J. Plant Biotechnology(2000) *Vol. 2(3). pp.123~128*

Journal of Plant Biotechnology

The Factors on Somatic Embryogenesis of Soybean [*Glycine max*. (L.) Merrill]

Kyong Ho Kim[1]*, Hag Sin Kim[1], Young Jin Oh[1], Sug Kee Suh[1], Tae Soo Kim[1], Ho Kee Park[1], Moon Soo Park[1], Seok Dong Kim[1], Up Dong Yeo[2]

[1]*National Honam Agricultural Experiment Station R.D.A. Iksan, 570-080, Korea;* [2]*Faculty of Biological Science, College of Natural Sciences, Chonbuk National University, Chonju, 561-756, Korea.*

Key words: callus, somatic embryogenesis, soybean, 2,4-D, pH, gelrite, immature cotyledon, plantlet regeneration.

Abstract

To enhance *in vitro* plantlet regeneration efficency of soybean through embryogenesis, the culture conditions such as material part and size of immature seed, 2,4-D, pH and solidifying agents for somatic embryogenesis were investigated. Somatic embryogenesis was induced from the immature embryo, immature cotyledon and embryonic axis explants of the immature seed on MS medium supplemented with 2.0 mg/L 2,4-D. The highest rate (up to 22.9%) of somatic embryogenesis was obtained from the immature cotyledon, following embryonic axis and the immature embryo. The rate varied with the developmental stages of seed. The maximum rate (25.4%) of embryogenesis was obtained from 3-4 mm length of the seed (after 25 days of flowering). The optimum concentration of 2,4-D for embryogenesis was 10 mg/L. The optimum pH was at 5.8 and solidifying agent for medium was better with 0.4% gelrite than with agar. For rapid multiplication of shoot tips from the germinating somatic embryos, they were cultured on MS medium containing 2 mg/L indole-3-butyric acid (IBA) and 1 mg/L 6-benzyladenine (BA). After then somatic embryos with one and three cotyledons were transferred to the growth regulator free medium. The medium exhibited the higher rate (ca. 50%) of development than the multiplication medium.

Introduction

Soybean [*Glycine max* (L.) Merrill] has been regenerated *in vitro* from several explant sources by both somatic embryogenesis (Barwale et al., 1986; Ranch et al., 1985) and shoot differentiation (Barwale et al., 1986; Wright et al., 1986). Somatic embryos have been obtained from immature cotyledons of zygotic seed (Lippmann and Lippmann, 1984; Lazzeri et al., 1985; Barwale et al., 1986; Anna and Waclaw, 1994; Sohn and Bae, 1995; Rajasekaran and Pellow, 1997; Santarem et al., 1997), and immature embryos of zygotic seed (Ranch et al., 1985; Buchheim et al., 1989; Choi et al., 1994). The regeneration by shoot differentiation has been documented from the several explants such as the cotyledon and primary leaf nodes (Barwale et al., 1986; Wright et al., 1987; Kim et al., 1990). Compared with another species, it demanded a higher concentration of 2,4-D (Li et al., 1985; Ghazi et al., 1986; Tetu et al., 1987; Finer and Nagasawa, 1988; Wright et al., 1991; Santarem et al., 1997).

Somatic embryogenesis and shoot differentiation for the regeneration can be utilized for genetic transformation works. Tissues undergoing shoot differentiation have been transformed by using *Agrobacterium* (Hinchee et al., 1988) and particle bombardment (McCabe et al., 1988), while proliferous embryogenic cultures have been transformed only via particle bombardment (Finner and McMullen, 1991; Sato et al., 1993; Parrot et al., 1994). In spite of all these reports for soybean transformation, the methods are far from routine. A more useful and efficient system for soybean transformation may rely on a new system where the regeneration is proce-

* Corresponding author, E-mail; kimkh@nhaes.go.kr
Received Apr. 20, 2000; accepted Jun. 26, 2000

eding directly and rapidly. One soybean tissue culture system that has not been extensively evaluated in transformation studies is an induction of the somatic embryos. The proliferous embryogenic cultures will enhance the efficiency for transformation. The factors such as material part, immature seed size, medium pH and solidifying agent in media on somatic embryogenesis have not been fully investigated. In the present study, we provide an efficient method for regeneration of soybean plants through somatic embryogenesis.

