Journal of Plant Molecular Breeding (JPMB)
Vol. 3/No. 1/ June 2015/

High-throughput direct regeneration of Soybean mutant and common lines from cotyledonary node

M.Younessi-Hamzekhanlu[1], A. Izadi-Darbandi[1*], M.A Malboubi[2] & M. Ebrahimi[3]

1. Department of Agronomy and Plant Breeding Sciences, College of Aburaihan, University of Tehran, Tehran-Pakdasht, Iran. Postal code: 3391653755.
2. Department of Plant Biotechnology, National Institute of Genetic Engineering and Biotechnology,Tehran, Iran, P.O. Box: 14965/161.

*Corresponding Author, Email: aizady@ut.ac.ir

Received: January 2015 Accepted: April 2015

Abstract

Regeneration through mature cotyledonary node has set rapid regeneration of plants directly from explants which is more time-saving and presented as an effective strategy. So we have evaluated regeneration protocol through single shoot using cotyledonary node as a rapid and efficient protocol for two soybean cultivars and one mutant line. Cotyledonary nodes explants obtained from 7-day-old *in vitro* seedlings. After 28 days, the percent of regeneration and after 42 days, regeneration area were calculated. The results showed that percent of regeneration and regeneration area of mutant line was significantly more than two cultivars, L17 and Williams. After shoot induction, plants were transferred to shoot elongation medium followed by transferring plants to rooting medium. The percent of rooting was calculated during 5-14 days. The results showed that the percent of rooting was not significantly affected by genotypes. In another experiment to test kanamycin sensitivity of regenerated shoots, it was found that kanamaycin with 150 mg/L concentration is lethal for regeneration of soybean shoots from cotyledonary node explants. The results showed that regeneration efficiency of mutant line was significantly more than two other cultivars. Kanamaycin sensitivity of regenerated shoots showed that kanamycin at 150 mg/L or above can be used as a selective agent for all three tested cultivars transformation.

Key words: Soybean, Cotyledonary node, Regeneration, Mutant

Introduction:

Soybean [*Glycine max* (L.) Merrill] is one of the most important protein and oil crops worldwide (Ma and Wu, 2008). It is projected that at the current yield level, world soybean production will rise to 311.1 million metric tons by 2020 (Masuda and Goldsmith, 2009). With the rise in world population, food production has to be increased. In order to boost the production of crops, including soybean, a sustainable crop improvement is needed to overcome the challenges of biotic and abiotic stresses, such as salt, drought, water-logging, high and low temperatures, diseases, weeds and insect pests. Traditional methods of soybean improvement are

very lengthy and less effective against multiple stress factors. With the development of plant molecular biology and genetic engineering, its transformation has become one of the core issues in molecular breeding. First requirement for the successful application of biotechnology in crop improvement is to have efficient plant regeneration from cultured cells and tissues. (Haliloglu, 2006). *In vitro* and molecular methods are being devised and used to enhance crop improvement process by using plant tissue culture, recombinant technology and marker-assisted selection (Paz *et al*, 2006). Tissue culture and plant regeneration are required to generate transgenic plants, and these techniques open new possibilities for improving soybean (Kita *et al*. 2007; Paz, 2004). Improvement of crops through plant tissue culture can be accomplished by growing plants in a laboratory through callus initiation and regeneration of shoots and roots or regeneration of multiple plants directly from small pieces of leaves, shoots, seeds, embryos or cotyledons. Numerous methods have been introduced for soybean regeneration over the last several decades that are based on direct regeneration of shoots and roots from cotyledonary node (Arun *et al,* 2014; Barvale *et al*, 1986). Several factors affect the regeneration process including; selection of the explants, media composition, environmental conditions and the genotype of the soybean cultivar. Genotype plays a critical role in soybean regeneration through tissue culture along with the choice of different growth hormones it appears that selecting the proper medium can overcome genotype-associated problems with regeneration in the soybean whole cotyledonary node regeneration system (Ma and Wu, 2008; Paz, 2006). Gamma irradiation has been widely used in the biological study of plants, including tissue culture. Gamma ray had been reported to induce cytological, biochemical, genetical, physiological and morphogenetic changes in cells and tissue, thus influencing the growth and development of the plants (Mujar *et al*, 2014).

