African Journal of Biotechnology Vol. 11(84), pp. 14982-14987, 18 October, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.2249
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Development of an efficient plant regeneration protocol for sweet potato (*Ipomoea batatas* L.) cv. Blesbok

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Accepted 14 September, 2012

An efficient and reproducible plant regeneration protocol for the South African sweet potato (*Ipomoea batatas* Lam.) cultivar Blesbok was developed in this study. The effect of different hormone combinations and type of explant on shoot regeneration was evaluated in order to optimize the regeneration protocol. Explants in the form of stem sections, leaf discs, apical shoots and axillary buds derived from *in vitro* stock plant cultures were cultured on Murashige and Skoog (MS) media supplemented with 36 combinations of naphthalene acetic acid (NAA) (0, 0.01, 0.1, 0.2, 0.5, and 1 mg/L) and 6-benzylaminopurine (BAP) (0, 0.01, 0.1, 0.2, 0.5 and 1 mg/L). The highest percentage of shoot regeneration was obtained when apical shoot explants (31%) and axillary bud explants (22%) were cultivated on MS supplemented with 0.01 mg/L NAA + 1 mg/L BAP. Leaf discs and stem section explants produced highly recalcitrant callus that did not regenerate into shoots in shoot induction medium (SIM). Callus from apical shoots explants cultured on SIM developed into shoots. The shoots rooted readily on root induction medium (RIM) and then in hormone free MS medium. Regenerated plants appeared normal and showed a 100% survival rate when transferred to soil. The regeneration protocol described in this study will be used in a plant transformation protocol to produce transgenic sweet potato with broad virus resistance.

Key words: Tissue culture, regeneration, sweet potato, genetic transformation.

INTRODUCTION

Crop improvement using transgenic approaches is dependent on the development of an efficient and reproducible plant transformation protocol (Liu and Cantliffe, 1984). The ability to regenerate a whole plant from isolated plant cells or tissues which have been genetically transformed underpins most plant transformation systems. Therefore, tissue culture and plant regeneration are integral parts of most plant transformation strategies, and can often prove to be the most challenging and critical aspects of a plant transformation protocol. The basis of the success of integrating plant tissue culture into a plant transformation protocol is the development of a rapid and efficient plant regeneration protocol that delivers a high frequency of regenerable cells that are accessible to gene transfer (Altman and Hasegawa, 2012).

In broad terms, somatic embryogenesis and organogenesis are the two methods of plant regeneration that are widely used in plant transformation studies. In somatic embryogenesis, embryo-like structures are formed either directly or indirectly from somatic tissues. These somatic embryos can develop into whole plants. In direct somatic embryogenesis, the embryo is formed directly from a cell or group of cells without the production of an unorganized, growing and dividing mass of cells known as callus (Thorpe, 1994). However, in the more commonly occurring indirect somatic embryogenesis, embryos are produced from callus tissue or from a cell suspension derived from that callus. Organogenesis does not rely on embryo germination for plant regeneration. Instead, adventitious organs are produced either directly

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from an explant or from a callus culture (Altman and Hasegawa, 2012).

The tendency for organogenesis or somatic embryogenesis to occur in culture tends to be species specific and plant growth regulators (PGRs) play an important role in these two processes (Thorpe, 1994). The addition of PGRs as media components is critical in determining the developmental pathway of the plant cells. The PGRs used most commonly are plant hormones or their synthetic analogues. Auxins and cytokinins are the most frequently used PGRs in plant regeneration and when used in combination, the ratio of auxin to cytokinin dictates the type of culture established or regenerated. In general, a high auxin to cytokinin ratio favours root formation, whereas a low auxin to cytokinin ratio favours shoot formation. An intermediate ratio favours callus production. In the regeneration of sweet potato, the correct hormone combination ratio and source of plant part are critical factors to consider in obtaining high regeneration efficiency (Sihachakr et al., 1997; Gaba, 2005).

Studies on the regeneration of sweet potato have predominately focused on the somatic embryogenesis method for plant regeneration (Feng et al., 2011). To date, sweet potato somatic embryogenesis has been documented using different explants including anthers (Tsay and Tseng, 1979), leaves (Liu and Cantliffe, 1984), petioles (Zheng et al., 1996), shoots (Liu and Cantliffe, 1984), buds (Sihachakr et al., 1997) and meristems (Liu and Cantliffe, 1984; Cheé and Cantliffe, 1988; Liu et al., 2001). However, the production of responsive embryogenic tissues is a difficult task that requires considerable time. Once produced, high quality embryogenic tissues must be proliferated and maintained by frequent subculture without loss of their morphogenetic potential (Cheé and Cantliffe, 1988; Liu et al., 2001; Feng et al., 2011). In addition, production of suitable somatic embryogenic tissues is very commonly cultivar-specific and many sweet potato cultivars were found to be still recalcitrant (González et al., 2008; Feng et al., 2011). Due to these difficulties, a few studies have focused on organogenesis in sweet potato regeneration with some success (Morán et al., 1998; Luo et al., 2006; González et al., 2008).

