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Improved plant regeneration in cowpea through shoot meristem

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Abstract

Cowpea is a highly recalcitrant nutrient-rich leguminous vegetable crop. Efforts to genetically transform cowpea with insect-resistant genes remains a challenging task due to lack of an efficient regeneration system. We have established an efficient regeneration system in cowpea through shoot meristem. Shoot meristems were isolated from embryos that were precultured for 3-5 days on Murashige and Skoog (MS) medium containing 8.9 µM benzylaminopurine (BA). The isolated shoot meristems were cultured on MS medium containing 0.89 µM BA. After 3-4 weeks, multiple shoots were separated from the explant and cultured on half-strength MS medium for elongation and rooting. More than 90% of the regenerants formed roots. The rooted plantlets were transferred first to peat pellets and subsequently to the greenhouse. The plants were allowed to flower and set seed. The efficiency of regeneration in all four cultivars ranged from 76-87%, demonstrating a significant improvement over the published protocols (1-32%). At least six to seven plantlets were obtained from each meristem. The protocol using shoot meristems is simple, efficient, rapid and genotype-independent and may be amenable for transformation through particle bombardment.

Key words: *Vigna unguiculata*, shoot meristem, regeneration, transformation, legumes

Introduction

Cowpea (*Vigna unguiculata* L. Walp.), an annual vegetable, is one of the world's important legume food crops. Cowpea grain contains about 25% protein, especially rich in folate, potassium, iron, magnesium, and the essential amino acids lysine and tryptophan. Cowpea is also rich in phytochemicals that may help prevent chronic diseases such as cardiovascular disease, cancer and diabetes. In addition, cowpea is a good source of fiber. A diet high in fiber can help lower blood cholesterol levels, which can reduce risk of heart disease (www.mayoclinic.com/health/legumes/NU00260)

Cowpea is severely infected by insects such as thrips (*Megalurothrips sjostedti*), aphids (*Aphis craccivora*), curculio (*Chalcodermus aeneus*), pod borer (*Maruca vitrata*), weevils (*Callosobruchus maculatus*) etc. that cause significant damage to crop production and yield (Singh *et al.*, 1990). However, current cultivars do not offer protection against insect damage. Efforts to develop durable insect resistance did not succeed because the genome of cowpea may be devoid of major resistance genes to many insect pests that attack cowpea. Also, attempts to bring insect resistance into cowpea from wild *Vigna* species have failed because of high genetic barriers between wild *Vigna* and cultivated cowpea (Singh *et al.*, 1997).

Cowpea is an ideal vegetable crop for the application of genetic engineering technologies for developing insect resistance. However, cowpea is highly recalcitrant to tissue culture and therefore genetic transformation is difficult to achieve. There have been a few reports of plant regeneration through organogenesis and somatic embryogenesis (Muthukumar *et al.*, 1995; Pellegrineschi, 1997; Brar *et al.*, 1999a, b; Anand *et al.*, 2000, 2001; Ramakrishnan *et al.*, 2005). The efficiency of regeneration in these reports is too low (1-32%) to reliably obtain transgenic plants. Consequently, efforts to transform cowpea were mostly unsuccessful or resulted in very few transgenic plants (Garcia *et al.*, 1986, 1987; Penza *et al.*, 1991; Muthukumar *et al.*, 1996; Ikea *et al.*, 2003). In recent years, regeneration of shoots from cotyledon nodes or from other meristematic explants has emerged as a rapid and relatively efficient method of transformation in a number of legumes that are highly recalcitrant in tissue culture (Oger *et al.*, 1996; Trieu and Harrison, 1996; Larkin *et al.*, 1996; Olhoft *et al.*, 2001). In cowpea, transgenic plants were regenerated using cotyledon nodes containing axillary meristems (Popelka *et al.*, 2006), although at low frequency (0.05-0.15%), demonstrating the feasibility of using meristems as an alternate source for genetic transformation.

In this report, we present a simple, efficient, rapid and genotype-independent regeneration system for cowpea plants from four cultivars by using shoot meristems. The regeneration method has potential use in transforming cowpea with insect resistant genes.

Materials and methods

Four cowpea cultivars (Early Scarlet, Coronet, Quick Pick and AR87-435-68) were selected for regeneration. Mature seeds (kindly provided by Dr. S. Okiror, University of Arkansas at Pine Bluff, USA) were surface sterilized in 70% alcohol for 5 minutes, rinsed in sterile water and placed in 0.2% sodium hypochlorite solution. After 1 h, the seeds were rinsed thrice with sterile water. Finally, the seeds were allowed to soak in sterile water overnight. Murashige and Skoog (1962) medium (MS) supplemented with 3% (w/v) sucrose (Sigma, USA) and various concentrations of growth regulators were used for tissue culture and regeneration. The media were adjusted to pH 5.8 with 1 N NaOH or 1 N HCl,

