J. Plant Biotechnology(2004) Vol. 6(1). pp. 39~43

Journal of Plant Biotechnology

Factors Affecting Organogenesis from Mature Cotyledon Explants and Regeneration in Soybean

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Abstract

A successful, efficient system for multiple shoot induction and plant regeneration of soybean (*Glycine max*) was established. Four soybean genotypes were compared for organogenic responses on various media cultured under light conditions. The adventitious shoots (98%, 2.6 shoots/cotyledon) directly from one-day-old cotyledon after germination induced by the hormone treatment and its efficiency was higher than any other conditions. The optimal medium for the induction of multiple shoots from cotyledon in Pungsannamulkong (shoot formation rate, 98%), Lx 16 (83%) and Ilpumgeomjeongkong (63%) was MS medium supplemented with 2 mg/L BAP, but for Alchankong (75%), MS medium supplemented with 1 mg/L zeatin and 1 mg/L IAA, 3% sucrose, 4% Phytagel. Higher root induction (88%) was observed from the shoots placed on rooting medium (hormone-free MS basal). Plantlets were transferred onto the same medium supplemented with 1% activated charcoal for further development. With this treatment, regenerated plantlets were obtained within 7-8 weeks (shoot induction for 4 weeks, rooting and shoot elongation for 3-4 weeks).

Key words: cotyledon, organogenesis, regeneration, soybean

Introduction

Soybean [*Glycine max* (L.) Merrill] is one of the most important agronomic crops in respect of protein and oil. A great deal of effort has been made towards the development of new cultivars of soybean with increased nutritional value along with improved yield and disease and pest resistance. However, traditional soybean breeding programs have been limited to varietal improvement due to the extremely narrow germplasms and its incapability of free transfer of useful genes from species to species. But the application of tissue culture techniques may lead to new avenues of crop improvement through genetic transformation. Since the first report of regeneration *in vitro* of soybean was issued by Cheng et al. (1980) and Christianson et al. (1983), new methods on soybean regeneration have been published either through organogenesis (Barwale et al. 1986) or somatic embryogenesis (Ranch et al. 1986; Finer, 1988).

Hinchee et al. (1988) found that numerous shoots were produced via organogenesis from the cotyledonary node of soybean. McCabe et al. (1988) also induced multiple shoots on high cytokinin medium (Reddy et al. 1998). Soybean shoot organogenesis also occurs from tissues such as cotyledonary nodes (Cheng et al. 1980; Barwale et al. 1986; Wright et al. 1986) as well as embryos (Chyuan and Yeh, 1991), embryonic axes (Reddy et al. 1998), hypocotyls (Dan and Reichert, 1998), epicotyls (Wright et al. 1987), primary leaf node (Kim et al. 1990), and primary leaves (Wright et al. 1987). An effective organogenesis system was developed by Dan and Reichert (1998) where hypocotyls from soybean seedlings were placed on a cytokinin-containing medium and the shoots elongated on a second medium. However, these methods had some general problems that require a considerable amount of time and many kinds of media before a whole plant can be obtained.

Hence, an efficient time-saving system for plant regeneration by organogenesis from mature cotyledon in soybean

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Received Nov. 14, 2003; Accepted Feb. 25, 2004

has to be established for gene transformation in future.

Materials and Methods

Seed germination

Plant materials used for this experiment were soybean variety Pungsannamulkong, Alchankong, Ilpumgeomjeongkong and Lx 16 harvested from the field of National Honam Agricultural Experiment Station, RDA. Mature seeds were surface-sterilized with 70% ethanol for 2 min and washed. After that, seeds were soaked in 1% sodium hypochlorite (NaOCl) solution with continuous shaking at 150 rpm for 20 min. Seeds were rinsed thoroughly 3 times with double distilled sterile water, and then were aseptically germinated on hormone-free MS basal medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.4% Phytagel (Sigma) in petri dishes (Figure 2A). The seeds were maintained under the light intensity of 30 $\mu molm^{-2}s^{-1}$, 16 hr photoperiod at 27℃ from 12 hr to 5 days.

