Micropropagation of F_1 Hybrids Soybean [*Glycine max* (L.) Merrill] Tolerance to Aluminum

Titin Handayani and Budhi Priyanto

Agency for the Assessment and Application of Technology, Jakarta, Indonesia

ABSTRACT

A reproducible method for multiplication of shoot from cotyledone node of soybean (*Glycine max*) has been developed. Progeny from seed recovered from regenerated plants appear normal. The technique for micropropagation of F_1 hybrid soybean plants is described. By culturing cotyledone node obtained from 5 day old seedlings on modified B5 medium, average success multiply shoot were obtained. Best regeneration was from cotyledone node explants obtained on modified B5 medium with NAA and picloram. The optimal concentration of NAA for the induction of regeneration to be 0.1 mg/l. A few buds were induced in the 0.01 mg/l picloram treatment but at the higher levels the entire explant callused. When the explant was placed directly on B5 medium standard, the production of buds and small shoots was not inhibited. However, when the explants were placed directly onto B5 medium with BAP, resulting buds and shoots had a low rate of survival. No multiplication shoot occurred when coconut milk was added on B5 medium. The cotyledone nodes explant were continued to grow but the growth showed faster than on B5 medium standard. Plantlets were grown to maturity under greenhouse condition.

Key words: Soybean, hybridization, micropropagation, B5 medium, coconut milk

INTRODUCTION

The cultivated soybean [*Glycine max* (L.) Merrill] has small flowers and their emasculation for crossing requires special care (Fehr, 1980). The number of seeds per pod is naturally low, varying from one to three, and artificial hybridization usually lowers the number still further. These factors make the production of hybrid seed labour intensive. Moreover, the reproductive development of the soybean plant is sensitive to changes in day length, and the environment of the scarce hybrid plants is critical in determining the number F_2 seeds produced. In many cases a large number is required in order to obtain a desired genetic combination.

A possible method of overcoming the problems is to grow the parent plants in artificially prolonged days to ensure that they accumulate a large biomass, then expose them to short days to induce flowering. This procedure will produce many flowers, but the opportunity to make crosses is limited to the period of flowering and by the quality of the flowers. Under short days, genotypes adapted to high latitudes produce small and cleistogamous flowers (Fehr, 1980). Another tactic is to grow F_1 plants under long-day regime to generated a larger F_2 population, but seed production is still dependent on the number of successful crosses. A possible alternative solution which reduces these difficulties is to propagate the F_1 hybrid plants vegetatively.

Several recent reports have described the formation of somatic embryos and/or plants from tissue cultures of *Glycine max* (Christianson *et al.*, 1983; Lippmann and Lippmann, 1984; Ranch *et al.*, 1985; Barwale *et al.*, 1986; Wright *et al.*, 1986; 19867). In these studies, immature embryos, cotyledonary nodes, and epicotyls were used as primary explants. Regeneration from leaf explants may be preferable since the leaf disc regeneration method has been used successfully for genetic transformation of several species of the Solanaceae (McCormick *et al.*, 1986).

Successful shoot proliferation techniques have been developed for cotyledonary nodes or epicotyl (Cheng *et al.*, 1980). A procedure for soybean regeneration from cotyledonary node has been described. Three (3) mm sections of the first to the fourth trifoliate leaves of soybean seedlings were used. Callus was obtained from less than half of the explants with the youngest leaves producing the best callus. A single bud was obtained that eventually differentiated into a plantlet from hundreds of cultures initiated.

In this report we describe a technique for the multiplication system from cotyledone node of F_1 hybrid soybean tolerance to aluminum.

MATERIALS AND METHODS

Plant Material

Plant material in this study were soybeans (*Glycine max*. L. Merr. 2n = 2x = 40) cultivars Ceneng (sensitive to aluminum) as a female parent and Sindoro (tolerance to aluminum) as male parent. The soybean used in the crossing were grown in a green house at PUSPIPTEK Serpong, Tangerang.

Crosses

Crosses were made between the parents and reciprocal. The young buds were emasculated 2 or 3 days before anthesis and immediately pollinated with pollen from the newly opened flowers of the male parents. To encourage the retention and growth of the pods, the hybridized gynocia were sprayed daily with giberellic acid (100 mg/l) for 20 days (Singh *et al.*, 1987; Chung and Kim, 1990).

Seed Culture

Mature putative hybrid pods were harvested between 50-60 days after pollination (DAP). Pods were surface-disinfected with 70% ethanol for 1 min followed by a 20-min soaking in 1% sodium hypochlorite with a trace amount of Tween. After being rinsed with three changes of sterile, distilled water, the pods were disected and the seeds were excised and cultured. The seed size were measured during dissection. The culturing procedure was divided into the following two stages: (a) germination and seedling development, (b) cotyledone node multiplication, and (c) maintain culture.

