

Full Length Research Paper

Tissue culture regeneration of three Nigerian cultivars of tomatoes

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The *in-vitro* response of three Nigerian cultivars of tomato - Ibadan local (IbL), Ife and JM94/46 (JM) - were tested using cotyledons as explants for direct shoot regeneration. The cultivars showed variable responses in the two media used. In the medium consisting of an initial culture on pre-culture medium made up of Murashige and Skoog (MS) with 1.0 mg L⁻¹ naphthaleneacetic acid (NAA) and 1.0 mg L⁻¹ 6-benzylaminopurine (BAP) and subsequent culture on shoot regeneration medium consisting of MS with 0.1 mg L⁻¹ thiamine, 0.5 mg L⁻¹ indoleacetic acid (IAA) and 0.5 mg L⁻¹ zeatin. Results showed that 15.65% of the cultured explants of IbL cultivar produced shoots, while Ife and JM cultivars did not produce any shoots. In the second medium consisting of MS with 0.1 mg L⁻¹ IAA and 1.0 mg L⁻¹ zeatin, 64 - 97% of the cultured cotyledon explants from the three cultivars produced shoots. Rooting medium was MS with 0.1 mg L⁻¹ NAA. Statistical analysis of regeneration data using Student Newman Keul's (SNK) mean values for cultivar-medium interaction showed that Ife and JM94/46 were not significantly different (P>0.05) for shooting explants and number of shoots. However, both were significantly different from IbL P<0.05 for shooting explants and number of shoots produced. The least significant difference among the cultivars for shooting explants and number of shoots on the shoot regeneration medium was 0.0005 and 0.001, respectively. SNK mean values for cultivar-media interaction for shooting explants and number of shoots produced were not significantly different (P>0.05) among the three cultivars.

Key words: Nigeria, tomato, tissue culture, regeneration.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is a fleshy vegetable used all over the world. It belongs to the family Solanaceae, and is the world's most widely cultivated vegetable (Villarael, 1980) with an estimated global production of over 120 million metric tons (F.A.O. 2007). Tomato fruits are a significant source of nutrition for substantial portions of the world's human population because this vegetable crop is widely cultivated and consumed extensively as both fresh vegetable and concentrated processed products (Hamner and Maynard,

1942; Beecher, 1998). In tropical Africa, the area used for tomato cultivation is about 300,000 ha, with an estimated annual production of 2.3 million tonnes. Nigeria is the largest producer, accounting for 126,000 ha and an annual production of 879,000 tonnes (Van der Vossen et al., 2004). In developing countries like Nigeria, efficient storage, packaging, transport and handling techniques are practically non-existent with perishable crops (Babalola et al., 2010), resulting in considerable loss of produce. Therefore improvements such as longer shelf-life, resistance to biotic and abiotic stresses, nutrient enhancement, higher soluble solids, etc are desirable in the local cultivars of tomato. However, the introduction of genes that confer these qualities to commercial cultivars by conventional breeding techniques often encounters

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serious difficulties due to high incompatibility barriers to hybridization (Kaul, 1991). To overcome these problems more recent approaches of gene manipulation might be required.

The establishment of an efficient tissue culture protocol is an essential pre-requisite for harnessing the advantage of cell and tissue culture for genetic improvement. Meanwhile, the lack of a good regeneration protocol limits the efficiency or percentage of the transformants obtainable. There is, however, paucity of documented work on the *in-vitro* regeneration of Nigerian cultivars of tomato; such work would provide the background work for the application of genetic engineering in solving these problems. The development of protocols for *in-vitro* selection can provide new advances for the production of stress tolerant cultivars (Bhatia et al., 2004). There is therefore an urgent need to domesticate these technologies for the improvement of Nigerian indigenous cultivars of tomatoes. Efficient plantlet regeneration in tomato was reported from the leaf (Behki and Lesley, 1976; Kartha et al., 1976; Padmanabhan et al., 1974). The success in tomato regeneration response has been found to depend largely on genotype, explant and plant growth regulators used in the culture medium (Praveen and Rama Swamy, 2011).

In vitro regeneration of cultivated tomato (*S. lycopersicum*) has been a subject of research because of the commercial value of the crop and its potential of amenability to further improvement through genetic engineering strategy (Evans, 1989). The choice of cultivars used in this study is based on agronomic studies carried out at the National Institute for Horticultural Research and Training (NIHORT). Ibadan local and Ife cultivars are farmer preferred varieties in the south-western part of Nigeria, which are reported to be resistant to certain diseases and relatively high yielding (Badra et al., 1984; Anno-Nyako and Ladunni, 1984). ICS-Nigeria (Anonymous, 2000) also reported Ife cultivar to be high yielding with fruits and is a determinate bushy plant; and that other local cultivars are fairly resistant to virus, have round and irregularly shaped fruits that are soft and prone to cracking.