Mutation breeding in crop plants is an effective tool in the hands of plant breeders especially in crops having narrow genetic base. Many mutants have been identified as donors of desirable traits in breeding program. Mutation breeding works in soybean resulted in identification of many mutant lines with desirable traits like high germination and survival percent (Younessi-Hamzekhanlu *et al.*, 2011). Induced mutation may broaden genetic variants and provide materials for plant improvement and facilitate tissue culture response of plants such as soybean. Since mutant line would be a great source of useful germplasm in high efficiency regeneration of soybean from cotyledonary node; the objectives of this study were assessment of gamma irradiation effect on soybean regeneration from cotyledonary node, optimization of direct regeneration for soybean mutant line (M7) and L17 with William cultivar and verifying the lethal

level of kanamycin as a selected marker on soybean explants.

Materials and methods

Plant material

Two soybean cultivars (L17 and William) and one M_7 generation of soybean mutant line were utilized in these experiments. Mutant line obtained from Agricultural, Medical and Industrial Research School, Nuclear Science and Technology Research Institute, Atomic Energy Organization of Iran that previously evolved by gamma ray (cobalt-60) from cultivar L17 irradiated with doses of 250 Gray (absorbed dose). In our previous work (Younessi-Hamzekhanlu *et al.*, 2011) this mutant line was compared with other mutant lines (32 mutant lines) and parental cultivar L17 in view of some important traits such as number of grain per plant, number of pod per plant, plant dry weight (shoot dry weight), root dry weight, harvest index, number of nodule per plant, nodule dry weight. The results showed that this mutant line is better than others in view of mentioned traits. In other experiment this mutant line showed high N2 fixation character thus because of good characters of this mutant line we used that in this experiment.

Seed sterilization and germination

Soybean seeds were surface sterilized for 24 hours using chlorine gas produced by mixing 3.5 ml of 12N HCl with 100 ml commercial bleach; 5% sodium hypochlorite (Di *et al.*, 1996). Sterilized seeds were germinated on full-strength B5 major and minor salts, MSIII iron, pH 5.8 that were solidified with 0.6% agar. Fifteen soybean seeds were germinated in a 100×25 mm petri dish with the hilum proximal to the media. Dishes were wrapped with parafilm to prevent from opening as the seedlings grow thus, avoiding contamination. Germination plates were incubated in a growth chamber under 18-hr photoperiod at 140 μmoles sec^{-1} m^{-2} light intensity (24 ˚C) for 4-7 days, or until the cotyledons became green and seed coat split opened, but before the first leaves expanded to the length of the cotyledons (Olhoft *et al*. 2003).

Explants preparation and regeneration

Roots and the major portion of the hypocotyls approximately 3-5 mm below the cotyledonary node on the hypocotyls were removed, separating the cotyledons. A vertical cut through the remaining hypocotyls was made with a surgical blade. The epicotyl was subsequently removed and 30 such explants from two cultivars and one mutant line were plated adaxial side on shoot induction medium (SIM) (full strength B5 medium supplemented with 3% sucrose, 0.8% agar and pH 5.6) having constant concentration of BAP (1.67 mg L^{-1}). The explants were kept for 14 days under above mentioned culture conditions. Shoots were counted after 14 days of culture. After 28 days in SIM, photo was taken from regenerated explants and the regenerated area calculated using MATLAB software. The green parts of the regenerated explants were considered as a regenerated area. Explants with shoot buds were