In general, a number of reports have been published on the regeneration of sweet potato, using various tissues from different cultivars with varying levels of reproducibility and efficiency. However, there is a severe limitation of genotypes for regeneration and efficiency on the whole is still relatively low (Otani and Shimada, 2002). In addition, sweet potato is considered highly recalcitrant to regeneration and transformation and this has limited its application in genetic engineering technologies (González et al., 2008). An efficient and practicable regeneration method of sweet potato is still needed for the plant transformation to meet the requirements of effective genetic improvement of this crop. In this study, an efficient and reproducible method for the regeneration of a South African sweet potato cv. Blesbok is reported. The effect of different hormone combinations and explant types on shoot regeneration efficiency was studied in order to determine an efficient protocol for optimal plant regeneration.

MATERIALS AND METHODS

Sweet potato cultivar 'Blesbok', a popular variety in South Africa (SA), was obtained from Dr. P. Shanahan (Plant Breeder, Department of Horticultural Sciences, School of Agribusiness and Agricultural Sciences, University of KwaZulu-Natal, Scottsville 3209, SA) and used in this study. Vine cuttings of plants were potted in sterilized potting medium and maintained at 25°C in an insect-proof greenhouse in the Controlled Environment Research Unit (CERU) at the University of KwaZulu-Natal (UKZN-PMB). These plants were fertilized weekly with 3:1:3 (nitrogen: phosphate: potassium) and used as stock plants for the establishment of virus-free *in vitro* plantlets. Stock plants were maintained in the greenhouse for up to one year and were kept in highly vegetative state by constantly cutting back of stems.

Establishment of *in vitro* plantlets

To establish *in vitro* cultures, shoot tips 5 cm in length were excised from the greenhouse-grown stock plants and rinsed in sterile distilled water. The shoot tips were cut down to 3 cm in length and immersed in 70% (v/v) ethanol for 2 min. Shoots were surface-sterilized in 5% (v/v) sodium hypochlorite solution for 20 min and then rinsed three times in sterile distilled water. They were cultured in 9 cm Petri dishes containing 25 ml of shoot induction medium (SIM) consisting of a basic medium (BM) supplemented with 1 mg/L 6-benzylaminopurine (BAP, Sigma-Aldrich, UK) and 0.01 mg/L naphthalene acetic acid (NAA, Sigma-Aldrich, UK). The BM consisted of Murashige and Skoog (1962) inorganic medium (MS, Sigma-Aldrich, UK) supplemented with 30% (w/v) sucrose, myoinositol (100 mg/L), thiamine (0.1 mg/L), pyroxidine (0.5 mg/L), nicotinitic acid (0.5 mg/L), glycine (2 mg/L) and 0.6% (w/v) agar. The pH was adjusted to 5.8 with NaOH prior to autoclaving at 121°C for 20 min. The cultures were grown in a growth room at 25 +/- 2°C under a 16 h photoperiod with a light intensity of 54 $\mu Es^{-1}m^{-2}$ provided by white fluorescent tubes. After three to four weeks, excess callus was removed and the elongating shoot tips were cultured in glass culture vessels (Sigma-Aldrich, UK) containing 50 ml of root induction medium (RIM) supplemented with 1 mg/L NAA for root development. After two weeks, plantlets were singly cultured in Magenta GA7 vessels (Sigma-Aldrich, UK), each containing 100 ml of hormone-free BM. These *in vitro* plantlets were maintained in the same environmental conditions as the starting dishes and were used to provide axenic explant material for propagation and subsequent studies.

Explant preparation and culture

For rapid and efficient shoot regeneration of sweet potato cv Blesbok, an optimum hormone combination and explant type was determined. In this experiment, four different explant types (leaf discs, stem portions, axillary buds and apical tips) were subjected to 36 different hormone concentrations of auxin (NAA) and cytokinin (BAP).

