 using 1 M NaOH before autoclaving for 15 minutes at 121^{o}C and 100 kPa. The experiment was set up in a completely randomized design, with three replications of five Magenta jars, each containing 5 explants. The cultures were maintained in darkness for up to 12 months and the experiment was repeated once.

The induced somatic embryos were transferred to germination medium, comprising full strength MS (1962) basal salts medium, supplemented with 0.2 g/L thiamine, 0.1 g/L nicotinic acid, 0.1 g/L pyridoxine, 20 g/L sucrose, 100 mg/L myo-inositol and 3 g/L gelrite, and maintained at 25±2^{o}C temperature and 16 h light for up to 8 weeks for shoot growth and development.

Table 1. Pedigree of Ruiru 11 Sibs regenerated

SN	Sib	Parentage	Sib characteristic
1	11	CAT 86 x [SL28 x B 3.97) = (SL28 x RS) (B x HDT)]	Progeny B 3.97 from 4-way crosses of male parents backcrossed to SL28 then crossed with Catimor 86
2	41	CAT 86 x [SL28 x B 3.116) = (SL28 x RS) (B x HDT)]	Progeny B 3.116 resulting from 4-way crosses of male parents backcrossed to SL28 then crossed with Catimor 86
3	71	CAT 86 x [SL28 x B 3.314) = (N39 x HDT) (SL4 x RS)]	Progeny B 3.314 resulting from 4-way crosses of male parents backcrossed to SL28 then crossed with Catimor 86
4	91	CAT 86 x [SL28 x B 3.863) = (SL34 x RS) HDT]	Progeny B 3.863 resulting from 4-way crosses of male parents backcrossed to SL28 then crossed with Catimor 86
5	121	CAT 86 x [SL28 x B 3.887) = (SL34 x RS) HDT]	Progeny B 3.887 resulting from 4-way crosses of male parents backcrossed to SL28 then crossed with Catimor 86
6	131	CAT 86 x [SL28 x B 3.879) = (SL34 x RS) HDT]	Progeny B 3.97 resulting from 4-way crosses of male parents backcrossed to SL28 then crossed with Catimor 86
7	52	CAT 88 x [SL28 x B 3.185) = (K7 x RS) (SL34 x HDT)]	Progeny B 3.185 resulting from 4-way crosses of male parents backcrossed to SL28 then crossed with Catimor 88
8	142	CAT 88 x [SL28 x B 4.54) = (SL28 (SL28 x RS)]	Progeny B 4.54 resulting from 3-way crosses of male parents backcrossed to SL28 then crossed with Catimor 88
9	93	CAT 90 x [SL28 x B 3.863) = (SL34 x RS) HDT]	Progeny B 3.863 resulting from 3-way crosses of male parents backcrossed to SL28 then crossed with Catimor 90

10	137	CAT 128 x [SL34 x B 3.879) = (SL34 x RS) HDT]	Progeny B 3.879 resulting from 3-way crosses of male parents backcrossed to SL28 then crossed with Catimor 128
11	100	CAT 134 x [SL28 x B 3.863) = (SL34 x RS) HDT]	Progeny B 3.863 resulting from 3-way crosses of male parents backcrossed to SL28 then crossed with Catimor 134

Key: CAT- Catimor, HDT- Hibrido de Timor, RS- Rume Sudan, B- Bourbon. Source: Gichimu (2013)

Germinated shoots were transferred to rooting medium, comprising half strength MS (1962) basal salts medium supplemented with 1 ml/L naphthalene acetic acid, 0.2 g/L thiamine, 0.1 g/L nicotinic acid, 0.1 g/L pyridoxine, 100 mg/L myo-inositol, 20 g/L sucrose and 3 g/L gelrite. The germinated shoots were maintained at 25±2^{o}C temperature and 16 h light for up to 6 weeks.

The rooted plantlets were removed from the rooting medium using forceps, agar washed off roots under running tap water and plantlets soaked in 10% Ridomil fungicide for 1 to 2 hours. The rooting mixture comprised 3:2:1 v/v top soil, sand and composite manure in pots that were left to soak water until the potting mixture saturated. The plantlets were removed from the fungicide and transplanted into pots that were covered completely, opened gradually to reduce humidity, and opened fully after 4 weeks. The weaned plantlets were transferred to similar potting mixture.