transferred onto B5 medium for shoot elongation (SEM) (full B5 medium supplemented with 3% sucrose, 0.1 mg L^{-1}IAA, 0.5 mg L^{-1}GA3, 1 mg L^{-1}ZR, solidified with 0.8% agar) and explants were kept for 14 days under above-mentioned culture conditions. The explants were subcultured onto the same fresh shoot elongation medium for next 14 days. Elongated shoots roughly 3–4 cm in length were detached and rooted on root induction medium (RIM) (1⁄2-strength B5 major and minor salts, full-strength MSIII iron, 2% sucrose, 1 mg L^{-1}IBA, adjusted to pH 5.6 with 1*N* KOH and solidified with 0.8% Noble Agar) for 5 to 14 days and after this period rooted plants were counted. Once a small root was formed on the shoot, the plantlets were transferred directly to large pots (2.5 gallons or larger) filled with a mix of 1⁄2 pasteurized top soil (sandy loam):1/4 perlite:1/4 peat moss in the greenhouse. The greenhouse conditions were set to 28°C with a 16/8-h (light/dark) photoperiod at 140 μmoles sec^{-1} m^{-2} light intensity. To reduce moisture loss and to protect the shoot from the surrounding environment, the plantlets were covered with a clear plastic container and removed when the first set of new leaves opened.

Kanamycin sensitivity test

The explants derived from cultivars L17 and Williams and one mutant line were cultured in a factorial experiment based on completely randomized design (CRD). Different concentrations of kanamycin sulfate (0, 25, 50, 75, 100, 125 and 150 mg/l) were tested for 3 different soybean genotypes to screen optimal lethal level of kanamycin.

Experimental design and statistical analysis

Two cultivars L17 and Williams and one mutant line were supposed as a treatment. The experiment was repeated three times to compare each cultivar on efficiency of regeneration protocol. Data were analyzed using one-way analysis of variance (ANOVA) and the means were compared using Duncan's multiple range test.

Results

Comparisons among genotypes

Seeds of three cultivars were germinated on B5 medium for 6 days (Fig. 1a). The germination frequency of L17, Williams and one mutant line were 99.2, 98.8, and 99, respectively. There was no significant difference between genotypes in view of germination frequency at 5% level.

Sterile seedlings were collected after they germinated for 6 days on B5 medium. The whole cotyledonary node was detached from each seedling and transferred to shoot induction medium.

The influence of genotypes (L17 and Williams and one mutant line) was examined to determine their effects on shoot regeneration. All three genotypes induced shoot growth within 2 weeks. ANOVA showed that all three genotypes had significantly different effects on shoot regeneration from whole cotyledonary node ($P<0.01$) (Table 1).

These genotypes had various effects and in our study, genotype differences were

observed with regard to shoot induction formation. However, mutant line was the best at inducing shoot growth followed by L17, and Williams (Fig. 2). After 28 days in SIM, the regenerated area of genotypes was analyzed and the results showed that all genotypes had significantly different effects in view of regenerated area (Table 1). However, mutant line was the best in view of regenerated shoot area followed by L17, and Williams (Fig. 3).

After 28 days in SIM (Fig. 1b), the explants were placed in SEM (Fig. 1c). Individual elongated shoots (>3 cm) from cotyledonary node explants were separated and cultured on root induction medium (RIM) (Fig. 1d). ANOVA showed that all three genotypes did not significantly different effects on root induction regeneration from elongated shoots (Table 1).

Plantlets were planted in large pots filled with mixture of soil, coco peat and perlite (2:1:1) in greenhouse (Fig. 1e). We have found that a hardening-off period in a smaller pot is not necessary in our hands, and it is not worth the time it takes to do two transfers. We found that if environmental conditions be optimal, greater than 90% of the rooted shoots develop into healthy fertile plants (Fig. 1e). Results showed that all three genotypes had no significantly different effects on acclimatization of rooted plantlets.

Kanamycin sensitivity test results

Different concentrations of kanamycin (25, 50, 75, 100, 125 and 150 mg/L) were checked during shoot induction and shoot elongation stages**.** Concentrations 25, 50, 75, 100 and 125 mg/L did not affect inducted shoots. Concentration 125 mg/L caused somewhat browning in regenerated leaves but concentration 150 mg/L caused complete browning in regenerated leaves as a result they did not continued their development .

Of two cultivars and one mutant line tested, all of them had similar sensitivity to kanamycin (Table 2). The kanamycin lethal concentration (150 mg/l) indicated that cotyledonary-node explants, from all three cultivars shoots were unable to form any shoot after being treated with that lethal doses (Fig. 4). So, kanamycin at 150 mg/L or above can be used as a selective agent for all three tested cultivars transformation.

