Full Length Research Paper

Surface sterilant effect on the regeneration efficiency from cotyledon explants of groundnut (*Arachis hypogea* L.) varieties adapted to eastern and Southern Africa

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Five groundnut (*Arachis hypogea* L.) varieties - ICGV-12991, ICGV-99568, ICGV-90704, CG-2 and Chalimbana - that are adapted to Eastern and Southern Africa were compared to variety JL 24 for their regeneration response in tissue culture. Sodium hypochlorite and mercuric chloride were compared for efficiency as sterilizing agents and subsequent effect on regeneration. All five varieties formed shoot buds that elongated well on shoot elongation medium. ICGV-90704 and Chalimbana performed better than the other three varieties for shoot organogenesis although all varieties produced healthy rooted plants *in vitro* that were successfully transferred to the greenhouse where they exhibited normal growth, flowering and seed set. Both sterilizing agents were suitable, but mercuric chloride was less harmful than sodium hypochlorite. This study established a basis for genetic engineering activities on African groundnuts in the future.

Key words: Groundnuts, regeneration, genotype independent, Eastern and Southern Africa.

INTRODUCTION

Groundnut (*Arachis hypogea* L.) is an oil, food and fodder crop which plays an important role in the agricultural economies of countries of the semi-arid tropics. It contributes significantly to food security and alleviates poverty (Naidu et al., 1999) and as a legume, improves soil fertility by fixing nitrogen and increases productivity for smallholder farmers of the semi-arid cereal cropping systems (Giller et al., 2002; Smatt, 1994).

Several diseases and pests cause serious losses of groundnuts in Africa. The most important post-harvest constraint of groundnut is aflatoxins. Invasion of seeds by toxigenic strains of *Aspergilllus flavus* and consequent aflatoxin contamination is a serious problem in most countries where groundnuts are cultivated (Mehan and McDonald, 1984; Mehan et al., 1991). The most destructtive virus disease that affects groundnuts in Africa is the groundnut rosette disease of which outbreaks is sporadic and unpredictable, but can result in yield losses of up to 80% (Subrahmanyam et al., 2001). Current methods to control pests and diseases usually entail the use of chemicals which are unaffordable and often unavailable to the resource-poor farmers in Africa. Conventional breeding programs aimed at conferring genetic resistance have had limited success to date and more sources of

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Abbreviations: SIM, shoot induction medium; **BA,** benzylaminopurine; **2, 4-D,** 2, 4-dichlorophenoxyacetic acid; **SEM,** shoot elongation medium; **RIM,** root induction medium; **NAA,** α-naphthalene acetic acid.

resistance in the groundnut germplasm need to be identified (Naidu et al., 1999). The International Crops Research Institute for the Semi Arid Tropics (ICRISAT) has identified some wild relatives of groundnuts with rosette resistance (www.icrisat.org/gt-bt/ResearchBriefs/rgrdiwas.htm). However, most of the resistant varieties released to date, require a long growing season (150 - 180 days), exposing them to terminal drought (Subrahmanyam et al., 2001).

Genetic engineering provides unique possibilities to introduce foreign DNA into plant cells to alleviate the above constraints (Torres, 1989; Sharma and Ortiz, 2000). A number of tissue culture protocols and transformation systems from diverse explants of groundnuts have been published of which the ones that report direct organogenesis proved to be the most promising for genetic transformation (Cheng et al., 1992; Li et al., 1994; Kanyand et al., 1997; Venkatchalam et al., 1999; Bhatnagar-Mathur et al., 2008). In 2000, Sharma and Anjaiah reported a genetic transformation protocol based on direct organogenesis from cotyledon explants of mature seeds of groundnut. This protocol was shown to work well with six cultivars cultivated in India and was successfully applied to variety JL 24 to engineer resistance to the Indian peanut clump virus by introducing the coat protein gene. The potential of this development to genetically transform groundnut to obtain resistance to viruses and other biotic and abiotic constraints can positively impact groundnut productivity, especially in the resource poor agricultural systems of the semi-arid tropics (Sharma and Anjaiah, 2000; Bhatnagar-Mathur et al., 2007).

To date, reported tissue culture and genetic engineering research in groundnut have been carried out in India and the USA. To prepare the way for genetic engineering for the improvement of groundnuts in Africa, we evaluated the regeneration response from five varieties that are adapted to a wide range of environments in Eastern and Southern Africa using the method of Sharma and Anjaiah (2000) to determine if this protocol is genotype-independent and applicable to African groundnuts.