Germinating and Seedling Development

The first stage, seeds were cultured in medium containing 1/2 of standard B5 medium (Gamborg *et al.*, 1968). The cultures were incubated at 27°C under 12 hour light/12 hour dark cycle.

Cotyledonary Node Multiplication

The second stage, after 5 days germination, cotyledone nodes were excised and transfered to shoot multiplication medium containing modified B5 medium.

a. Experiment 1

The following auxin were added to B5 medium: NAA and picloram (PC). The cultures were incubated 4 weeks followed by transfer to B5 medium added by 5 mg/l BAP. After 4 weeks on this medium, the cultures were scored for callus proliferation and regeneration.

b. Experiment 2

To test the effect of cytokinin in the induction medium, cotyledone nodes from 5 days old germinated seedlings were incubated in glass culture on the B5 medium containing 5, 7.5, and 10 mg/l BAP; and 50, 100, 150 ml/l coconut milk. The cultures were maintained at 25±1°C under a 16 hour photoperiod. After 4 weeks the cultures were scored for callus proliferation and regeneration.

Maintain Medium for Regenerated Shoot

The third stage, regenerated shoot were excised and transfered to B5/BAP medium with 20% sucrose and 0.8% agar. The cultures were maintained at 25±1°C under a 16 hour photoperiod. After 20-30 days plantlets with well developed root systems were transplanted to 250 ml plastic pots containing a 3 : 1 compos and steriled soil.

RESULTS AND DISCUSSION

The protocol described (Figure 1) is essentially a modification and expansion of the procedure described by Reynolds *et al.* (1982). They reported embryoid formation from cultured hypocotyls, roots, and primary leaves but did not obtained plantlets.

Crosses and Seed Culture

The hybridization were successfully obtained hybrid pods. From approximately 400 crosses, 46 putative hybrid pods were harvested and 97 seeds cultured. All of the seed survived the initial stage I cultured (Figure 2).

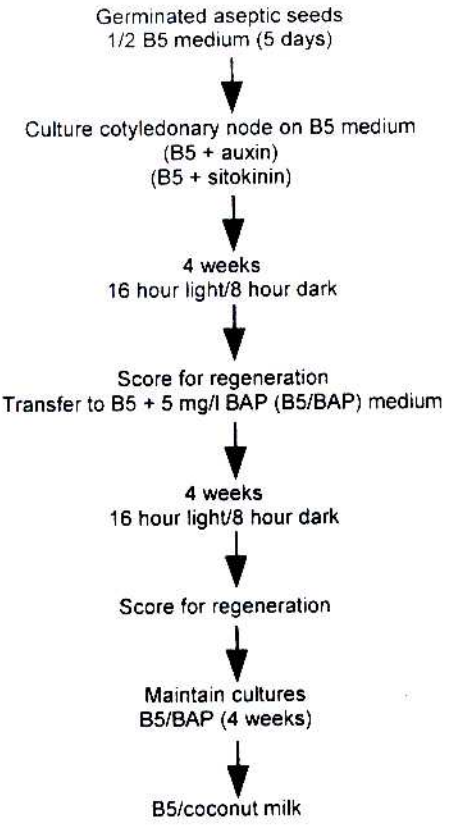
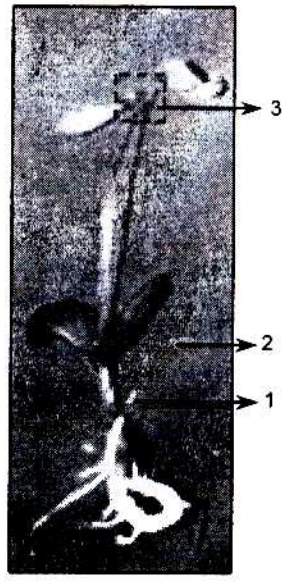


Figure 1. Protocol for the regeneration of soybean from cotyledonary nodes

Cotyledone Node Multiplication

Experiment 1

After 4 weeks the cotyledone nodes (Figure 3) cultured on B5 medium experiment 1, the shoot continued to grow. Callus was initiated at the base of the petiole. During the 4 weeks on this medium, green leaf-like structures resembling small. After 4 weeks induction on the auxin-containing medium, transfer of the callus and remaining cotyledone node to BAP-containing medium supported continued bud, shoot and callus proliferation. BAP has previously been shown to be required for soybean organogenesis (Wright *et al.*, 1986). Root were removed before transfer to the B5/BAP medium as root formation reduces regeneration.