Discussion

Researchers have proved that cotyledonary node is a suitable candidate explants for shoot regeneration in soybean cultivars (Oholf *et al*. 2007). Two cultivars (L17 and Williams) and one soybean mutant line were used in the present study to standardize the regeneration protocol, which showed that the protocol is genotype independent. This study showed that explants obtained from mutant line are more prone to regenerate from cotyledonary node. Radiation mutation breeding combined with tissue culture has made a significant contribution to plant breeding. They have introduced new techniques for inducing genetic variation, by improving selection technology and accelerating breeding

time. Higher efficiencies in root formation and regeneration of explants were observed in mutant line compared to other two genotypes. Mutation induction has become a recognized tool in crop improvement to supplement conventional breeding efforts to improve cultivars in certain specific traits (Patade and Suprasanna, 2008). More than 2500 improved mutant varieties have been released for commercial cultivation in different crop species demonstrating the economic value of the mutation breeding technology. Plant mutagenesis has the ability to generate great interest in creating novel genetic variability such as good agronomic traits and other traits like a good response to tissue culture conditions (Hasbullah *et al*, 2012). Addition of suitable tissue culture related traits such as higher frequency regeneration ability by using mutation and followed by *in vitro* regeneration of transformed plants using gene transferring methods can lead to high frequency transformation.

The positive response of soybean mutant line explants to gamma irradiation agrees with the hypothesis proposed by Fowler and MacQueen (1972), implying that most of the stimulatory effects of irradiation result in increased regeneration and root formation. Measured increases of genetic diversity and some other characteristics in tissue culture due to irradiation have also been reported by Kang *et al* (2013), Yaycili and Alikemanoglu (2012) and Maghraby (1987) for chrysanthemum, potato and soybean, respectively. The increased shoot regeneration and root formation caused by mutant explants could be explained by stimulation of biosynthesis of some amino acids modification and increase in primary biochemical processes, uptake of mineral nutrients and photosynthesis (Charbaji and Nabulsi, 1999).

A research done on two commercial red peppers found that gamma irradiations increased the photosynthetic rate in plant tissue culture.18 Internal metabolism in plant depend highly on photosynthesis because photosynthesis is the fundamental source of energy for growth and development. If the photosynthesis rate increases, so does the plant metabolism. However, this only applies for a low dose of gamma irradiation because photosynthesis process is sensitive toward heavy metal, where it disturbs the chloroplast function rendering it useless in CO_2 fixation. (Mujar *et al*, 2014).

Mujar and *et al* (2014) found that another reason for increasing the regeneration of vanilla cultures by gamma irradiation is that radiation significantly influences the protein synthesis and cell metabolism in plant meristem cells. Plants respond to gamma irradiation by developing a defense mechanism that increases the total soluble protein content by altering the protein metabolism, thus influencing the plant cell metabolism and development (Humera, 2006).

Use of X-ray radiation on *in vitro* grown shoots of olive was elaborated. According to the measured morphological responses, treatments caused an increase in the growth

parameters such as shoot weight, shoot height, and rooting efficiency (Orazem *et al* 2013).

The response of soybean cultivars to kanamycin concetration agrees with the work done by Anita *et al* (2012), they used sub-lethal dose of kanamycin (100–175 mg/L). They found that the transformed plants were recovered with a success rate of 0.91%.

This was the first study on the comparison regeneration of mutant line and two soybean cultivars that are commonly cultivated in Iran using cotyledonary node as explants. Some differences were observed in genotypes with regard to the shoot regeneration and root formation.

Overall, the regeneration period has been shortened to only 40-45 days, which is the shortest duration of soybean regeneration. Beside other previous works this standardized regeneration system will be compatible with Agrobacterium-mediated soybean transformations, and offer newer strategies for the soybean transformation.

Acknowledgements

This work has been supported by the Center for International Scientific Studies and Collaboration (CISSC), Authors would like deeply appreciate for their support.