Materials and Methods

Experimental material

The seeds of soybean [*Glycine max* (L.) Merrill cv. Iksannamulkong] were obtained from National Honam Agricultural Experiment Station R.D.A.. Soybean plants were cultivated in a greenhouse under 14h-light/10h-dark photoperiod at 25℃. The immature pods containing embryos (1.5-6 mm in length) were harvested 15 to 35 days after flowering.

Culture conditions

The seeds ranging a 1.5 to 6 mm in length were excised from pods which had been surface sterilized with 70% ethanol for 1 min and with 1% sodium hypochlorite solution for 10 min, and then subsequently rinsed 4 times with sterile deionized water. The immature seeds were taken by taking the seed coat off the ovules by cutting next to the hilum which insured an intact embryo. The MS (Murashige and Skoog, 1962) media supplemented with 2,4-D (0, 0.5, 1, 2, 5, 10, 20, 40 mg/L) and 3% sucrose, and with either agar (0.6, 0.8, 1.0, 1.2%) or gelrite (0.2, 0.3, 0.4, 0.5%) as the solidifying agent adjusted to pH 5.0, 5.8, 7.0, 8.0 were used. The explant were cultured in the dark condition at 25±1℃ for eight weeks. The somatic embryos formed as a result of direct embryogenesis on somatic embryo forming medium. The shoots formed on shoot forming medium supplemented with 2 mg/L IBA and 1 mg/L BA and the roots formed on root forming medium were transferred to the regenerating media. The cultures were maintained for 4 weeks at 25±1℃ under 16:8 h (light : dark) photoperiod with a light intensity of 80 μE $m^{-2}s^{-1}$ provided by white fluorescent lamps in an environmentally controlled incubation room. After the shoots were grown at a height of about 1 cm, they were transferred to culture bottles containing 30 mL of the growth requlator free medium for the rootings.

Analysis of embryo formation

Primary somatic embryos were clearly distinguished on the surface of the explant materials (Figure 6 ABCD). Means of somatic embryogenesis rates were calculated on the basis of induction medium after 6 weeks. Each value represented 50-70 replicates, and each experiment was repeated at five times.

Results and Discussion

Comparison of somatic embryogenesis rates among immature embryo, embryonic axis, and immature cotyledon explants

The rates of somatic embryogenesis induced from material parts (immature embryo, immature cotyledon and embryonic axis) of soybean seed on MS solidify medium containing 2.0 mg/L 2,4-D, 0.8% agar, and 3% sucrose were compared (Figure 1). After inoculation for 2 to 3 weeks, the pale yellow and friable callus was formed and then, the somatic embryos were formed on the callus. The embryogenesis rate (22.9%) of immature cotyledon was the highest, followed embryogenic axis (17.6%) and immature embryo (5.5%). It has been reported that the immature embryo has a higher potential of embryogenesis than other explants (Choi et al., 1994; Li et al., 1985). On the other hand, it has been reported that immature cotyledon has a higher potential of embryogenesis than other explants (Anna and Maclaw, 1994; Bailey et al., 1993; Buchheim et al., 1989; Santarem et al., 1997). These results may due to physiological background of the explant tissue according to the aging.

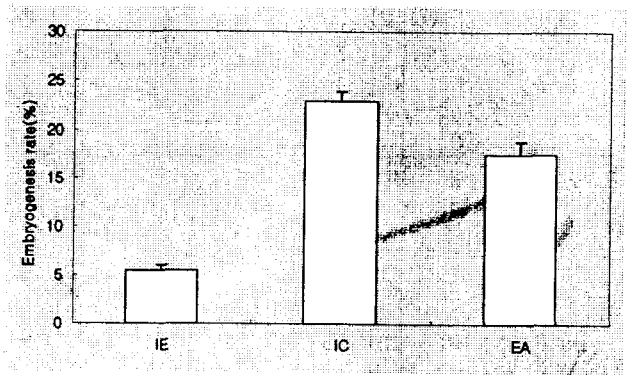

Figure 1. Effect of the material parts on callus and somatic embryogenesis from immature soybean seed. IE: immature embryo; IC: immature cotyledon; EA: embryonic axis. Vertical bars represent SEs (n=5).