Explant preparation for shoot induction

Explants for shoot induction were taken from 12 hr to 5-day-old seedlings after inoculation for germination. Seed coats after surface-sterilization were peeled, cotyledon was split longitudinally into two pieces. The cotyledon after removing a radicle and a plumule (shoot axis) was placed on media (Figure 2B), i.e. MS medium [MS salts and B_5 vitamin (Gamborg et al. 1968) containing 0.5-4 mg/L 6-benzyl-aminopurine (BAP) or 1 mg/L zeatin + 1 mg/L indol-3-acetic acid (IAA) depending on the genotypes, with 3% sucrose, 0.4 % phytagel and pH 5.8] with the flat side up at 27℃ under the light intensity of 30 $\mu molm^{-2}s^{-1}$, 16 hr photoperiod for 4 weeks. Most effective plant growth regulators (PGRs) among BAP, IAA, IBA (indol-3-butyric acid), NAA (α-naphtalene acetic acid), and zeatin for shoot induction were selected from the results of preliminary experiment.

Shoot elongation and plant regeneration

Induced individual shoots were excised from the cotyledon and placed on a shoot elongation and root induction medium composed of hormone-free MS basal salts and vitamins, 3% sucrose, 4% Phytagel and pH 5.8. They were cultured on this medium for 3-4 weeks at 27℃ under a 16 hr photoperiod. Rooted shoots longer than 5 mm were subcultured on the same medium supplemented with 1% activated charcoal in Magenta GA7 box (Sigma) and culture bottles at 27℃ under 23 hr photoperiod. For acclimation, well grown plantlets were transferred to vermiculite in small plastic pots and these pots were placed in a plastic container containing nutrient solution. The lid of plastic container was gradually opened to harden the plantlets prior to transplanting into soil. The plants were maintained in a glass house for flowering and seed set.

Three replications of experiment were conducted for each of three separate experiments. After 6 weeks in culture, the effects were determined in terms of shoot formation rate (number of cotyledons responding/inoculated cotyledon number×100), the number of independent shoots produced per cotyledon plated and root formation rate. The data were analyzed with DMRT (Duncan's multiple range test) test at 5% level.

Results and Discussion

Mature seeds were germinated and then inoculated on the medium containing plant growth regulators. After 10 days, most of cotyledon explants were remained green, and some of them showed shoot formation from the cut edge of the cotyledons (Figure 2). However, some cotyledons were turned from light to dark brown, and did not induce multiple shoots.

A comparison of the age of explant of mature soybean seeds showed the highest shoot formation of 96% at 24 hr after germination, and the shorter the days after germination the higher the shoot formation rate except 12 hr period (Figure 1). However, contradictory results have also been reported by other researchers. Kaneda et al. (1997) used cotyledonary nodes and upper hypocotyl segments at the age of 14 days after seed germination. They obtained lower frequency (less than 60%) of shoot formation from those materials. Yuqing et al. (1995) used cotyledonary nodes taken from 4-day-old seedlings and shoot induction frequency was 45-80%. Meurer et al. (1998) also used cotyledonary nodes with 5 mm of radical taken from 4-day-old seedlings.

The highest frequence of shoot formation was obtained on the MS medium supplemented with 2 mg/L BAP. Multiple shoots were induced on the same medium containing 4 mg/L BAP. The frequency of adventitious multiple shoot formation tended to increase when BAP concentration was increased. However, the highest root formation (97%) after shooting obtained from the medium with 2 mg/L BAP, whereas the medium with 4 mg/L BAP gave a little decreased

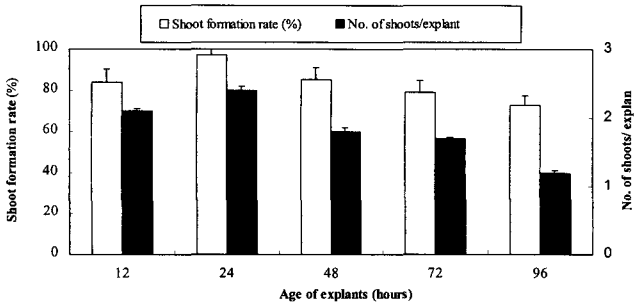

Figure 1. Effect of age of explant on shoot formation rate and number of shoots via organogenesis in soybean (cv. Pungsannamulkong). Values represent ±SE for n=30.

frequency to 92% (Table 1). Wright et al. (1986) and Shetty et al. (1992) reported that MS basal medium had minimal effects on shoot formation. Kaneda et al. (1997) found that 1/2 B_5 and 1/2 L2 media more stimulate shoot organogenesis efficiently than MS medium from hypocotyl segments.