MATERIALS AND METHODS

In-vitro regeneration of the tomato cultivars

In-vitro regeneration study of three local cultivars of tomato by shoot organogenesis was carried out in two shoot regeneration media (SRM₁ and SRM₂) using complete randomized design (CRD) in replicated experiments. The seeds of three local cultivars of tomatoes namely Ibadan local, Ife and JM94/46, were obtained from the National Institute for Horticultural Research and Training (NIHORT), Ibadan, and tested for germinability. Different combinations of surface seed sterilization treatments with varying concentration and duration of application of sodium hypochlorite (NaOCl) and 70% ethanol were carried out in triplicates on the tomato seeds. Optimum sterilization protocol for seeds of the tomato cultivars was treatment with NaOCl (3.5%) for 20 min without an ethanol treatment. A drop of Tween 20 was added and

the seeds were rinsed with sterile distilled water at least three times.

Media and sterilization

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) was prepared and adjusted to pH 5.8, filled into culture bottles and sterilized by autoclaving at 121°C at 15 psi for 15 min. The efficiency of sterilization was ascertained using Bowie-Dick autoclave tape which changed from blue to white. About 50-100 ml of the medium was dispensed into each previously sterilized culture jar while still hot under the laminar flow hood and allowed to cool. The seeds were inoculated onto sterile, cooled and solidified germination medium (GM). The various media used are:

Germination medium (GM) = MS + 30 g L⁻¹ sucrose + 8 g L⁻¹ agar gel (Cortina et al., 2004)

SRM₁ consisted of an initial culture in preculture medium for two days

Preculture medium (PM) = MS + 30 g L⁻¹ sucrose + 8 g L⁻¹ agar gel + 1 mg L⁻¹ naphthaleneacetic acid (NAA) + 1 mg L⁻¹ 6-benzylaminopurine (BAP) (Cortina et al., 2004)

SRM₁ = MS + 30 g L⁻¹ sucrose + 8 g L⁻¹ agar gel + 0.1 mg L⁻¹ thiamine + 0.5 mg L⁻¹ indoleacetic acid (IAA) + 0.5 mg L⁻¹ zeatin (Cortina et al., 2004)

SRM₂ = MS + 0.1 mg L⁻¹ IAA + 1 mg L⁻¹ zeatin + 8 g L⁻¹ agar gel (Tingting et al., 2005)

Rooting medium (RM) = MS + 15 g L⁻¹ sucrose + 8 g L⁻¹ agar gel + 0.1 mg L⁻¹ NAA (Davis et al., 1994)

In-vitro tomato seed germination

In-vitro tomato seedlings were produced according to the methods described by Cortina et al. (2004). Surface-sterilized tomato seeds were inoculated on germination medium (GM). Each culture jar containing GM was inoculated with ten sterilized seeds and were placed in the dark at 25 ± 2°C for 3 - 5 days to germinate and then brought to light for a 16 h photoperiod with light intensity of 1500 lux for 7 - 10 days at the same temperature.

Regeneration through direct shoot organogenesis

Cotyledon explants (5 × 5 mm²) from 10 - 13 days old *in-vitro* seedlings of tomato seedlings were cut off, the tips removed and sectioned transversely with a sterile scalpel. Fifteen pieces of cotyledon explants from each cultivar were cultured per Petri dish in triplicates. Shoot regeneration was established in SRM₁, which consisted of an initial culture in preculture medium (PM) for 2 days before being transferred to SRM₁ (Cortina et al., 2004) and in SRM₂ with the explant pieces placed upside down. After about 3 - 4 weeks in the shoot regeneration media, regenerated shoots were subcultured into modified rooting medium (RM) and after 10 days in RM, rooted plants were transferred to the screen house for hardening.

Seedling establishment

According to methods described by Raj et al. (2005), rooted plants were transferred to hardening medium consisting of coconut fibre

Table 1. Regeneration data in SRM₁.

Cultivar	No. of explant	Survived explant	% survival	Mean survival ± S.E.	Shoot explant	% Shoot explant	Mean shoot explant ± S.E.	No. shoots	Average shoot /explant
IbL	45	37	82.22	0.822 ± 0.06	7	15.65	0.156 ± 0.051	15	2.14
Ife	45	39	86.67	0.867 ± 0.05	0	0	0	0	0
JM	45	33	73.33	0.733 ± 0.07	0	0	0	0	0

Explant type: cotyledon.

Table 2. Regeneration data in SRM₂.