Notes: 1. Hypocotyl, 2. cotyledone node, 3. shoot tip

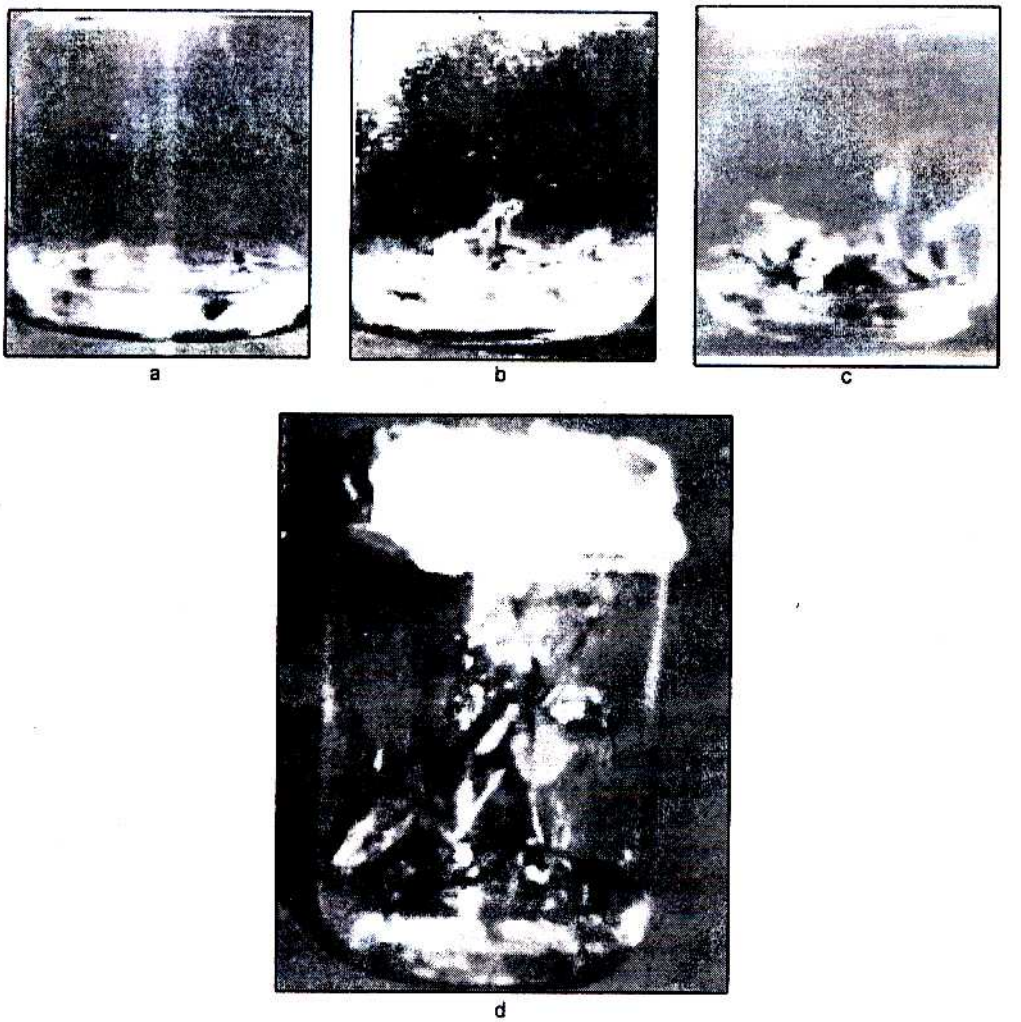
Figure 2. Germination from F_1 hybrid soybean

The results of experiment 1 (Table 1) confirmed both the requirement for NAA for the induction of regeneration and demonstrated the optimal concentration to be 0.1 mg/l. At the highest level tested, 1.0 mg/l, the entire explant callused and no regenerative structures were observed while the lowest concentration tested, 0.01 mg/l, gave a reduced response and produced a small amount of callus or no callus. A few buds were induced in the 0.01 mg/l picloram treatment but at the higher levels the entire explant callused. None of the other auxins tested produced regenerative structures. After 4 weeks on B5/BAP, the embryoid calllus from cotyledonary nodes produce adventious shoots (Table 2).

Plantlets were removed from the callus to hormone-free rooting medium. The regenerated plants were grown to maturity under standard greenhouse condition and appeared normal. The progeny evaluations were performed in the field but the data has not been analysed.

Experiment 2

The results in Table 3 confirm the promotive effect of BAP on soybean regeneration note previously (Wright *et al.*, 1986). When the explant was placed directly on B5 medium standard, the production of buds and small shoots was not inhibited. However, when the explants were placed directly onto B5 medium with BAP, resulting multiple buds and shoots had a low rate of survival. Without the growth of the associated callus the number of buds and shoots obtained from individual cultures was limited. After 4



Notes: a. Callus produced at the base of the cotyledone node after 4 weeks on B5 medium with NAA, b. regenerating callus after several transfers to fresh B5/BAP medium, c. shoot multiplication from cotyledone node, d. plantlet on B5 medium with coconut milk

Figure 3. Regeneration from F_1 hybrid soybean cotyledone nodes

weeks cotyledone node cultured on B5/BAP, multiple shoots were exciced and transfer to maintain medium.