The explants were observed weekly and total number of embryos per sib, number of explants with somatic embryos and somatic embryos per explant counted after 12 months in culture for all sibs. The number of shoots and average shoot length were recorded after 6 weeks in culture for all sibs. Following transfer of germinated shoots to rooting medium, the number of roots and average shoot length were recorded after 6 weeks in culture for all sibs. Acclimatized and hardened plantlets were counted per sib and data expressed as a percentage.

The SAS 9.2 computer software was used to analyze data using the General Linear Model for a completely randomized design (SAS, 2008). The linear model fitted was: $Yij = \mu + Ti + \varepsilon ij$, Where: i = 1, 2, 3...10, 11; j = 1, 2, 3; μ = grand mean, Ti = ith 'Ruiru 11' sib and εij is random error component, normally and independently distributed about zero mean with a common variance σ^2. Differences between treatment means were separated using LSD test at $\alpha = 0.5$.

3. . Results

3.1. Sterilization of explants

There was significant (P<0.05) effect of 3.85 v/v sodium hypochlorite (NaOCl) concentration and time on the survival rate of clean explants (Table 2). Sterilizing with 20% NaOCl for 30 minutes had 55.3% clean and surviving explants. A similar trend was observed for 25% NaOCl, where the highest clean and surviving explants resulted after 30 minutes and the lowest resulted after 25 minutes, whereas sterilizing for 25 minutes with 25% NaOCl resulted in 11%, which was the lowest rate of clean and surviving explants. For 30% NaOCl,

the highest 64.3% rate of clean and surviving explants resulted after sterilizing for 20 minutes. Sterilization for 30 minutes using 30% NaOCl had the lowest percentage of clean and surviving explants, which was 14.3%.

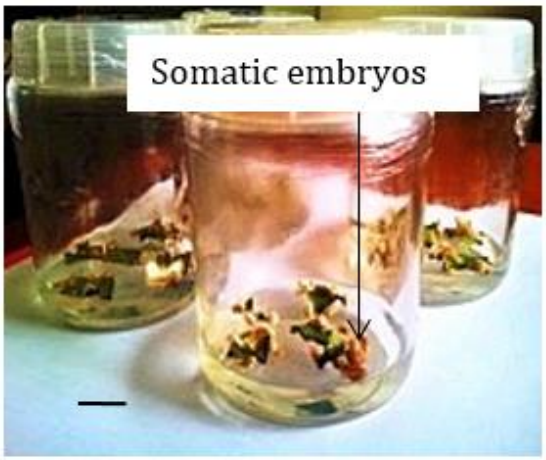
Table 2. Survival rate (%) of sterilized field-grown *Coffea arabica* 'Ruiru' 11 sib 121

Time (minutes)	20% NaOCl	25% NaOCl	30% NaOCl
20	35.0±5.7^{b}*	20.0±0.0^{b}	64.3±0.3^{a}
25	20.7±3.0^{c}	11.0±1.0^{c}	36.3±1.9^{b}
30	55.3±2.6^{a}	30.0±2.9^{a}	14.3±0.7^{c}
CV (%)	16.3	16.1	12.1
LSD $_{(0.05)}$	12.0	6.5	9.3

**Values followed by the same letter within each column are not significantly different according to the LSD test at α = 0.05.*

3.2. Induction rate of somatic embryos in 'Ruiru 11' sibs

The sibs started developing white protrusions at the cut ends of the leaf discs after 1 month of culture. Formation of white globular masses started after 6 months in culture. The sibs that produced embryos first were 137, 93 and 71 after 10 months in culture (Plate 1). Since embryo development was not uniform among the Sibs, the cultures were maintained for 12 months to allow for regeneration of embryos on all the Sibs. Sibs 52, 91, 131 and 142 did not produce any embryos after one year in culture.



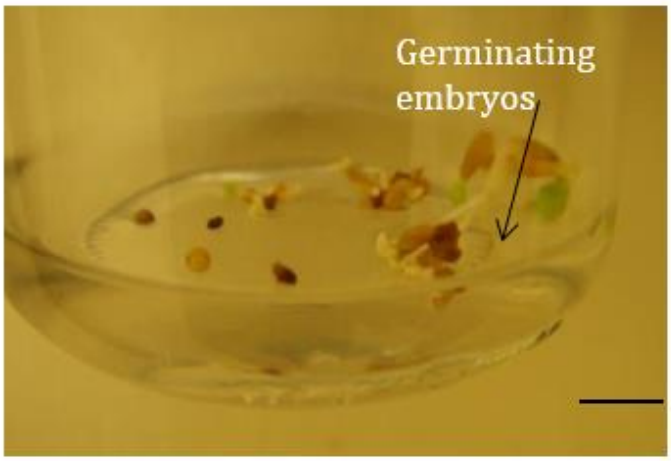


Plate 1. Induced somatic embryos of sib 137. Bar = 1 cm **Plate 2.** Germinating embryos of sib 71. Bar = 1 cm