References

Anita, R., Kuldeep, V. and Raman, S. 2012. Recovery of fertile transgenic plants via Agrobacterium tumefaciensmediated transformation in Indian soybean (Glycine max L. Merrill) cultivar. *Indian Journal of Genetics and Plant Breeding* 72(3): 325-331.

Arum, M., Subramanyam, k., Theboral, J. and Ganpathi, A. 2014. Optimized shoot regeneration for Indian soybean: the influence of exogenous polyamines. *Plant Cell Tiss Organ Cult, 117(2): 305-309.*

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

Charbaji, T. and Nablusi, I. 1999. Effect of low doses of gamma irradiation on in vitro growth of grapevine. *Plant Cell Tiss Organ Cult*, 57:129-132.

Di, R., V. Purcell, G.B. Collins & S.A. Ghabrial, 1996. Production of transgenic soybean lines expressing the bean pod mottle virus coat protein precursor gene. *Plant Cell Rep* 15: 746–750.

Fowlere, D.B. and Macqueen, K.F. 1972. Effect of low doses of gamma radiation on yield and other agronomic characters of spring wheat (Triticum aestivum). *Radiat. Bot.* 12~ 349-353.

K. Haliloglu. 2006. Efficient regeneration system from wheat leaf base segments . *Biologia Plantrum* 50 (3): 326-330.

Hasbullah, N.A., Taha, R.M., Saleh, A. and Mahamad, N. 2012. Irradiation effect on *in vitro* organogenesis, callus growth and plantlet development of Gerbera jamesonii. *Horticultura Brasileira 30:* 252-257.

Humera, A. 2006. Biochemical and Molecular Markers of Somaclonal Variants and Induced Mutants of Potato (*Solanum tuberosum* L.). An unpublished PhD dissertation.University of the Punjab Lahore, Pakistan.

Kang, E.J., Lee, Y.M., Sung, S.Y., Ha, B.K., Kim, S.H., Kim, D.S., Kim, J.B. and Kang, S.Y. 2013. Analysis of the Genetic Relationship of Gamma-

irradiated in Vitro Mutants Derived from Standard-type *Chrysanthemum* cv. Migok. *Hort. Environ. Biotechnol*. 54(1):76-81.

Kita, Y., Nishizawa, K., Takahashi, M., Kitayama, M. and Ishimoto, M. 2007. Genetic improvement and regeneration in soybean and transformation of the improved breeding lines. *Plant Cell Rep* 26: 439-447.

Ma, X.H. and Wu, T.L. 2008. Rapid and efficient regeneration in soybean [*Glycine max* (L.) Merrill] from whole cotyledonary node explants. *Acta Physiol Plant* 30:209–216.

Maghraby, G.M. 1987. Effect of radiation and some micronutrients on some legumes. Thesis submitted in partial fulfilment of the requirements for the degree of Master of Science. Zagazig University (Banha Branch).

Masuda, T. and Goldsmith, P.D. 2009. World soybean production: Area harvested, yield, and long-term projections. *International Food and Agribusiness Management Review* 12: 143-162.

Mujar, E.K., Sidlik, N.J., Sulong, N.A., Jappar, S.S. and Othman, M.H. 2014. Effect of Low Gamma Radiation and Methyl Jasmonate on Vanilla planifolia Tissue Culture. *Int. J. Pharm. Sci. Rev. Res*., 27(1): 163-167.

Olhoft, P.M., Flagel, L.E., Donovan, C.M. and Somers, D.A. 2003. Efficient soybean transformation using hygromycin B selection in the cotyledonary-node method. *Planta*, 216(5): 723-735.

Orazem, P., Stajner, N. and Bohanec, B. 2013. Effect of X-ray irradiation on olive shoot culture evaluated by morphological measurements, nuclear DNA content and SSR and AFLP markers. *Trees*, 27:1587–1595.

Patade, V.Y. and Suprasanna, P. 2008. Radiation induced in vitro mutagenesis for sugarcane improvement. *Sugar Tech* (2008) 10(1): 14-19.