Leaf (lamina with petiole, 1 to 1.5 cm), stem (3 cm), axillary buds (stem with bud, 2 cm) and apical shoots (2 cm) were excised from

the apical portions of five week old *in vitro* stock plants and surface sterilized as previously mentioned. Leaf explants were cut into 1 cm^2 discs and stem portions were cut transversely into 1 cm sections. Shoot tips from apical shoots and axillary buds were trimmed to 3 to 5 mm long with four to five primordia. Stem sections, leaf discs, apical shoots and axillary buds were cultured in 9 cm Petri dishes containing 25 ml BM supplemented with 36 hormone combinations of NAA (0, 0.01, 0.1, 0.2, 0.5, and 1 mg/L) and BAP (0, 0.01, 0.1, 0.2, 0.5, and 1 mg/L) and grown in the growth room under the conditions described above. After 3 to 4 weeks of culture, emerging shoots were counted and the percentage shoot generation was computed as the proportion of explants showing shoots, multiplied by 100.

Plant regeneration and morphological analysis

Surviving shoots were placed in root induction medium (RIM) supplemented with 1 mg/L NAA and fully regenerated into plantlets in hormone-free BM. The regenerated plantlets with fully developed roots were transferred to 8 cm plastic pots filled with sterilized seedling mix (Growmor, Cato Ridge), and placed in a growth chamber at 25 +/- 2°C and 80% relative humidity (RH) under a 16 h photoperiod. After 2 weeks, plants were transferred to bigger pots (20 cm) and placed in the greenhouse for further development of tuberous roots and morphological observations, including plant stature, growth habit, leaf size, shape and colour, and rooting capacity.

RESULTS

Effect of hormone concentration and explant type on shoot regeneration

The highest percentage shoot regeneration of 31% was obtained from apical shoot explants generated on 0.01 mg/L NAA + 1 mg/L BAP (Table 1). Responsive axillary bud explants showed 22% shoot regeneration, and also on 0.01 mg/L NAA + 1 mg/L BAP. Therefore, the hormone concentrations of 0.01 mg/L NAA and 1 mg/L BAP was shown to be the best combination in the regeneration of shoots. When apical shoot and axillary bud explants were cultured in NAA concentrations of 0.2 mg/L or higher, only callus derived roots were produced. Leaf discs and stem section explants produced only highly resistant callus with a compact structure that did not regenerate into shoots.

Plant regeneration and morphological analysis

Greenhouse-grown stock plants (Figure 1A) provided the apical shoot explants needed for the establishment of *in vitro* stock plantlets (Figure 1B). Apical shoots derived from the *in vitro* stock plantlets were cultured on SIM (Figure 1C). Responsive apical shoots exhibited a swelling at the base that developed into callus after two to three weeks of incubation. At three to four weeks of culture, *de novo* organogenesis regeneration of shoots from callus was observed (Figure 1D). Organogenic further three to four weeks of culture (Figure 1E).

Plantlets were fully regenerated on hormone-free BM (Figure 1B) and then transferred to sterilized soil. Once regenerated plants were transferred to soil; they showed shoots were able to regenerate roots on RIM after a a 100% survival rate. The plants grew normally (Figure 1A) and rapidly in 20 cm pots and produced storage roots after three months of cultivation in the greenhouse. Plants derived from tissue culture were phenotypically indistinguishable from donor plants and showed no obvious variations in leaf shape, plant stature and root formation.

DISCUSSION

A simple, efficient and reproducible method for plant regeneration of sweet potato was developed in this study. This protocol was designed for the popular commercial variety - Blesbok, and is the first regeneration procedure based on a commercial South African sweet potato cultivar. Studies have shown sweet potato regeneration to be highly genotype-dependent because of the variation among cultivar responses to *in vitro* treatments (Dessai et al., 1995; González et al., 2008). Due to these noted genotype- dependent responses, a customized regeneration protocol was designed specifically for the cv. Blesbok using hormone concentration and explant type as parameters for optimization.

Results of this study demonstrated that apical shoot tips and axillary bud explants can be readily regenerated to form shoots (Table 1). Shoot tips produced callus that regenerated shoots at a higher frequency (31%) than axillary buds (22%). Stem and leaf derived explants resulted in the production of compact callus that remained unresponsive to shoot regeneration (Table 1). Studies on the regeneration of sweet potato have also noted low regeneration frequencies for various stem and leaf explants (Newell et al., 1995; Morán et al., 1998). The major influence on tissue culture response appears to be genetic, with culture requirements varying between cultivars. Consequently, the regenerative capability of various explants may be dependent on the plant genotype. As a result, the low regenerative capacity of stem and leaf explants from the cv. Blesbok has been demonstrated by this study. In addition to a genotype dependent response, the presence of meristematic tissues in apical shoot tips may have contributed to higher shoot regeneration. Meristem tissues contain actively dividing cells that are responsible for length extension of the plant body and therefore have a greater capacity for regeneration. Moreover, viruses do not invade the meristem and virus-free plants can be produced from cultures of meristematic explants (Evans et al., 2003). This proved to be of benefit in the establishment of virus-free *in vitro* stock cultures that, in turn, provided virus-free explant material for culture.