Effect of the size (aging) of immature seed on embryogenesis

According to immature seed size at developmental stage after flowering, the cotyledon explants were innoculated on the solid (agar, 0.8%) MS medium and cultured for one month. The embryogenesis rate (25.4%) of the 3-4 mm explant (after 25 days of flowering) was higher than that (18.7%) of the 1.5-3 mm explant (after 15-20 days of flowering), but the rates exhibited a decreasing tendency according to the size (after 30 days of flowering) (Figure 2). This result is coincident with other reports (Barwale et al., 1986; Buchheim et al., 1989; Choi et al., 1994; Rajasekaran and Pellow, 1997; Santarem et al., 1997; Sohn and Bae, 1995). The difference in the rates may due to the structural turnover of cell-wall polysaccharides during somatic embryogenesis of celery (Yeo et al., 1999). It also suggests that the difference is related with the degree of differentiation of embryogenic cells.

Effect of 2,4-D on callus formation and embryogenesis

Soybean embryogenesis demands a high 2,4-D (Ghazi et al., 1986; Liu et al., 1992; Rajasekaran and Pellow, 1997; Santarem et al., 1997). To understand the relationship between differentiation and embryogenesis, the cotyledon explants were innoculated on the media supplemented with different concentrations of 2,4-D and cultured for one month. The effects on callus formation and embryogenesis were investigated (Figure 3). The low concentrations (0.5-5.0 mg/L) exhibited a high callus formation rate (above 93%), while the high concentrations (10-40 mg/L) exhibited a low callus formation rate. However, the embryogenesis rate (37.5%) was the highest in the high (10 mg/L) medium, although the medium had lower callus formation rate (82%). In a soybean immature cotyledon culture, 2,4-D demand of this variety (Iksannamulkong) is different greatly from that of other varieties, which demands a low concentration (1-2 mg/L) of 2,4-D (Choi et al., 1994; Sohn and Bae, 1995). The difference in 2,4-D demand further will be investigated.

Effect of pH on embryogenesis

Effect of pH on the embryogenesis rate was investigated (Figure 4). The immature cotyledon explant was innoculated on the medium adjusted to pH 5.0, 5.8, 7.0, 8.0 for one month. The em-

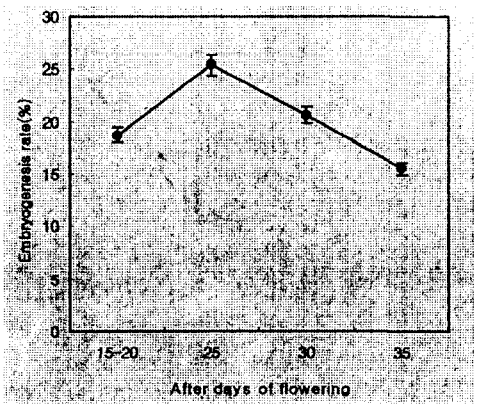

Figure 2. Effect of the developmental flowering stages of seeds serving as explant sources on somatic embryogenesis from immature cotyledons of soybean seed. Vertical bars represent SEs (n=5).

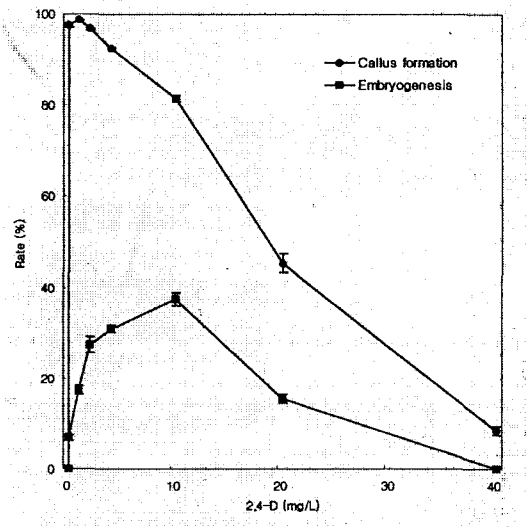

Figure 3. Effect of 2,4-D on callus formation and embryogenesis from immature cotyledons of soybean seed. Vertical bars represent SEs (n=5).

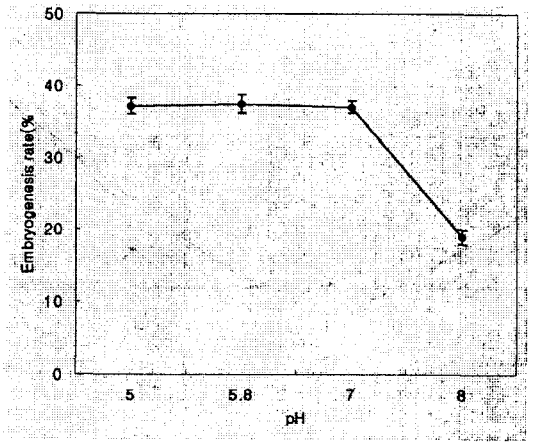

Figure 4. Effect of media pH on somatic embryogenesis from immature cotyledons of soybean seed. Vertical bars represent SEs (n=5).

bryogenesis rates were similar at pH 5.0 to 7.0, showing ca. 37%, while pH 8.0, weak alkali repressed to a half (19%). This result means that the soybean is one of weak acidic crops. Santaren et al. (1997) had reported that optimum pH for the soybean somatic embryogenesis was 7.0.