Cultivar	No. of explant	Survived explant	% survival	Mean survival ± S.E.	Shoot explant	% Shoot explant	Mean shoot explant ± S.E.	No. shoots	Average shoot /explant
IbL	45	44	97.78	0.978 ± 0.022	44	97.78	0.978 ± 0.022	125	2.84
Ife	45	41	91.11	0.911 ± 0.043	37	82.22	0.822 ± 0.058	134	3.62
JM	45	36	80.0	0.8 ± 0.060	29	64.44	0.644 ± 0.072	97	3.35

Explant type: cotyledon.

Table 3. SNK test for mean values in SRM₁ - cultivar*media.

Cultivar	IbL	Ife	JM
Survive	0.822 ^a	0.867 ^a	0.733 ^a
shoot explant	0.156 ^a	0 ^b	0 ^b
Shoots	0.333 ^a	0 ^b	0 ^b

Mean values with same letters on the same row are not significantly different at $\alpha = 0.05$.

pellets (peat) that were soaked in water for about 3 h to loosen up and vermiculite was added to make the medium sturdy enough to keep the plants upright. The mixture was poured into polythene bags and the rooting plants from RM were transferred into the medium. They were kept in humidity chamber for 2 weeks before planting in soil, still under humidity chamber and gradually acclimatized in the screen house environment.

Statistical analysis of regeneration data

Pooled data from the regeneration experiments was subjected to analysis of variance (ANOVA) using the general linear model (GLM) procedure of statistical analysis software (SAS Institute, Cary, NC). Least significant difference (LSD) test was based on Student Newman Keuls' (SNK) procedure after a significant F-test in the analysis of variance.

RESULTS AND DISCUSSION

Sterilisation and *in vitro* germination of tomato seeds

Sterilization treatment with 2% hypochlorite for 10 min, 70% ethanol for 1 min and 3.5% hypochlorite for 20 min without the ethanol treatment gave optimum sterilization

of the explants. Cultivar JM germinated 3 days after planting (d.a.p.), while Ife and IbL germinated from 5 d.a.p.

Direct shoot regeneration

Only 15.65% of the cultured IbL cultivar explants produced shoots in SRM₁ (Table 1) with average shoots per explant of 2.14. Ife and JM cultivars did not produce any shoots from the cotyledon explants in SRM₁. However, all the cultivars survived in the media for the eight week period they were on SRM₁ at 82.22, 86.67 and 73.33% for IbL, Ife and JM cultivars respectively (Figure 1). In SRM₂, 64 - 97% of cultured explants produced shoots across the three cultivars (Table 2). The highest number of shooting explants was observed in IbL (97.78%) and the highest average shoot per explant was recorded in cultivar Ife at 3.62. Higher percentage survival was also recorded in SRM₂ for all cultivar types. Student-Newman-Keuls (SNK) mean values for cultivar-medium interaction in SRM₁ (Table 3) showed there was no significant difference in the survival rate among the three cultivars ($P > 0.05$); while Ife and JM were not significantly different ($P > 0.05$) for shooting explants and number of shoots. They were both significantly different from IbL $P < 0.05$ for shooting explants and number of shoots produced. The least significant difference among the cultivars for survival, shooting explants and number of shoots on SRM was 0.268, 0.0005 and 0.001 respectively. Furthermore, the SNK values for cultivar-medium interaction in SRM₂ (Table 4) showed there was no significant difference ($P > 0.05$) among the three cultivars for number of calli, shooting explants and number of shoots per explants.

Table 4. SNK test for mean values in SRM₂ - cultivar*media.

Cultivar	IbL	Ife	JM
No calli	0.833 ^a	0.811 ^a	0.878 ^a
shoot calli	0.644 ^a	0.644 ^a	0.711 ^a
Shoots	1.711 ^a	2.011 ^a	2.156 ^a

Mean values with same letters on the same row are not significantly different at $\alpha = 0.05$.