A successful tissue culture protocol starts with effective explant sterilization (Dodds and Roberts, 1985). In this study a simple and fast protocol using commercial bleach (sodium hypochlorite, NaOCl) was evaluated for explant sterilization and in vitro establishment in comparison to mercuric chloride ($HgCl_2$) which is mostly used in reported groundnut tissue culture studies (Cheng et al., 1992; Li et al., 1994; Kanyand et al., 1997; Venkatchalam et al., 1999; Sharma and Anjaiah, 2000). Bleach is affordable and widely available in shops and supermarkets, including in developing countries. Compared to $HgCl_2$, it is less toxic and does not require special handling and waste-disposal precau-tions, making it a safer option for both researchers and the environment. Optimization of this protocol was an important aspect in this study to ensure that large numbers of clean explants survived sterilization.

MATERIALS AND METHODS

Mature seeds of ICGV-12991, ICGV-99568, ICGV-90704, CG-2 and Chalimbana (Monyo, 2008) were used as starting material and JL 24 was included as control. All seeds were obtained from the ICRISAT breeding station in Malawi except for those of variety CG-2 which was obtained through Leldet (Ltd.) Seed Company from farmers' fields in Kenya.

Surface sterilization entailed rinsing seeds in 70% ethanol (1 min) followed by treatment with various combinations of different durations (5, 10, 15, 30 min and overnight) and concentrations of NaOCl (3.5% (w/v) in commercial bleach) ranging from 0.018, 0.35, 0.53, 0.70 to 1.05% (w/v) NaOCl. This was compared to sterilization with 0.1% (w/v) aqueous mercuric chloride ($HgCl_2$) for 8 min (Sharma and Anjaiah, 2000). All treatments included a few drops of Tween 20. Sterilization was followed by thorough washes with sterile water and subsequent soaking for 2 h before use.

Explant preparation and in vitro culture was performed according to Sharma and Anjaiah (2000). Cotyledon explants were cultured on shoot induction medium (SIM) containing MS salts (Murashige and Skoog, 1962) supplemented with B5 vitamins (Gamborg et al., 1968), 20 µM benzylaminopurine (BA), 10 µM 2,4-dichlorophen-oxyacetic acid (2,4-D) and 3% (w/v) sucrose at pH 5.8 and 0.8% (w/v) agar. Thirty five explants, 5 per plate, were cultured per treatment at 26 ± 1℃ and 16 h light/8 h dark cycle. Shoot buds were transferred after 14 to 28 days to shoot elongation medium (SEM) containing MS salts, B5 vitamins, 2 µM BA, 3% (w/v) sucrose and 0.8% (w/v) agar at pH 5.8 for two to three passages of 4 weeks each. Elongated shoots, 3 - 5 cm long, were transferred to root induction medium (RIM) containing MS salts, B5 vitamins, 5 µM α-naphthalene acetic acid (NAA), 3% (w/v) sucrose and 0.8% (w/v) agar at pH 5.8 for 28 days. Rooted plants that were 6 to 8 cm tall were transplanted to sand: soil (1:1) in a greenhouse and gradually hardened over 10 days. The plants were routinely irrigated with tap water and maintained for harvesting the seeds. Data were analysed with analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Explant preparation, sterilization and establishment in vitro

For the optimization of sterilization of explants and in vitro establishment, the responses measured were (i) no reaction (explants that showed no physical damage after cultivation on SIM), (ii) survival (explants turning green, producing callus or developing shoot buds), and (iii) pathogen contamination. Greening of explants and callus formation indicated survival of the explants; however, the type of callus produced was important as shoot buds formed mainly from hard, compact callus and not from white, soft callus.