Effects of solidifying agents on embryogenesis rate

Effects of solidifying agents (agar; 0.6, 0.8, 1.0, 1. 2% and gelrite; 0.2, 0.3, 0.4, 0.5%) of the medium on the embryogenesis rate were investigated (Figure 5). Concentrations of agar 0.8 to 1.0% exhibited the higher embryogenesis rates (37.4 and 39.1%) than low agar (0.6%) and high agar (1.2%). On the other hand, concentrations of gelrite 0.3 to 0.4% exhibited the higher embryogenesis rates (45.2 and 45.3%, respectively) than that low gelrite (29.3%) and high gelrite (35.7%). Gelrite was more affective than agar for embryogenesis ($P<0.05$). The callus formation rates were similar to the concentrations of each solidifying agent. Huang et al. (1995) and Santaren et al. (1997) reported that gelrite was also more affective than agar. It also suggests that the embryogenesis and development are related to the water contents of the callus.

Plant regeneration

Rapid multiplication of shoot tips from the germinating somatic embryos was achieved on MS medium supplemented with 2 mg/L IBA and 1 mg/L BA (Townsend and Thomas, 1996). When somatic embryos with one to three cotyledons were transferred to the plant growth regulator free medium, those with cotyledons converted to the plantlets at a higher frequency (50%) than in the others media. It took 4 to 6 weeks for shoots to appear on the somatic embryos under selection, but it took only one month under non-selective conditions. The roots were differentiated after 2-3 weeks (Figrue 6H). When the shoots were longer than 5 mm, they were transferred to the rooting medium and regenerated to the plantlets (Figure 6I).

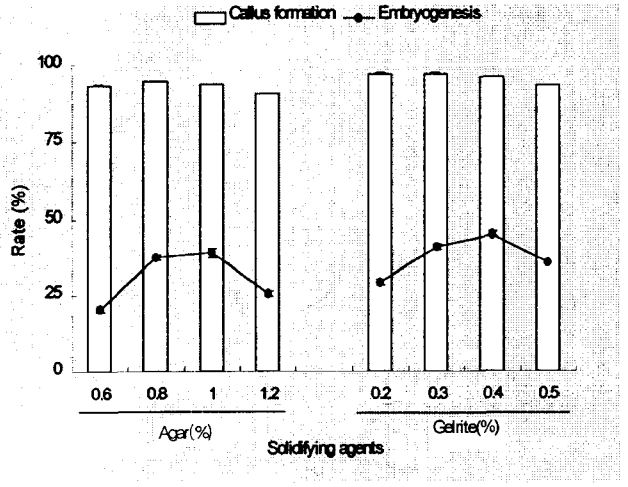

Figure 5. The effect of solidifying agent and concentration on somatic embryogenesis from the immature cotyledons of soybean seed. Vertical bars represent SEs (n=5).

References

Anna NO, Waclaw O (1994) New aspects of soybean somatic embryogenesis. Euphytica 80: 137-143.

Bailey MA, Boerma HR, Parrott WA (1993) Genotype-specific optimization of plant regeneration from somatic embryos of soybean. Plant Sci 93: 117-120.

Barwale UB, Kerns HR, Widholm JM (1986) Plant regeneration from callus cultures of several soybean genotypes via embryogenesis and organogenesis. Planta 167: 473-481.

Buchheim JA, Colburn SM, Ranch JP (1989) Maturation of soybean somatic embryos and the transition to plantlet growth. Plant Physiol 89: 768-775.

Choi PS, Soh WY, Cho DY, Liu JR (1994) Somatic embryogenesis in immature zygotic embryo cultures of Korean soybean (*Glycine max* L.) cultivars and effect of 2,4-D on somatic embryo morphology. Korean J Plant Tissue Culture 21: 7-13.

Finer JJ, McMullen MD (1991) Transformation of soybean via particle bombardment of embryogenic suspension culture tissue. *In Vitro* Cell Dev Biol 27: 175-182.