It was observed that optimum shoot regeneration was achieved when shoot tip explants were cultured in

Table 1. Effect of hormone (naphthalene acetic acid [NAA] and 6-benzylaminopurine [BAP]) concentration combinations and type of explant on shoot regeneration of sweet potato (*Ipomoea batatas* Lam.) cv. Blesbok.

Hormone combination (mg/L)	Explant type			
	Stem section	Leaf disc	Apical shoot	Axillary bud
0 BAP + 0NAA	0	0	0	0
0.01BAP + 0 NAA	0	0	0	0
0.1 BAP + 0NAA	C	C	C	0
0.2 BAP + 0 NAA	C	C	3	C
0.5 BAP + 0 NAA	C	C	C	C
1 BAP + 0 NAA	C	C	5	C
0 BAP + 0.01 NAA	C	C	C	C
0.01 BAP + 0.01 NAA	C	C	2	C
0.1 BAP + 0.01 NAA	C	C	3	2
0.2 BAP + 0.01 NAA	C	C	11	5
0.5 BAP + 0.01 NAA	C	C	15	12
1 BAP + 0.01 NAA	C	C	31	22
0 BAP + 0.1 NAA	C	C	C	C
0.01 BAP + 0.1 NAA	C	C	C	C
0.1 BAP + 0.1 NAA	C	C	2	C
0.2 BAP + 0.1 NAA	C	C	5	C
0.5 BAP + 0.1 NAA	C	C	6	3
1 BAP + 0.1 NAA	C	C	5	3
0 BAP + 0.2 NAA	0	0	CR	C
0.01 BAP + 0.2 NAA	C	C	CR	C
0.1 BAP + 0.2 NAA	C	C	CR	C
0.2 BAP + 0.2 NAA	C	C	CR	C
0.5 BAP + 0.2 NAA	C	C	CR	C
1 BAP + 0.2 NAA	C	C	CR	C
0 BAP + 0.5 NAA	C	C	CR	C
0.01 BAP + 0.5 NAA	C	C	CR	CR
0.1 BAP + 0.5NAA	C	C	CR	CR
0.2 BAP + 0.5 NAA	C	C	CR	CR
0.5 BAP + 0.5 NAA	C	C	CR	CR
1 BAP + 0.5 NAA	C	C	CR	CR
0 BAP + 1 NAA	C	C	CR	CR
0.01 BAP + 1 NAA	C	C	CR	CR
0.1 BAP + 1 NAA	C	C	CR	CR
0.2 BAP + 1 NAA	C	C	CR	CR
0.5 BAP + 1 NAA	C	C	CR	CR
1 BAP + 1 NAA	C	C	CR	CR

Values represent percentage shoot regeneration: proportion of explants showing shoots, multiplied by 100.
C= non- regenerable callus; CR= callus derived root formation.

medium containing 0.01 mg/L NAA and 1 mg/L BAP (Table 1). This data indicate that 1 mg/L BAP interacts with 0.01 mg/L NAA in a synergistic manner to promote optimal shoot regeneration via the organogenetic pattern of plant regeneration. However, when the concentration of NAA in the medium was increased to 0.2 mg/L, this synergy ceased and callus regenerated roots instead of shoots. Therefore, results indicated that concentrations of NAA in the lower range of 0.01 to 0.1 mg/L was critical to the development of shoots and concentrations exceeding this threshold resulted in root formation. The distinctive limit of NAA needed to promote shoot regeneration is probably due to the endogenous concentrations of auxin present in this specific genotype.