Significant (P<0.05) differences resulted in the total number of embryos formed in the various Ruiru 11 Sibs tested (Table 3). The control (greenhouse-grown Ruiru 11) had the highest 75.3% total embryo regeneration rate. Sibs 11 had 3.7 had the least total embryo formation, although it was not significantly different from 4.3 of sib 41. Significant differences (P<0.05) resulted in percentage explants forming somatic embryos (Table 3).

The control had the highest 73.3% frequency of somatic embryogenesis. Sibs 11, 41 and 121 had the lowest frequency of explants that formed somatic embryos, which were 11.67%, 13.33% and 25%, respectively. The mean number of embryos formed per embryogenic explant was significantly different, with the control having

6.9 (Table 3). Sibs 11 and 41 explants had the lowest mean embryo formation rate, although not significantly different from each other.

Table 3. Induction and regeneration rate of 'Ruiru 11' sibs

	Embryos per 5 explants	Embryogenic cultures (%)	Embryos per explant
Sib 11	3.7±0.7^{f*}	11.7±1.7^{g}	1.6±0.1^{d}
Sib 41	4.3±0.3^{ef}	13.3±1.7^{g}	1.7±0.2^{d}
Sib 52	0.0±0.0^{f}	0.0±0.0^{h}	0.0±0.0^{e}
Sib 71	29.0±1.7^{c}	42.5±4.3^{d}	3.4±0.1^{b}
Sib 91	0.0±0.0^{f}	0.0±0.0^{h}	0.0±0.0^{e}
Sib 93	14.0±3.0^{d}	33.3±11.7^{e}	2.4±0.6^{c}
Sib 100	25.0±6.4^{c}	50.0±8.7^{c}	2.4±0.2^{c}
Sib 121	10.7±0.9^{de}	25.0±5.0^{f}	2.2±0.3^{cd}
Sib 131	0.0±0.0^{f}	0.0±0.0^{h}	0.0±0.0^{e}
Sib 137	46.3±1.3^{b}	63.3±3.3^{b}	3.7±0.1^{b}
Sib 142	0.0±0.0^{f}	0.0±0.0^{h}	0.0±0.0^{e}
Control	75.3±2.8^{a}	73.3±4.1^{a}	6.9±0.4^{a}
CV (%)	18.7	14.2	21.8
LSD $_{(0.05)}$	8.5	6.23	0.75

**Values followed by the same letter within each column are not significantly different according to the LSD test at α = 0.05.*

3.3. Germination rate of somatic embryos of Ruiru 11 Sibs

After transfer of embryos to hormone-free media and incubation at 25±2^{o}C and 16 h light, the embryos started synthesizing photosynthetic pigments after 2 weeks (Plate 2). This phase was also characterized by development of the polar axis on the germinating shoots, which was evidenced by the formation of shoot-like structures with two leaflets at each shoot tip (Plate 2). The germinating shoots were maintained for 8 weeks to grow to uniformity (Plate 2). Sib 71 had a significantly (P<0.05) longest shoot length of 1.7 cm, although not significantly different from that of Sibs 137, 100, 93 and the control (Table 4). Similarly, the percentage of embryos with shoot development was not significantly different among the four sibs and the control, although the control had highest 85.3% shoot formation rate. Sib 121 had the shortest shoots of 0.7 cm and resulted in the least 46.7% rate of embryo shoot development.

Table 4. Germination rate of 'Ruiru 11' sib embryos

Sib	Shoot length (cm)	Shoot formation (%)
Sib 11	0.0±0.0^{e}	0.0±0.0^{e}
Sib 41	0.0±0.0^{e}	0.0±0.0^{e}
Sib 52	0.0±0.0^{e}	0.0±0.0^{e}
Sib 71	1.7±0.4^{a}	73.3±13.3^{b}
Sib 91	0.0±0.0^{e}	0.0±0.0^{e}
Sib 93	1.1±0.3^{c}	65.0±3.8^{c}
Sib 100	1.3±0.2^{bc}	80±0.0^{ab}
Sib 121	0.7±0.1^{d}	46. 7±17.6^{d}
Sib 131	0.0±0.0^{e}	0.0±0.0^{e}
Sib 137	1.3±0.1^{bc}	84.3±7.2^{a}
Sib 142	0.0±0.0^{e}	0.0±0.0^{e}