Effect of coconut milk on multiplying of cotyledonary nodes was not induced regenerative structures. The cotyledone nodes explant were continued to grow. Although 55% of the cultures treated with 50 ml/l coconut milk responded, they were morphologically different from cultures on B5 medium standard. The leaves and stems of the cotyledone node were thick, dark green, and contained very little callus. After 4

Table 1. Regenerative response of culture F_1 hybrid soybean cotyledone nodes to varied concentrations of auxins

Auxin	Concentration (mg/l)	Number of explant	Percent cultures regenerating	
			4 weeks incubation	8 weeks incubation
NAA	0.01	8	0	0.5
	0.05	8	0	0
	0.10	5	0	0
PC	0.01	8	0.9	30
	0.05	8	0	0.6
	1.00	8	0	0

Table 2. Regeneration of embryoid callus from B5 medium with picloram

Sitokinin	Concentration mg/l	Number of explant	Percent callus regeneration		Number regenerant per explant	
			4 weeks	8 weeks	shoot	plantlet
BAP	5	4	37.5	50	>5	0
	7.5	4	50	50	>5	0
	10	4	50	50	>5	0
Coconut milk	50	4	25	25	2-3	2-3
	100	4	50	50	2-5	2-5
	150	4	50	50	>5	>5

Table 3. Effect of sitokinin on soybean regeneration utilizing the cotyledone node system

Sitokinin	Concentration (mg/l)	Number of explant	Percent cultures regeneration		Number of shoot per explant
			4 weeks	8 weeks	
BAP	5	8	75	100	>5
	7.5	8	100	100	>5
	10	8	50	50	>5
Coconut milk	50	8	100	100	1
	100	8	100	100	1
	150	8	100	100	1

weeks plantlet obtained from cotyledone node cultured on B5 medium with coconut milk, transfered to B5 medium standard to induced root. Cotyledone node explants were placed on B5 medium with 100 and 150 ml/l coconut milk showed higher and thicker than explant on B5 standard. Plantlets were grown to maturity under greenhouse condition.

ACKNOWLEDGEMENT

The authors wish to acknowledge the technical expertise of Sati Suyanti and Atang.

REFERENCES

Barwale, U.B., H.R. Kerns, and J.M. Widholm. 1986. Plant regeneration from callus cultures of several soybean genotypes via embryogenesis and organogenesis. Planta 167:473-481.

Cheng, T.Y., H. Saka, and Voqui-Dinh. 1980. Plant regeneration from soybean cotyledonary node segments in culture. Plant Sci. Lett. 19:91-99.

Christianson, M.L., D.A. Warnick, and P.S. Carlson. 1983. A morphogenetically competent soybean suspension culture. Science 222:632-634.

Chung, G.H., and J.H. Kim. 1990. Production of intergeneric hybrids between *Glycine max* and *G. tomentella* through embryo culture. Euphytica 48:97-110.

Fehr, W.R. 1980. Soybean. *In* Fehr, W.R. and H.H. Hadley (*Eds.*). Hybridization of crop plants. Amer. Soc. of Agronomy, Madison, Wisconsin. p. 589-599.

Gamborg, O.L., R.A. Miller, and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cell. Exp. Cell Res. 50:151-158.

Lippmann, G., and G. Lippmann. 1984. Induction of somatic embryos in cotyledonary tissue of soybean, *Glycine max* L. Merr. Plant Cell Report 3:213-218.

McCormick, S., J. Niedermeyer, J. Fry, A. Barnason, R. Horsch, and R. Fraley. 1986. Plant Cell Report 5:81-84.

Ranch, J.P., L. Oglesby, and A.C. Zielinski. 1985. Plant regeneration from embryo-derived tissue cultures of soybeans. *In Vitro* Cell Dev. Biol. 21:653-658.

Reynolds, B.D., W.J. Blackmon, and A.M. Lawrence. 1982. Hort. Sci. 17:60.

Singh, R.J., K.P. Kollipara, and T. Hymowitz. 1987. Intersubgeneric hybridization of soybeans with a wild perrenial species *Glycine cladestina* Wendl. Theor. Appl. Genet. 74:391-396.

Wright, M.S., S.M. Koehler, M.A. Hinchee, and M.G. Carnes. 1986. Plant regeneration by organogenesis in *Glycine max*. Plant Cell Report 5:150-154.

Wright, M.S., D.V. Ward, M.A. Hinchee, M.G. Carnes, and R.J. Kaufman. 1987. Initiation and propagation of *Glycine max* L. Merr.: Plants from tissue cultured epicotyls. Plant Cell Report 6:83-89.