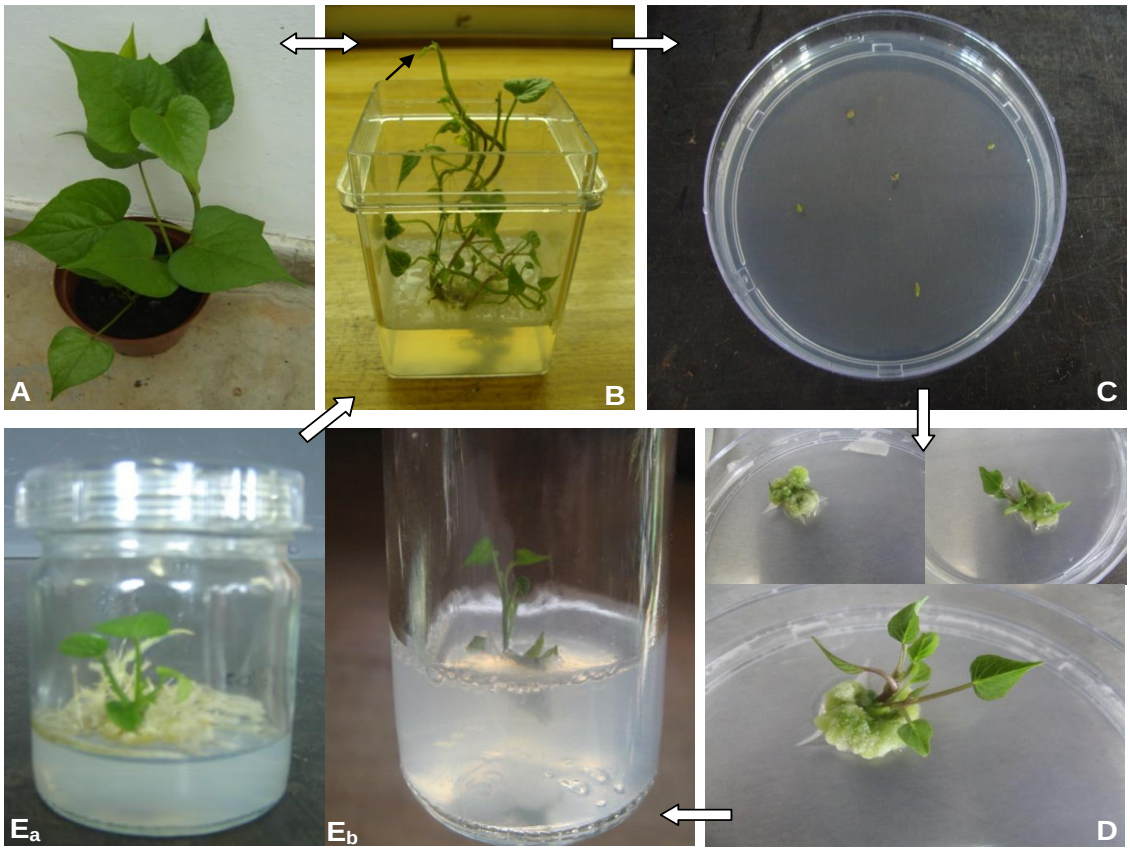

Figure 1. Schematic illustration of plant regeneration system for sweet potato (*Ipomoea batatas* Lam.) cv. Blesbok. (A) Greenhouse-grown stock plant in pot provides starting material for the establishment of *in vitro* stock cultures. (B) *In vitro* stock plants for apical shoots (black arrow) for culture. (C) Apical shoots explants cultured on shoot induction medium (SIM) gave rise to the (D) *de novo* organogenic regeneration of shoots from callus after 2 (top left), 3 (top right) and 4 (bottom) weeks of culture. (E) Organogenic shoots (right) were able to regenerate roots (left) on root induction medium (RIM) after a further three to four weeks of culture. Plantlets were fully regenerated on hormone-free basal medium (BM) and then in sterilized soil.

This result is in agreement with the classic report by Skoog and Miller (1957), who demonstrated how cytokinin and auxin interact to produce different morphological responses, depending on the concentrations of endogenous hormones and the ratio of the concentrations of exogenous auxin to cytokinin. In general, they showed that when cytokinins and auxins were combined at low concentrations, the regeneration of tobacco tissues was enhanced. However, combinations of auxin and cytokinin at high concentrations reduced the specific effects of endogenous hormones resulting in the formation of unresponsive callus. For sweet potato, regeneration has also been documented using combined treatments of auxin and cytokinin (Gosukonda et al., 1995; González et al., 2008; Xing et al., 2008).

Earlier studies have shown that shoot organogenesis can be achieved from the petiole, stem and leaf explants (Gosukonda et al., 1995; Morán et al., 1998; Luo et al., 2006). The present study showed that *de novo* organogenesis from apical tip derived callus can also occur (Figure 1). Organogenic shoot cultures obtained from culture in SIM were superior in quality and rooted easily when transferred to RIM. Regenerated plantlets produced using the described methods in this study appeared normal and were successfully transferred to the greenhouse.

Results of this study indicate that an acceptable percentage of shoot regeneration was obtained when apical shoot tips were cultured on medium containing the combined presence of 0.01 mg/L NAA and 1 mg/L BAP. We have developed an efficient and reproducible regeneration system that will provide the initial framework in the development of a transgenic sweet potato cultivar.

ACKNOWLEDGEMENTS

BJS was sponsored by the National Research Foundation (NRF), South Africa for the entire duration of her studies for which she is eternally grateful.

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