ANOVA revealed significant differences among the numbers of explants that survived the various NaOCl treatments. Exposure time also affected the ability of the explants to survive. However, there was no significant difference in the number of explants that survived among varieties (results not shown) which indicated that survival rates after each treatment was not dependent on the
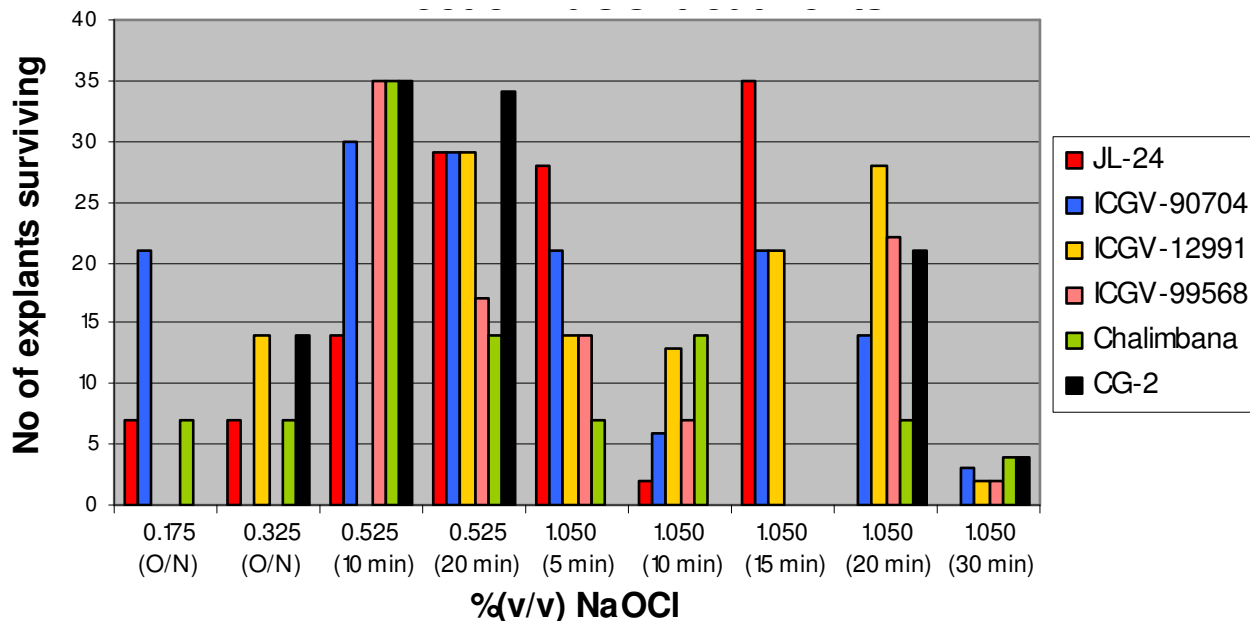
Figure 1. Effect of NaOCl treatment on explant survival.

genotype.

Using NaOCl, all six varieties responded well compared to the JL 24 control. The optimal concentration for each variety was selected based on explants that showed the highest rate of survival and had the least contamination. There was a significant difference in the number of explants that survived the various NaOCl treatments as shown in Figure 1. The best treatments ranged between 0.525 and 1.050% of NaOCl for 10 to 20 min, indicating efficient sterilization without excessive damage to the explants (Davies and Dale, 1979; Dodds and Roberts, 1985). Explants of CG-2 contaminated routinely regard-less of the sterilizing treatment used which necessitated the use of a second sterilization step after removal of the seed coat with 0.175% NaOCl for 5 min, which worked well. This persistent contamination could be ascribed to the source of the seed, which were obtained from farmer fields where few pest control measures were taken, while the other five varieties were obtained from the ICRISAT breeding station in Malawi where they undergo rigorous plant husbandry (Monyo, 2008).

Based on these results, the two best NaOCl treatments for each variety were repeated. The optimal treatment for each variety was (i) ICGV-12991; 1.050% applied for 20 min, (ii) ICGV-90704; 0.525% for 10 min, (iii) JL-24; 1.050% for 15 min, (iv) ICGV- 99568; 1.050% for 20 min, (v) Chalimbana; 0.525% for 10 min and (vi) CG-2; 1.050% for 20 min. Sterilization worked best for ICGV-99568 (73% survival at 1.050%) and was worst for ICGV-90704 (no survival at 0.175%) as summarized in Table 1. These results are in agreement with that of Khosh-Khui and Sink (1982) who reported that for most legume species, the effective NaOCl concentration ranged from

0.350 - 1.050% for 10 - 20 min.

In comparison, explant survival rates from all varieties were generally higher following $HgCl_2$ sterilization ranging from 82% for CG-2 to 100% for Chalimbana (Table 1). This result was confirmed when explants from the same variety were subjected to both treatments on the same date and subsequently cultivated on SIM. Results showed that $HgCl_2$ allowed better initiation of regeneration than NaOCl as illustrated in Figure 2B.

Cultivation of explants

The best regeneration response was obtained with variety JL 24 (used as control variety in this study), regardless of the sterilizing agent used. All other varieties, with the exception of CG-2, responded better when $HgCl_2$ was used for sterilization. It was less damaging to the explants and allowed better survival and subsequent shoot bud regeneration as was also reported previously by Christianson and Warnick (1988). Although ICGV-90704 and Chalimbana responded almost as well as JL 24, successful regeneration was accomplished for all the tested varieties regardless of which chemical was used for sterilization. In most experiments, CG-2 did not perform well due to contamination but when this was improved through a second seed sterilization step, this variety was also highly regenerable.

Shoot buds formed at the proximal cut end (where the embryo was removed, Figure 2A) of the surviving explants (Figure 2C) within 14 to 28 days of cultivation on SIM in all tested varieties. After about 4 weeks, the well-formed shoots were removed from the cotyledon explants and

Table 1. Explant contamination and survival rates following the two best treatments with NaOCl as well as 0.1% $HgCl_2$.