Paz, M.M., Martinez, J.C., Kalvig, A.B., Fonger, T.M., and Wang, K. 2006. Improved cotyledonary node method using an alternative explant derived from mature seed for efficient Agrobacterium-mediated soybean transformation. *Plant Cell Reports*, 25(3): 206-213.

Paz, M.M., Shou, H., Guo, Z., Zhang, Z., Banerjee, A. K., and Wang, K. 2004. Assessment of conditions affecting Agrobacterium-mediated soybean transformation using the cotyledonary node explant. *Euphytica*, 136(2): 167-179.

Yaycili, O. and Alikamanoglu, S. 2012. Induction of salt-tolerant potato (*Solanum tuberosum* L.) mutants with gamma irradiation and characterization of genetic variations via RAPD-PCR analysis. *Turk J Biol*, 36:405-412.

Younessi-Hamzekhanlu M., Izadi-Darbandi, A., Pirvali-Beiranvand, N., Halljian, M.T. and Majdabadi, A. 2011. Phenotypic and molecular analysis of M7 generation of soybean mutant lines through random amplified polymorphic DNA (RAPD) marker and some morphological traits. *African Journal of Agricultural Research*. 6(7)1779-1785.

باززایی مستقیم با کارآیی بالا در لاین‌های تجاری و جهش یافته سویا با استفاده از ریزنمونه گره لپه‌ای

مهدی یونسی حمزه‌خانلو[1]، علی ایزدی دربندی*[1]، محمد علی ملبوبی[2] و محسن ابراهیمیان[1]

۱- گروه علوم زراعی و اصلاح نباتات، پردیس ابوریحان، دانشگاه تهران، ایران

۲- گروه زیست‌فناوری گیاهی، پژوهشگاه ملی مهندسی ژنتیک و زیست‌فناوری، تهران ، ایران

*نویسنده مسئول: aizady@ut.ac.ir

چکیده

باززایی از طریق گره لپه‌ای منجر به یک سیستم باززایی مستقیم سریع در گیاهان شده است که نیاز به زمان کمتری داشته و یک روش موثر می‌باشد. در ا ین تحقیق پروتوکول باززایی اندام هوایی با استفاده از ریزنمونه گره لپه‌ای به عنوان پروتوکول سریع و کارآمد برای دو رقم تجاری و یک لاین جهش یافته سویا ارزیابی گردید. ریزنمونه‌های گره‌های لپه‌ای از گیاهچه‌های ۷روزه به دست آمدند که در شرایط درون شیشه‌ای رشد یافته بودند. بعد از ۲۸ روز، درصد باززایی و بعد از ۴۲ روز، سطح باززایی محاسبه شد. نتایج نشان داد که درصد باززایی و سطح باززایی لاین جهش یافته به طور معنی‌داری بیشتر از دو رقم تجاری سویا، L17 و ویلیامز می‌باشد. بعد از القای شاخه‌زایی، گیاهان به محیط طویل شدن شاخه و در ادامه آنها به محیط القای ریشه منتقل شدند. درصد ریشه‌زایی طی ۵ الی ۱۴ روز محاسبه شد. نتایج نشان داد که درصد ریشه‌زایی به طور معنی‌داری تحت تأثیر ژنوتیپ قرار نمی‌گیرد. در آزمایش جداگانه دیگری برای آزمون حساسیت شاخه‌های باززا شده به کانامایسین، مشخص گردید که غلظت ۱۵۰ میلی گرم در لیتر کانامایسین برای شاخه‌های باززا شده از ریزنمونه گره لپه‌ای کشنده می‌باشد. نتایج نشان داد که کارایی باززایی لاین جهش یافته به طور معنی‌داری بیشتر از دو رقم تجاری دیگر می‌باشد. حساسیت به کانامایسین شاخه‌های باززا شده نشان داد که غلظت‌های بالای ۱۵۰ میلی گرم در لیتر کانامایسین می‌تواند به عنوان عامل انتخابی در تراریزش لاین جهش یافته و دو رقم تجاری دیگر استفاده شود.

کلمات کلیدی: سویا، گره لپه‌ای، باززایی، جهش یافته.