Adventitious shoots were usually directly induced from cotyledon nodes, near the basal-excised portion of cotyledons without intervening callus formation (Figure 2C-E) and 98% to 63% of the explants produced shoots 4 weeks after innoculation (Table 2). And some explants showed callus induction later and developed root (Figure 2F,G). Among all genotypes tested, Pungsannamulkong showed the highest frequency of shoot formation on MS medium supplemented with 2 mg/L BAP. However, Ilpumgeomjeongkong had the lowest shoot formation on the same medium.

To enhance the frequency of organogenesis, we used two basal media such as MS and B_5, and plant growth regulators (PGRs), i.e. BAP, IAA, IBA (indol-3-butyric acid), NAA (α-naphtalene acetic acid), zeatin in the preliminary experiment. The frequency of shoot formation depended on the genotypes and media. After 6 weeks of culture, the highest frequency of shoot formation and root formation reached up to 98% and 99%, respectively, using MS medium supplemented with 2 mg/L BAP in Pungsannamulkong, and 75% and 89% on MS medium containing 1 mg/L zeatin and 1 mg/L IAA in Alchankong (Table 2). In the preliminary experiment, other effective media for induction of shoot and root were B5B3N4 (Gamborg et al. medium plus 3 mg/L BAP and 4 mg/L NAA), B_5B2I5 (2 mg/L BAP and 5 mg/L IAA), B_5B1I5 (1 mg/L BAP and 5 mg/L IAA) and B_5B5I5 (5 mg/L BAP and 1 mg/L IAA). Kaneda et al. (1997) reported that thidiazuron (TDZ) induced adventitious shoots more efficiently than BA and the optimal TDZ concentrations for shoot organogenesis from hypocotyl segments were 1-2 mg/L.

Regeneration of whole plants from mature cotyledon explant shows on the MS medium supplemented with 2 mg/L BAP and induction of roots and growth of regenerants on the hormone-free MS medium in soybean (cv. Pung-

Table 1. Effect of 6-benzyl-aminopurine on the responsiveness of cotyledons and shoots formation via organogenesis on MS basal medium in soybean (cv. Pungsannamulkong).

BAP (mg/L)	Number of cotyledons	Cotyledon responding (%)	Number of shoots/cotyledon	Root formation rate (%) [b]
0.5	13	5 (38) c[a]	0.4	83
1	13	8 (62) b	1.4	89
2	13	12 (92) a	2.8	97
4	13	10 (77) a	2.9	92

[a] Numbers followed by the same letter are not significantly different at 5% level of probability. [b] (Number of root formed/number of shoot inducted)×100.

Table 2. Effect of soybean genotypes and plant growth regulators on shoot and root formation in 1-day-old germinating soybean seeds 6 weeks after culture initiation.

Genotype	Nnumber of explants inoculated	Cotyledon responding (%)	Number of shoots/explant	Root formation rate (%) [c]
Pungsannamulkong [a]	60	59 (98)	2.6	99
Alchankong [b]	60	45 (75)	1.4	89
Ilpumgeomjeongkong [a]	60	38 (63)	1.3	88
Lx16 [a]	60	50 (83)	2.3	93

[a] MS medium+ 2 mg/L BAP, [b] MS medium+ 1 mg/L zeatin+ 1 mg/L IAA.
[c] (Number of root formed/number of shoot inducted)×100.