Variety	Treatment	Experiment 1		Experiment 2*	
		% survival	% contamination	% surviving explants with shoots	No. of regenerated plants (± SD)
ICGV-12991	0.525%NaOCl; 20 min	40	3	N/D	N/D
	1.050%NaOCl; 20 min	66	31	6	10 ± 3
	0.1% $HgCl_2$	89	0	**16**	31 ± 6
ICGV-90704	0.175%NaOCl; O/N	0	40	N/D	N/D
	0.525%NaOCl; 10 min	60	6	12	12 ± 3
	0.1% $HgCl_2$	94	0	**39**	39 ± 5
JL 24	0.525%NaOCl; 20 min	40	17	N/D	N/D
	1.050%NaOCl; 15 min	60	0	**29**	43 ± 26
	0.1% $HgCl_2$	94	0	28	40 ± 6
ICGV-99568	0.525%NaOCl; 10 min	9	20	N/D	N/D
	1.050%NaOCl; 20 min	73	17	7	15 ± 4
	0.1% $HgCl_2$	94	0	**17**	24 ± 5
Chalimbana	1.050%NaOCl; 10 min	23	0	N/D	N/D
	0.525%NaOCl; 20 min	63	0	2	15 ± 5
	0.1% $HgCl_2$	100	0	**35**	24 ± 6
CG-2	0.525%; 10 min	34	0	N/D	N/D
	1.050%NaOCl; 20 min	57	3	**29**	19 ± 5
	0.1% $HgCl_2$	82	0	20	23 ± 5

Bold figures indicate the optimal NaOCl treatment for each variety from the first experiment that was used in subsequent regeneration experiments. The last two columns indicate explant survival and regeneration. *Results are the average of 3 experiments.

transferred to SEM (Figure 2D) where they continued to elongate and often formed clusters of multiple shoots. On average, 4 to 8 shoots were recovered from each explant, although more than 10 shoots could be recovered from explants that were sub-cultured for 2 to 3 additional cycles of 2 to 4 weeks on SEM. In a number of cases, the healthy shoots were also contaminated in SEM, probably due to latent endogenous contaminants. Elongated shoots of about 3 cm were transferred to RIM (Figure 2E), where healthy roots developed within 14 days in all the tested varieties. Rooted plants acclimatized well within 10 days of transplanting from test tubes (Figures 2F and G), appeared phenotypically normal and produced normal flowers within two months followed by pods with healthy seeds within four months (Figure 2H).

Throughout the study, single explants produced shoot buds from the proximal cut end of the cotyledon explants in accordance to what was previously reported as the area with highest regeneration potential (Guerra and Handro, 1988) due to the presence of highly meristematic cells. Similar results were also reported by Hisajima (1982) who found that up to 10 million shoots of almond species could be obtained from a single seed explant within a year after several subcultures. This type of response has been initiated from the seeds of many species, particularly legumes (Vasanth et al., 2004).

In the study presented here, the most challenging step for the successful shoot regeneration was the shoot initiation from individual explants. Once shoot buds had formed, these could be nurtured into strong elongated shoots that rooted without problems.

Conclusion

The regeneration protocol described by Sharma and Anjaiah (2000) is also suitable for groundnut varieties adapted to Eastern and Southern Africa. All the varieties tested regenerated well from cotyledon explants upon *in vitro* culture. $HgCl_2$ was found to be more effective as sterilizing agent compared to NaOCl. However, efficient regeneration was also achieved following sterilization with commercial bleach, which can therefore be used for tissue culture purposes, especially when $HgCl_2$ is not available or when appropriate disposal facilities for mercury-containing waste products are not in place. This cotyledon regeneration system can be applied to obtain large numbers of groundnut plants over a relatively short period of 7 to 9 months. Shoot formation from all varieties was rapid and prolific and developed into normal fertile

Figure 2. Regeneration of multiple shoots and plants from the cotyledon explants of groundnut. **A**) Preparation of cotyledon explants for *in vitro* culture. Aseptic removal of the embryo from a surface sterilized, uncoated seed of ICGV 90704. **B**) Comparison of NaOCl and HgCl$_2$ surface sterilization on the regeneration response in JL 24 after 14 days in culture. **C**) Shoot bud formation at the proximal end of explants of ICGV-99568 within 28 days of cultivation on SIM. **D**) Elongated shoots from two different varieties in SEM. **E**) Rooted plants of all 5 varieties used in this study on RIM. **F** and **G**) Acclimatized plants growing in the greenhouse and outside in soil. **H**) Mature pods harvested 4 months after hardening. About 30 - 40 seeds were recovered from each plant.

plants. Hence, the protocol is genotype independent and any of the varieties tested in this study will be suitable for future transformation studies with African groundnuts.

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