solidified with 3 g L^{-1} phytagel (Sigma, USA) and autoclaved at 1 kg cm^{-2} for 20 min. Media (50 mL) were dispensed into 20- by 100-mm sterile Petri dishes (Falcon, Becton Dickinson Labware, USA). The cultures were maintained at 25 ± 2 ^{0}C with a 16-h photoperiod (25-40 µmol cm^{-2} s^{-1}). All growth regulators were filter sterilized before adding to the media. Embryos were isolated and precultured either on MS basal medium or MS medium containing BA (8.9 µM) (Table 1). After 3-5 days, the shoot meristems (0.5-1 mm) were carefully isolated and cultured on MS media with different concentrations of BA (0.4-22.2 µM) for three week. After three weeks, the regenerated multiple shoots were transferred to elongation and rooting medium (half-strength MS with no growth regulators). Plantlets with well-developed roots were removed from the culture medium, the roots washed thoroughly with tap water, and transferred to peat pellets [Jiffy-7, Jiffy Products (N.B.) Ltd., Shippagan, Canada] for initial acclimatization. The plantlets were covered with plastic wrap to maintain high humidity for first few days. Gradually the humidity was reduced by slowly removing the plastic wrap and the hardened plants were transferred to the greenhouse [24-28°C, 16/8 h (day/night) photoperiod supplemented by sodium halide lights]. Plants were allowed to flower and set seed. The root tips of the regenerated plants were collected in cold distilled water, kept at 4^{0}C for 24 h and then fixed in Farmer's fixative (3:1 95% ethanol: glacial acetic acid). The root tips were squashed with carbol fuchsin and observed under a microscope.

Results

Shoot meristems (0.5-1 mm) were carefully isolated from precultured embryos and cultured on different concentrations of BA (Table 1). After 3-5 d of culture, newly formed multiple shoots could be seen from the cut end of the meristem. After 3-4 weeks, individual shoots were separated from each meristem and transferred to half-strength MS medium for elongation and rooting. Among the different concentrations tried, MS medium containing 0.89 µM BA produced more shoots (3.2 shoots per meristem) than other concentrations. To improve the number of multiple shoots, embryos were precultured on higher concentration of BA (8.9 µM) for 3-5 days and the isolated shoot meristems were cultured on different concentrations of BA (Table 1). Significantly, more plants were regenerated after transfer to MS medium containing 0.89 µM BA from all four cultivars tested, indicating the positive effect of preculture in inducing multiple shoots (Fig. 1A). More than six shoots per meristem were regenerated from all four cultivars with the preculture on BA (8.9 µM) medium. Increasing the concentration of BA beyond 0.89 µM in the culture media resulted in callus growth that significantly reduced the number of multiple shoots. The addition of other cytokinins such as zeatin and kinetin in the regeneration media also led to callus growth from shoot meristems, thereby significantly reducing the number of shoots (data not shown). Shoots were separated and transferred to half-strength MS medium for elongation (Fig. 1B) and rooting. More than 90% of the regenerants formed roots in the half-strength MS medium (Fig. 1C). The regenerated plants with roots were initially transferred to peat pellets for hardening. Only 50% of the plants survived during hardening. After 10-12 days of hardening, the surviving plants were transferred to the greenhouse (Fig. 1D). No phenotypic and chromosomal abnormalities (2n = 22) were noticed.

Table 1. Effects of benzylaminopurine (BA, µM) on shoot regeneration from cultured meristems in cowpea

Genotype	Preculture medium	Culture medium	Explants forming shoots (%)	Number of shoots per explant†
Early Scarlet	MS	MS	56.25	1.16 j
	MS	MS + 0.4 BA	59.37	2.62 f,g,h,i,j
	MS	MS + 0.9 BA	60.94	3.24 c,d,e,f,g
	MS	MS + 2.2 BA	59.37	2.83 e,f,g,h,i
	MS	MS + 4.4 BA	51.28	2.37 f,g,h,i,j
	MS	MS + 8.9 BA	69.58	2.16 g,h,i,j
	MS	MS + 13.3 BA	60.35	1.89 h,i,j
	MS	MS + 22.2 BA	68.71	1.22 j
	MS + 8.9 BA	MS + 0.4 BA	70.89	4.87 b,c
	MS + 8.9 BA	MS + 0.9 BA	72.33	6.73 a
	MS + 8.9 BA	MS + 2.2 BA	76.82	5.45 b
	MS + 8.9 BA	MS + 4.4 BA	74.02	4.21 c,d,e
Coronet	MS	MS	72.66	1.99 g,h,i,j
	MS	MS + 0.9 BA	73.08	3.34 d,e,f,g,h
	MS + 8.9 BA	MS + 0.9 BA	87.65	6.89 a
	MS + 8.9 BA	MS + 2.2 BA	86.23	5.32 b
Quick Pick	MS	MS	81.23	1.89 h,i,j
	MS	MS + 0.9 BA	83.78	3.87 c,d,e,f
	MS + 8.9 BA	MS + 0.9 BA	86.66	6.46 a
	MS + 8.9 BA	MS + 2.2 BA	85.62	5.12 b
AR87-43568	MS	MS	75.83	1.71 i,j
	MS	MS + 0.9 BA	79.21	3.43 c,d
	MS + 8.9 BA	MS + 0.9 BA	84.02	6.29 a
	MS + 8.9 BA	MS + 2.2 BA	82.17	5.18 b

† means followed by the same letters are not significantly different based on Duncan's multiple range test (P<0.05).