Finer JJ, Nagasawa A (1988) Development of an embryogenic suspension culture of [*Glycine max.* (L.) Merrill]. Plant Cell Tissue Organ Cult 15: 125-136.

Ghazi TD, Cheema HV, Nabors MW (1986) Somatic embryogenesis and plant regeneration from embryonic callus of soybean, *Glycine max* L. Plant Cell Reports 5: 452-456.

Hinchee MA, Connor-Ward DV, Newell CA (1988) Production of transgenic soybean plants using *Agrobacterium*-mediated DNA transfer. Biol/Technology 6: 915-922.

Huang LC, Kohashi C, Vangundy R (1995) Effects of common components on hardness of culture media prepared with Gelite™. *In Vitro* Cell Dev Biol 31: 84-89.

Kim JH, LaMotte GE, Hack E (1990) Plant regeneration in vitro from primary leaf nodes of soybean (*Glycine max*) seedlings. Plant Physiol 136: 664-669.

Lazzeri PA, Hildebrand DF, Collins GB (1985) A procedure for plant regeneration from immature cotyledon tissue of soybean. Plant Mol Biol Reports 3: 160-167.

Li BJ, Langridge WHR, Szalay AA (1985) Somatic embryogenesis and plantlet regeneration in the soybean *Glycine max*. Plant Cell Reports 4: 344-347.

Lippmann B and Lippmann G (1984) Induction of somatic embryos in cotyledonary tissue of soybean *Glycine max* L. Merr. Plant Cell Reports 3: 215-218.

Liu W, Moore PJ, Collins GB (1992) Somatic embryogenesis in soybean via somatic embryo cycling propagation. *In Vitro* Cell Dev Biol 28: 153-160.

McCabe DE, Swain WF, Mertinell BJ (1988) Stable transformation of soybean (*Glycine max*) by particle acceleration. Bio/Technology 6: 923-926.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco cultures. Physiol Plant 5: 473-479.