Control	1.4±0.0[b]	85.3±1.6[a]
CV (%)	17.5	12.5
LSD (0.05)	0.2	7.6

**Values followed by the same letter within each column are not significantly different according to the LSD test at α = 0.05.*

3.4. Rooting and survival rate of regenerated Ruiru 11 sib plantlets

After transfer of germinated embryos to rooting medium, a radical pole started to develop from the opposite end of the germinated shoots after 2 weeks (Plate 3) and well developed roots were formed after 6 weeks in culture (Plate 4). Sib 93 had the longest roots measuring 2.5 cm, although not significantly different from those of the control and Sib 137 (Table 5). Sib 121 and sib 100 had the shortest roots, although not significantly (P>0.05) different from each other. Sib 71 had significantly highest percentage root formation, although not significantly different from the control. Sib 71 had the highest survival rate of 91%, which was significantly (P<0.05) different from that of the other Ruiru 11 Sibs.

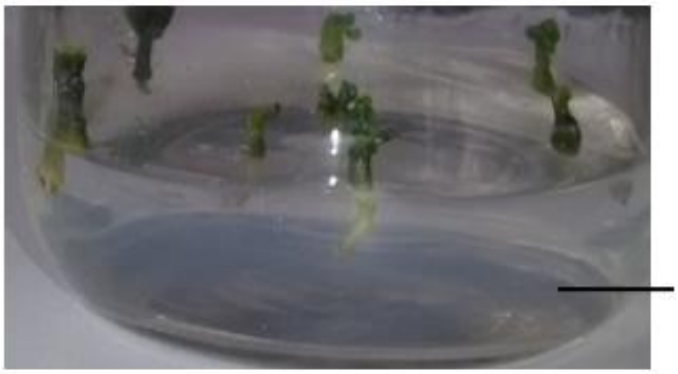


Plate 3. Developing radical poles of sib 137. Bar = 1 cm

Plate 4. Rooting of Ruiru 11 sib 71. Bar = 1 cm

Table 5. Rooting and survival rate of regenerated Ruiru 11 Sib plantlets

Sib	Root length (cm)	Root formation (%)	Survival (%)
Sib 11	0.0±0.0[d]	0.0±0.0[f]	0.0±0.0[f]
Sib 41	0.0±0.0[d]	0.0±0.0[f]	0.0±0.0[f]
Sib 52	0.0±0.0[d]	0.0±0.0[f]	0.0±0.0[f]
Sib 71	2.0±0.3[bc]	94.3±5.7[a]	91.0±1.2[a]
Sib 91	0.0±0.0[d]	0.0±0.0[f]	0.0±0.0[f]
Sib 93	2.5±0.3[a]	50.0±0.0[de]	78.3±1.5[b]
Sib 100	1.6±0.2[c]	56.3±7.2[cd]	50.0±1.2[d]
Sib 121	1.7±0.2[c]	41.7±8.3[e]	46.7±3.3[d]
Sib 131	0.0±0.0[d]	0.0±0.0[f]	0.0±0.0[f]
Sib 137	2.2±0.1[ab]	63.6±6.4[c]	80.0±1.2[b]
Sib 142	0.0±0.0[d]	0.0±0.0[f]	0.0±0.0[f]
Control	2.4±0.1[ab]	79.2±3.3[b]	61.0±2.1[c]
CV (%)	24.3	22.3	6.8
LSD (0.05)	0.42	12.08	3.91

*Values followed by the same letter within each column are not significantly different according to the LSD test at α = 0.05.

4. Discussion

4.1. Explant sterilization

In the present study, field-grown Ruiru 11 Sib 121 explants exhibited high contamination rate. Most of the contamination in the present study was fungal, arising from ubiquitous presence in the field-grown explants or introduction through poor culture handling. Establishment of field-grown plants *in vitro* is prone to contamination that causes massive losses (Tiwari et al., 2012). Explant contamination depends on plant- and environment-related factors such as species, age, source and prevailing weather conditions (Rout et al., 2000). Several authors have reported the difficulty in achieving clean *in vitro* plantlets while using field-grown plants as a source of explants (Odutayo et al., 2007; Oyebanji et al., 2009; Ndakidemi et al., 2013; Guma et al., 2015) since the plants are always at a high risk of internal and external contamination. Successful culture depends on removal of exogenous and endogenous microbes (Buckley and Reed, 1994).

