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BRIEF COMMUNICATION

Plant regeneration from immature embryo cultures of *Vigna unguiculata*

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Abstract

Mature and immature cotyledon explants of cowpea were cultured on Murashige and Skoog's (MS) medium supplemented with 0.1 - 2.0 mg dm^{-3} benzyladenine (BA). Shoot organogenesis was observed from the minimal greenish calli formed at proximal cutting edges of the immature cotyledon explants after 15 - 20 d of culture. Among whole immature zygotic embryo and seven explant types we tested, single whole cotyledon was suitable for shoot organogenesis. Nearly 67.5 % of the explant types produced adventitious shoots on MS medium containing with 1 mg dm^{-3} BA, and the shoot number (10.1) per explant was higher than other explant types. From the histological studies, the shoot primordia originated from the procambial strands of immature cotyledon explants. When the shoots were transferred to 1/2 MS basal medium, formed roots within 20 d of culture. The rooted plants were subsequently transferred to the pots and to the field.

Additional key words: cowpea, zygotic embryo explants, procambial strands, whole cotyledon, shoot organogenesis.

Cowpea is an important grain legume crop grown in Africa. The grain is a secondary carbohydrate staple and provides an important source of dietary protein especially for the population in the West Africa (Bressani 1985). Since cowpea in tissue culture has been regenerated from shoot and root meristem explants (Subramaniam *et al.* 1968; Kartha *et al.* 1981), the plant regeneration *via* organogenesis was reported from immature zygotic embryos (Pellegrineschi *et al.* 1997), cotyledon explant of mature zygotic embryos and primary leaves of seedling (Muthukumar *et al.* 1995). In addition, *Agrobacterium tumefaciens*-mediated genetic transformation of cowpea has been reported (Penza *et al.* 1991, Muthukumar *et al.* 1996, Lurquin *et al.* 2002), but it is presently no evidence that stable transgenic cowpea obtained by agroinfection. Accordingly, the improvement of cowpea regeneration frequency is a prerequisite for their use in the production of transgenic cowpea. In this study, some factors that influenced cowpea shoot organogenesis such as optimal concentration of benzyladenine and explant type were studied. An efficient and reproducible plant regeneration system *via* shoot organogenesis was developed.

Cowpea seeds (*Vigna unguiculata* L. Walp, cv. Magnolia Blackeye) purchased from Nam-dae Moon market were surface sterilized in 70 % ethanol for 1 min followed by immersion in 1 % sodium hypochlorite solution for 15 min. Seeds were rinsed four times with sterile distilled water and mature cotyledons excised from them were used as explants. Also, plants were grown in a greenhouse under the natural light in constant temperature at 25 °C. Pods were collected approximately 3 weeks post-anthesis, surface sterilized in 70 % ethanol for 1 min. Cotyledons were excised from the immature zygotic embryos (4 - 6 mm) within these pods and used as explants. Each mature and immature cotyledon explants was placed onto 25 cm^3 of solid culture medium to induce shoot organogenesis. The shoot induction medium consisted of Murashige and Skoog's (1962, MS)

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Abbreviations: MS - Murashige and Skoog's medium, BA - benzyladenine.
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medium supplemented with various concentrations of BA (0.1, 0.5, 1.0, 2.0 mg dm^{-3}). In addition, immature zygotic embryos were taken from a seed and sliced into seven explant types: single whole cotyledon, one proximal half, one distal half, embryo axis, one whole cotyledon with embryo axis, proximal half of one cotyledon with embryo axis, proximal half of two cotyledons with embryo axis. A whole immature embryo and seven explant types were placed onto MS medium supplemented with 1.0 mg dm^{-3} BA. All media were adjusted to 5.8 before adding 8 g dm^{-3} agar and then autoclaved at 121 °C for 15 min. Five explants were placed in each Petri dish and sealed with Parafilm. The cultures were maintained at 25 °C under 16-h photoperiod at irradiance of 46 μmol $m^{-2}s^{-1}$, provided by cool-white fluorescent lamps. After six weeks of culture, the shoot organogenesis and shoot numbers per explant were recorded, respectively. Three replicates were prepared for each treatment and each cotyledon explant. For histological studies, the immature cotyledons cultured on the MS medium supplemented with 1 mg dm^{-3} BA were isolated from the induction medium at 0, 4, 8, 12 and 15 d of culture, and then were fixed in a solution of formaldehyde acetic acid (FAA) for 24 h, dehydrated in a tertiary-butanol series and embedded in paraplast. Serial sections were cut at 10 μm and stained with 0.5 % hematoxylin and 1 % safranin (Sass 1971). The sections were observed under light microscope (*Meiji ML8000*, Tokyo, Japan).