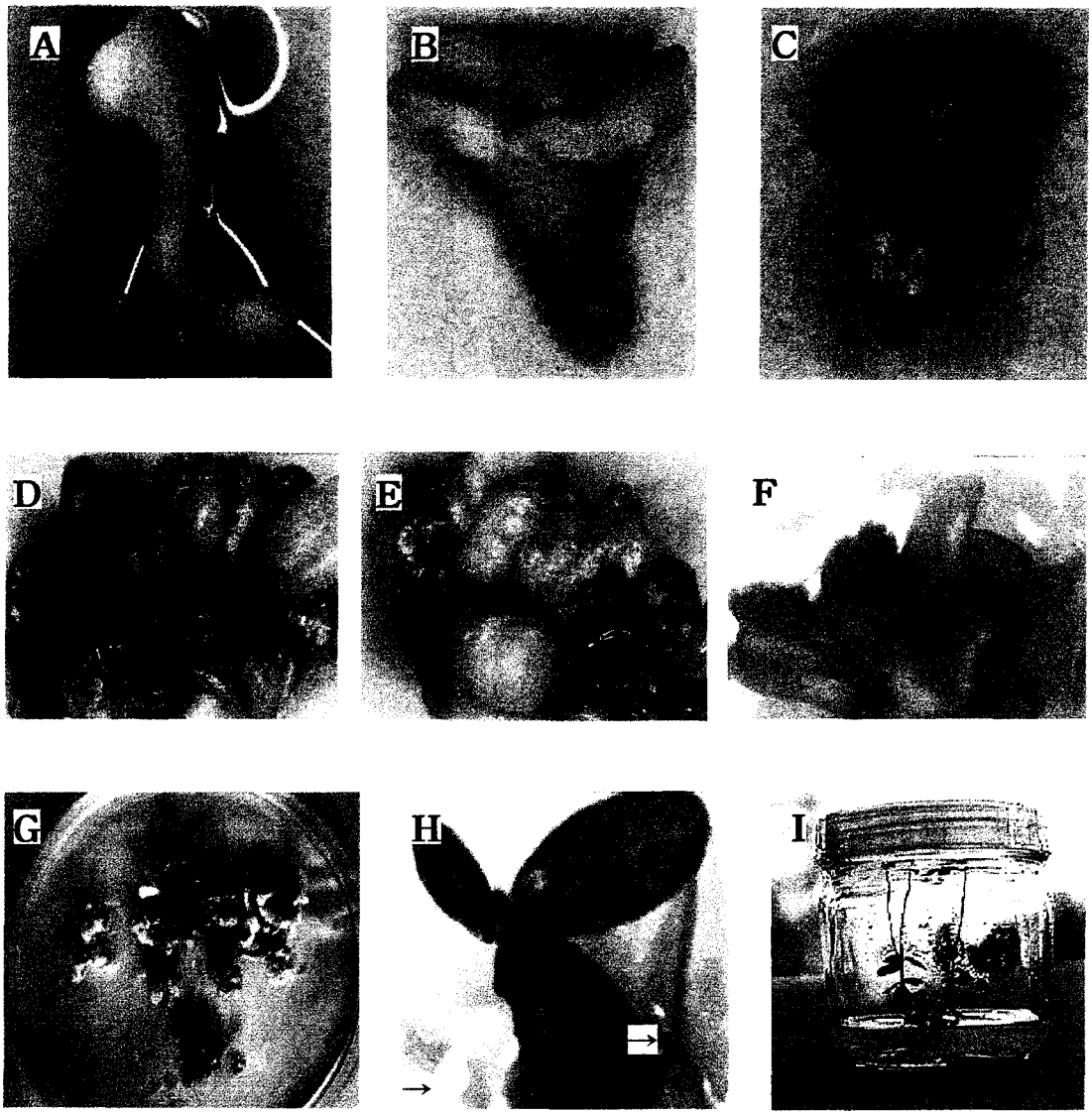

Figure 6. Somatic embryogenesis and plant regeneration in tissue cultures of the immature soybean (*Giycine max* cv. Iksannamulkong) seed. A: Somatic embryo with one cotyledon; B: Somatic embryo with two cotyledons; C: Somatic embryo with three cotyledons; D: Somatic embryos at the globular stage; E: Secondary globular embryos forming on the primary somatic embryo explants; F: Secondary somatic embryo at the cotyledonary stage; G: Germination of the somatic embryos; H: Rooting (arrows) of the regenerated shoot; I: Regenerated plant.

Noggle GR, Fritz GJ (1983) Introductory Plant Physiology. Second Edition. Prentice-Hall p372.

Parrot WA, All JN, Adang MJ (1994) Recovery and evaluation of soybean plants transgenic for a *Bacillus thuringiensis* var. Kurstaki insecticidal gene. *In Vitro* Cell Dev Biol 30: 144-149.

Rajasekaran K, Pellow JW (1997) Somatic embryogenesis from cultured epicotyls and primary leaves of soybean [*Glycine max*. (L.) Merrill]. *In Vitro* Cell Dev Biol-Plant 33: 88-91.

Ranch JP, Oglesby L, Zielinski AC (1985) Plant regeneration from embryo-derived tissue cultures of soybean. *In Vitro* 21: 653-658.

Santarem ER, Pelissier B, Finer JJ (1997) Effect of explant orientation, pH, solidifying agent and wounding on initiation of soybean somatic embryos. *In Vitro* Cell Dev Biol-Plant 33: 13-19.

Sato S, Newell C, Kolacz K (1993) Stable transformation via particle bombardment in two different soybean regeneration systems. Plant Cell Reports 12: 408-413.

Sohn JK, Bae JS (1995) Somatic embryogenesis and plant regeneration from in vitro cultured immature cotyledons of soybean. Korean J Breeding 27: 339-344.

Tetu T, Sangwan-Norreel B, Sangwan R (1987) *In vitro* somatic embryogenesis and plant regeneration in three precocious varieties of soybean. CR Seances Acad Sci Ser 3: 613-617.

Townsend JA and Thomas LA (1996) Transformation and re-

generation via cotyledonary nodes. http://mars.cropsoil.uga.edu/homesoybean/cotprot.htm.

Wright MS, Koehler SM, Hinchee MA (1986) Plant regeneration by organogenesis in *Glycine max*. Plant Cell Reports 5: 150-154.

Wright MS, Launis KL, Novitzky R (1991) A simple method for the recovery of multiplefertile plants from individual somatic embryos of soybean [*Glycine max*. (L.) Merrill]. *In Vitro* Cell Dev Biol 27: 153-157.

Wright MS, Ward DV, Hinchee MA (1987) Regeneration of soybean [*Glycine max*. (L.) Merrill] from cultured primary leaf tissue. Plant Cell Reports 6: 83-89.

Yeo UD, Han JY, Choi YE (1999) Turnover of cell-wall polysaccharides during somatic embryogenesis and development of celery (*Apium graveolens* L.). J Plant Biol 42: 8-15.