To ensure complete sterilization of explants, two step procedure using different sterilizing agents was performed in the laminar flow hood. In the present study, the explants were dipped quickly for about 30 seconds in 70% alcohol and rinsed 3 times in sterilized distilled water since ethanol is a powerful sterilizing agent but also phototoxic. Lubabali (2015) also reported a quick flush using 70% ethanol on greenhouse-grown coffee explants. Sodium hypochlorite (NaOCl), usually purchased as laundry bleach, is the most frequent choice for surface-sterilization. It is readily available and can be diluted to desired concentration. In the present study, 30% NaOCl sterilization for 20 minutes reduced contamination in field-grown explants, although it did not achieve total elimination of contaminants. Generally, contamination levels decreased with exposure time. However, in the present study exposure time of 25 minutes to 20% and 25% NaOCl resulted in higher contamination than exposure time of 20 minutes. This result was attributed to poor handling of the explants.

Generally, occurrence of high contamination rate of cultures at a relatively lower concentration and shorter exposure time is attributed to the insufficiency of sterilant concentration and exposure duration to remove or kill the contaminants, mainly fungi. Hailu et al. (2013) reported that the highest rate of contaminant-free culture (48.33±1.34) was obtained from treatment combinations of 1.5% and 2% NaOCl for 20 min and 25 min exposure duration for *Artemisia annua* shoot tip explants, respectively. For nodal explants, the authors found 1.5% concentration and 20 min exposure time to be the most effective treatment combination with mean average result of 81.7±1.34% contaminant-free cultures. Wetzstein *et al.* (2018) found that 1.2% NaOCl containing a few drops of Tween surfactant for 10 minutes with agitation was effective in sterilizing *Artemisia annua* explants. Our study with 30% NaOCl showed the percentage of clean and surviving explants decrease with exposure time. Longer exposure resulted in scorching of explants. The increase in sterilant concentration and exposure time above certain optimum limit cause loss of explants because of the oxidant chemical ingredient killing the plant tissues as well. Hence, the optimum treatment combination (concentration and time) for effective sterilization of explants should be determined based on the two aspects, namely a relatively minimum level of contamination as well as tissue death that gives the maximum percentage of clean cultures.

4.2. Direct somatic embryogenesis response

Embryo development was not uniform for the Ruiru 11 Sibs; hence the cultures were maintained for 12 months to allow for regeneration of embryos from the slow-forming sibs. Priyono et al. (2010), reported that time needed to observe the first coffee somatic embryo varies from one to over 12 months, depending on the protocol. Some sibs did not produce any embryos after one year in culture. Molina et al. (2002) reported that the variation in embryogenic capacity among *Coffea* genotypes ranges from 4.8% to 72.7%. The present study was in agreement with Kahia et al. (2016), who reported low embryogenic response and mean number of embryos per explant.

The growth of cultured tissues or organs, and morphogenesis *in vitro*, is more influenced by genotype than by any other factor (Elmeer, 2013). Genotype effects may interact with media or environmental conditions (Raimondi et al., 2001). Molina et al. (2002), observed some genotypes respond well, whereas others remained recalcitrant, which suggested that regeneration is genetically controlled. In a study on the effect of genotype and explant age on somatic embryogenesis of coffee, Molina et al. (2002) attributed differences between genotypes to the diverse ancestors, although the lines shared common ancestors during the early generations. Similarly, Aponte (1993) reported differences in the embryogenic response among genotypes of the same cross, but coming from different F2 plants. Cultivar plays a major role in somatic embryogenesis competency, out-weighing other factors such as explant source, donor plant conditions and even media composition (Popelka et al., 2003). In addition, Trolinder and Xhixian (1989) reported that genetic rather than culture components are the most critical factors in obtaining efficient regeneration protocols. Lelu-Walter et al. (2008) reported that the maternal effect was very strong relative to other effects during clonal plant production from self- and cross-pollinated seed families of *Pinus sylvestris* (L.) through somatic embryogenesis.