To regenerate whole plants, adventitious shoots formed from immature cotyledon explants were transferred to 1/2 MS basal medium and the regenerants were subjected to acclimation, transferred to potting soil, and maintained in field. Mature and immature cotyledons excised from whole zygotic embryos were cultured on MS medium containing 0.1 - 2.0 mg dm^{-3} BA. Shoot organogenesis was observed from the minimal greenish callus formed at proximal cutting edge of the immature cotyledon explants within 15 - 20 d of culture (Table 1). Nearly 67.5 % of the cotyledons were produced shoots on MS medium containing 1 mg dm^{-3} BA. The shoot number (10.1) per explant was also higher on MS medium supplemented with 1 mg dm^{-3} BA than with other BA concentrations. Whereas the shoot organogenesis were not observed from the mature cotyledon explants on any media. From these results, we concluded that 1.0 mg dm^{-3} BA was an optimal concentration for cowpea shoot organogenesis, and that shoot organogenic potential in cowpea is higher in the immature than in mature cotyledons. These results are in agreement with those reported that the immature cotyledons on MS medium containing 1.15 mg dm^{-3} BA or 2 mg dm^{-3} thidiazuron (TDZ) were found the most suitable for shoot organogenesis in cowpea (Pellegrineschi 1997, Pellegrineschi *et al.* 1997) and in many crop plants (Barwale *et al.* 1986, Wright *et al.* 1986, Gill and Saxena 1992, Malik and Saxena 1992).

Table 1. Shoot organogenesis from the whole cotyledon explants of immature zygotic embryos of *Vigna unguiculata* on MS medium containing various concentrations of BA. Data were collected after six weeks of culture. Means ± SE of three replicates with 15 immature zygotic embryos per replicate. No organogenesis from mature embryos was observed.

BA [mg dm^{-3}]	Frequency of organogenesis [%]	Number of shoots [$explant^{-1}$]
0.1	9.1 ± 1.1	0.2
0.5	49.9 ± 2.7	3.6
1.0	67.5 ± 2.9	10.1
2.0	15.4 ± 2.1	2.1

Table 2. The shoot organogenesis from different cotyledon explants of immature zygotic embryos of *Vigna unguiculata*. Data were collected after six weeks of culture on MS medium supplemented with 1.0 mg dm^{-3} BA. Means ± SE of three replicates with 15 immature zygotic embryos per replicate. No organogenesis from other explant types was observed.

Explant	Frequency of organogenesis [%]	Number of shoots [$explant^{-1}$]
Whole cotyledon	67.5 ± 2.9	10.1
Proximal half	50.1 ± 3.2	4.2
Distal half	30.3 ± 1.3	2.3

Whole immature zygotic embryo and seven explant types excised from them were cultured on MS medium containing 1 mg dm^{-3} BA that was an optimal BA concentration for shoot organogenesis. Adventitious shoots always arose from the greenish callus produced at the proximal cutting edge of excised cotyledons (Fig. 1*A-C*) and the highest frequency of shoot organogenesis was obtained from single whole cotyledon (67.5 %). However, the shoots were not formed from one or two cotyledon halves with embryo axis or embryo axis alone. When the single whole cotyledons were sliced into proximal or distal halves by transverse cuts, the proximal halves (50.1 %) showed better regeneration than the distal halves (30.1 %) (Table 2). Cotyledons with proximal cutting had earlier been shown to be potential source for plant regeneration in *Vigna unguiculata* (Brar *et al.* 1999), *Pyrus syriaca* (Rida *et al.* 2000), cucumber (Zhong *et al.* 1993) and crop plants (Vasil 1987). These results indicated that an embryonic axis, a cotyledon size and a proximal cutting edge of a cotyledon were critical factors in the success of shoot organogenesis of cowpea, and are almost comparable with those of Zhou *et al.* (1992), who found that the bud organogenesis rate of the larger section (62.5 %) was higher than the corresponding smaller section (14.4 %) in cucumber. Accordingly, we expect that this efficient and reliable plant regeneration