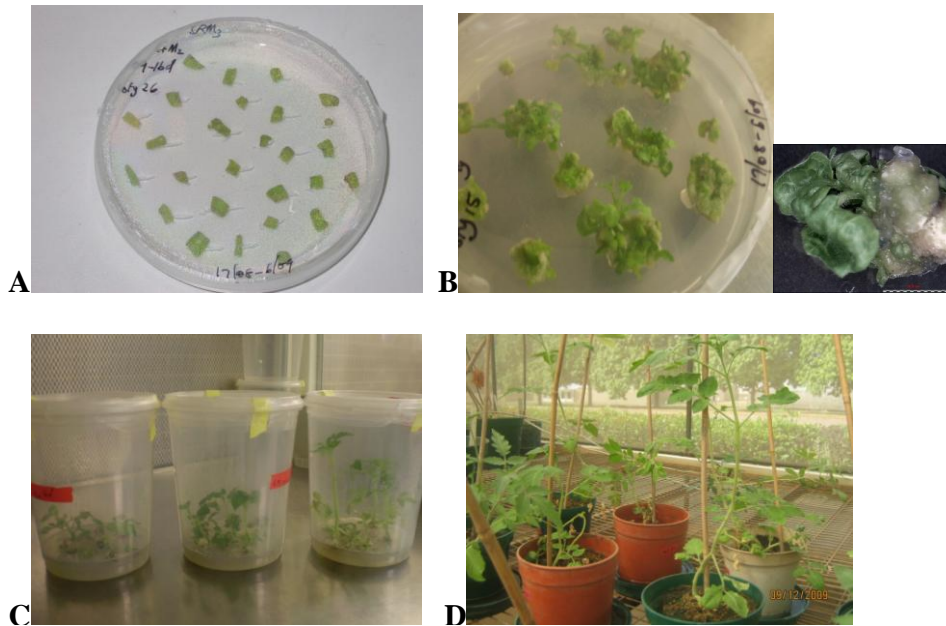


Figure 1. (A) Cotyledon explants - day 0; (B) cotyledon explants in SRM at 6 weeks (inset: close shot of shooting explants); (C) regenerated tomato seedlings in RM – 8 weeks; (D) rooted tissue culture tomato plants in top soil in screen house (4 months).

Rooting and hardening of regenerated shoots

Shoots were ready for rooting after 4 - 6 weeks in shoot regeneration media. The regenerated shoots were transferred to RM (Sun et al., 2006) without exogenous hormones. Regenerated shoots did not produce roots after three weeks in the RM. Mensuali-Sodi et al. (1995) also reported that tomato does not usually require any plant growth regulators (PGR) for rooting. The addition of 0.1 mg L⁻¹ of NAA to the RM however resulted in the development of roots after 10 days in the modified RM. Davis et al. (1994) and Compton and Veilleux (1991) reported the use of NAA at concentrations of 0.1 and 0.02 mg L⁻¹, respectively to induce rooting from tomato leaf and hypocotyl explants.

In tomato, adventitious shoot regeneration can be achieved either directly (Dwivedi et al., 1990) or indirectly through an intermediate callus phase (Behki and Lesley, 1980; Geetha et al., 1998). Bhatia (2003) reported that both callus and shoots may be produced together. The *in-vitro* morphogenic responses of cultured plant tissues

are affected by the different components of the culture media, especially by concentration of growth hormones as well as genotype, explant type and age. SRM₁ with 0.5 mg L⁻¹ zeatin did not support shoot induction in the cultivars/explants. BAP and IAA at the concentrations used in the PM did not also improve shoot regeneration in the cotyledon explants. However, in SRM₂ with concentrations of 0.1 mg L⁻¹ IAA and 1.0 mg L⁻¹ zeatin shoot regeneration of 64 - 97% of cultivated cotyledon explants was recorded across the three cultivars. Godishala et al. (2012) and Ting-ting et al. (2005) also reported that 1.0 mg L⁻¹ zeatin enhanced adventitious shoot regeneration in tomatoes. In addition, Jozef et al. (2004) reported that the addition of growth regulators in media influenced the number of shoots regenerated from tomato cotyledons.

Although previous studies demonstrated that 8 to 10 day-old cotyledons of tomato were superior to other source of explants, including hypocotyls, stems and leaves for promoting shoot organogenesis of tomato (Pino et al., 2010; Hamza and Chupeau, 1993; Van

Roekel et al., 1993; Ling et al., 1998), in these experiments 10 - 13 day-old cotyledons were used successfully as source of explants. Cortina et al. (2004) reported the effective use of 10 - 13 day-old cotyledons as source of explants. Godishala et al. (2012) also reported the successful use of 10 - 12 day-old tomato cotyledon as explants for tomato regeneration. Moreover, Zhang et al. (2012) reported that the most ideal explant for tomato *in-vitro* regeneration is the cotyledon. Nogueira et al. (2001) also observed high regeneration frequency 92 or 85% on cotyledonary explants of genotype Santa Clara or its natural mutant Firme, respectively, which corroborates the results obtained for IbL and lfe in SRM₂.

Based on the results of this study, SRM₂ is better suited for the *in-vitro* regeneration of the local tomato cultivars than SRM₁. This is probably due to the higher concentration of zeatin in SRM₂. Zeatin has been reported to support shoot regeneration in *in-vitro* tomato cultures (Pino et al., 2010). The results of these experiments in the two different media confirm that *in-vitro* regeneration responses in tomatoes are dependent on composition of media, explant and cultivar type. Also 10 - 13 day-old cotyledon explants are suitable for *in-vitro* regeneration of the three local cultivars.

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