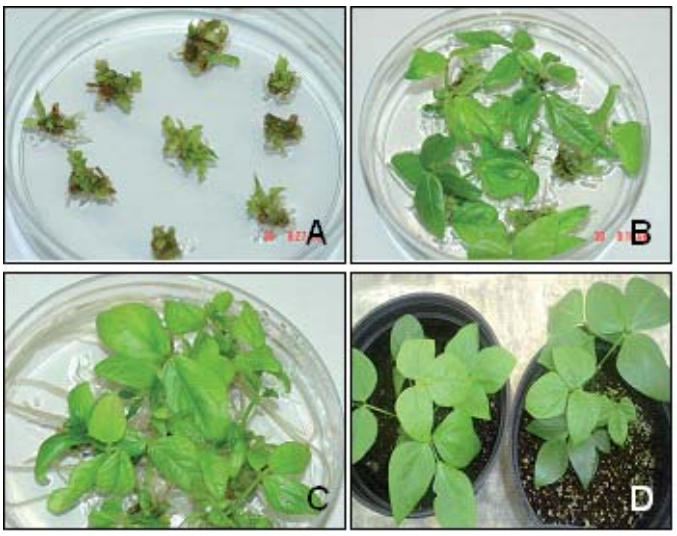

Fig.1. *In vitro* regeneration of cowpea plants cv Early Scarlet from shoot meristem. A. Development of multiple shoots from precultured shoot meristems on BA medium (0.89 µM); B. Multiple shoots, separated and transferred to rooting medium; C. Rooted plants in half-strength MS medium; and D. Fully established plants in the greenhouse.

Discussion

Cowpea is one of the most recalcitrant leguminous vegetable for regeneration and transformation (Popelka *et al.*, 2004). Consequently, well known insecticidal genes such as those for *Bacillus thuringiensis* (*Bt*) toxin, alpha-amylase inhibitor, plant lectins etc. could not be introduced into cowpea due to the lack of a simple, routine and reproducible regeneration system. Previously, regeneration of cowpea plants was achieved through organogenesis and somatic embryogenesis using explants such as cotyledons, hypocotyls, primary leaves or embryonal axes (Muthukumar *et al.*, 1995; Pellegrineschi, 1997; Brar *et al.*, 1999a, b; Anand *et al.*, 2000, 2001; Ramakrishnan *et al.*, 2005). However, the frequency of plant regeneration was too low (1-32%) to establish a routine and reproducible transformation system in cowpea.

In recent years, use of meristems as a source of totipotent cells for regeneration and transformation has emerged in a number of plants including legumes (Somers *et al.*, 2003). To date, the meristem-based transformation method has been successfully established in several species such as pea, sunflower, corn, tobacco, rice, and maize (Hussey *et al.*, 1989; Bidney *et al.*, 1992; Gould *et al.*, 1991a, b; Zimmerman and Scorza, 1996; Park *et al.*, 1996; Zhang *et al.*, 2002). Because inducing organogenesis or somatic embryogenesis is difficult, meristem-based direct regeneration may overcome bottlenecks in cowpea transformation.

In our study, shoot meristem culture was first established in the cultivar Early Scarlet. Embryos were cultured on MS basal medium for 3-5 days. Subsequently, shoot meristem was isolated and cultured on different concentrations BA. At least three shoots were regenerated from each meristem on medium containing 0.89 µM BA. Calli began to proliferate at the base of the meristems with increasing concentrations of BA (2.2-22.2 µM) thereby significantly reducing the number of shoots regenerated. Further, the number of multiple shoots significantly increased when shoot meristems were preconditioned on medium containing high BA (8.9 µM) before culturing on 0.89 µM BA, regenerating at least 6-7 shoots per meristem. The positive effect of preculturing explants on media containing cytokinins like BA on shoot regeneration has been reported in other legumes such as grain legume (*Vigna mungo*), *Phaseolus* sp. and *Cajanus cajan* (Shiv Prakash *et al.*, 1994; Santalla *et al.*, 1998; Saini and Jaiwal, 2005). Once the regeneration system for the cultivar Early Scarlet was optimized, the system was applied to the cultivars Coronet, Quick Pick and AR87-43568. The shoot meristems from these cultivars were isolated from the embryos precultured on 8.9 µM BA and cultured on 0.89 µM BA. All three cultivars produced six to seven shoots per meristem, demonstrating the applicability of the shoot meristem-based protocol to different genotypes. The regeneration efficiency of shoot meristems that produced multiple shoots ranged from 72-88% in the four cultivars compared to 1-32% obtained through organogenesis or somatic embryogenesis. In conclusion, we have successfully established a simple, rapid and efficient regeneration system in cowpea using shoot meristems. The regenerated plants exhibited no phenotypic and genotypic abnormalities. Moreover, the regeneration response of four cultivars to the shoot meristem based system was similar, demonstrating the genotype-independent nature and applicability of this protocol. The regeneration procedure developed from this study may be used to transform cowpea with insecticidal genes through biolistics transformation.

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