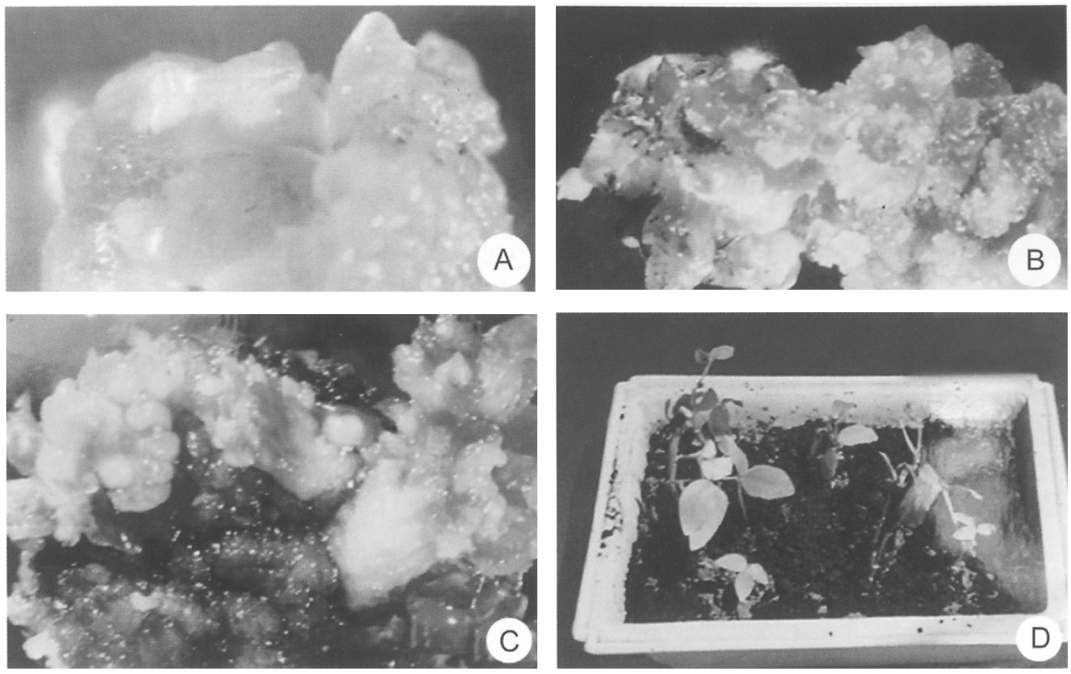

Fig. 1. Plant regeneration from cotyledon explants of immature zygotic embryos of *Vigna unguiculata* on MS medium containing 1.0 mg dm^{-3} BA: *A* - green spots; *B*, *C* - adventitious shoot primordia; *D* - plantlets in pot with vermiculite.

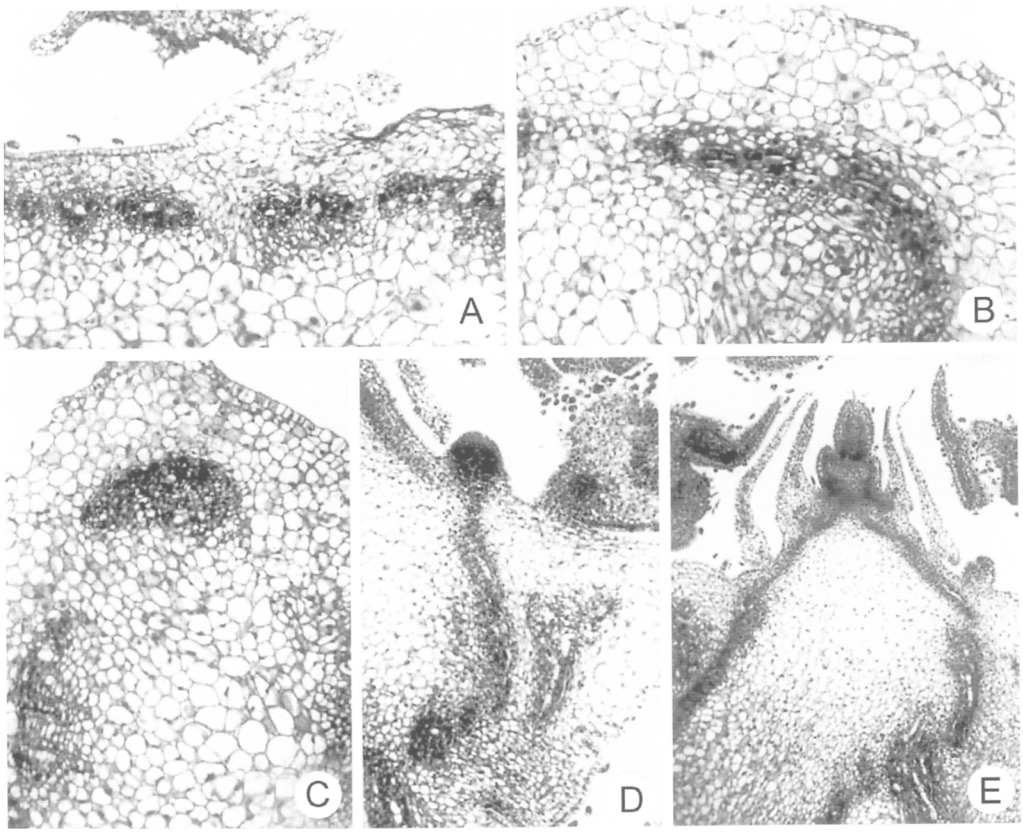

Fig. 2. Histological observations on the adventitious shoots formation from cotyledon explants of immature zygotic embryos of *Vigna unguiculata*: *A* - procambial cells at 5 d of culture; *B*, *C* - divisions of procambial cells; *D* - shoot primordium linked vascular tissue; *E* - shoot primodium.

system *via* shoot organogenesis can potentially be used effectively in producing transgenic cowpea. From the histological studies, the shoot primordia originated from the procambial strands of cotyledon of immature zygotic embryos, which were developed into a multiple shoots within 20 d (Fig. 2). The multiple shoots mechanically isolated from their tissue of origin, when transferred to hormone free 1/2 MS medium, formed roots within 20 d. The rooted plants were subsequently transferred to the pots and to the field (Fig. 1*D*).

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