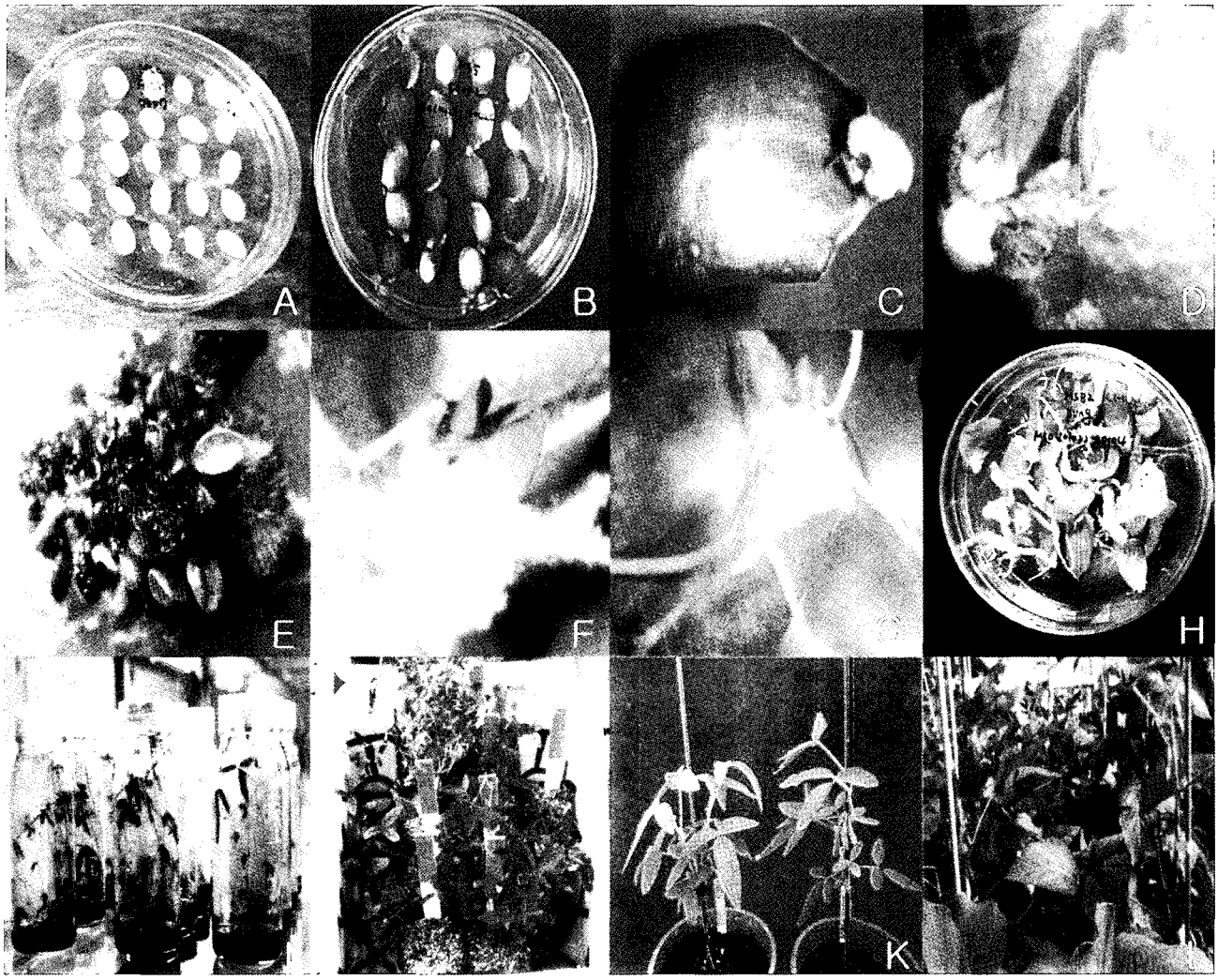

Figure 2. Plant regeneration through organogenesis from cotyledon and acclimation of regenerated pants. A.B: Seed inoculation for germination and inoculated cotyledon for shoot regeneration. C: Cotyledon 7 days after inoculation. D: Shoot induced directly from cotyledon. E : Multiple shoots induced on media containing high concentration (5 mg/L) of BAP. F. G: Shoot, callus and root induced from cotyledon. H. I: Regeneration of whole plants from mature cotyledon explant on MS medium supplemented with 2 mg/L BAP and growth of regenerants on the hormone-free MS medium in Pungsannamulkong. J-L: Acclimation, flowering and seed set of regenerated plants.

sannamulkong) (Figure 2H, I). After regeneration, plantlets were transferred to vermiculite in plastic pots and cultured for acclimation and hardening off in a nutrient solution at 23 hr photoperiod. Well-rooted plantlets were transplanted into soil in a glass house (Figure 2J- L). The regeneration system from mature seed via organogenesis was very simple and time-saving method and easily applicable to most of cultivars without genotype dependency.

Acknowledgements

This work was supported by a grant from BioGreen 21 Program, Rural Development Administration, Republic of Korea. S.J. Yun was supported by Research Center for Industrial Development of Biofood Materials in Chonbuk Nat'l Univ., Jeonju, Korea. The center is designated as a RRC appointed by the KOSEF, Jeonbuk Provincial Government and Chonbuk Nat'l, Univ., Korea.

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