Sibs tested from female parent 'Catimor 86' namely sibs 11, 41, 71 and 121 regenerated embryos with the exception of sibs 91 and 131 which did not regenerate somatic embryos. Sibs 52 and 142 from female parent 'Catimor 88' did not regenerate somatic embryos at all. Sibs from female parents 'Catimor 90, 128 and 134' regenerated somatic embryos using the existing protocol. Embryogenic potential of Ruiru 11 sibs was affected by difference in genotype and high variability for the induction of somatic embryos was evident with some sibs showing a higher embryogenic potential than others thus reinforcing the hypothesis that production of somatic embryos depends on genotype (Rezende et al., 2011).

As demonstrated by Molina et al. (2002) embryogenic capacity of *Coffea arabica* depends on the genotype, and that the character is fixed at the early generations. In the case of Ruiru 11, the progenies resulting from three-way crossing of the male parents 'SL34, Rume Sudan and Hibrido de Timor' may have had the character responsible for embryogenic capacity fixed. If this is the case, then non-regeneration of somatic embryos in Sibs 91 and 131 may have been due to other factors other than genealogy. Although general methodologies can be established, even closely related varieties can differ in their culture requirement as one sib may undergo regeneration in response to plant growth regulators used, while another sib remains unresponsive until the concentration of growth regulators is changed or a different regulatory compound is added. This could be what was required for Sibs 52 and 142 to generate somatic embryos.

It is commonly recognized that the efficacy of ontogenesis and embryo maturation phases is key to the success of conversion into plantlets. Cellular development and a buildup of storage products occur during embryo maturation, leading to increased size of developed embryos (Merkle et al., 1995) and this can be a good marker for monitoring the germination process. In the present study, germination of somatic embryos in full-strength hormone-free MS (1962) medium for a period of six weeks promoted shoot induction and growth frequencies. Lubabali (2015) also reported that use of full-strength hormone-free MS media promoted germination of somatic embryos in different *Coffea arabica* varieties.

Nonetheless, sibs 11 and 41 did not develop shoots due to desiccation of embryos in the germinating media. This response suggested that any genotype that becomes well-established up to shoot proliferation will probably survive for several months. Similar findings were reported by Niskanen et al. (2004) during a study of somatic embryogenesis in Scot pine, although these authors suggested that survival of embryogenic cultures partly depended on factors related to tissue culture technique manipulations, such as culture period and medium, which may not have been optimal for all the genotypes.

Induction of root formation in the germinated shoots on half-strength MS basal salts augmented with 1 ml/L NAA for six weeks was effective. In a similar study aimed at developing a protocol for somatic embryogenesis of the *C. arabica* 'Ababuna' hybrid, Ahmed et al. (2013) reported that thick roots formed at the bases of shoots on full-strength MS medium containing IBA plus NAA. The results of the present study agreed with Lubabali (2015) who reported that low levels of NAA induced a well-developed root system in different *Coffea arabica* varieties. However, it was noted that production of longer shoots and high percentage of shoot development was not always related to the best root development frequency, as was the case for Sib 100.

5. Conclusion and recommendations

Regeneration of different 'Ruiru 11' Sibs was achieved with the protocol tested, although wide variation in the competence of selected *Coffea arabica* 'Ruiru 11' sibs resulted by use of half-strength Murashige and Skoog (1962) basal salts augmented with thidiazuron. Field-grown explants were effectively sterilized using 30% sodium hypochlorite (3.85 v/v) for 20 minutes. There was genotypic variation among the 'Ruiru 11' sibs tested in this study. There was maternal effect on regeneration of 'Ruiru 11' where sibs 11, 41, 71 and 121 from the female parent 'Catimor 86' developed somatic embryos, whereas sibs 52 and 142 from female parent 'Catimor 88' did not develop somatic embryos. Sib 71 responded best to plantlet regeneration through direct somatic embryogenesis. Therefore, the regenerated plantlets of sib 71 should be used in the future as stocks for mother plants for mass propagation of 'Ruiru 11'. It is worth noting that the described direct somatic embryogenesis protocol requires maintenance of explants in media for a long period of time, almost 12 months to increase the embryo initiation and development. Future research should therefore explore how to hasten embryo initiation and development. In addition, not all sibs respond positively to direct somatic embryogenesis protocols and hence search for effective protocols should continue for recalcitrant sibs.

Acknowledgement

This study was co-financed by the Coffee Research Institute (CRI) and the National Council of Science, Technology and Innovation (NaCoSTI) grant reference number NACOSTI/RCD/ST&I 5th Call PhD/026. The authors thank the technical staff of the Crop Physiology Unit of CRI for their assistance. This paper is published with the permission of the Institute Director of the CRI on behalf of the Director General, Kenya Agricultural and Livestock Research Organization.

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