MICROPROPAGATION JILIN SMALL GRANULE SOY TRANSFERRED GmMYB12A

Nguyen Van Phong[1], Bui Thi Thu Huong[2], QingYu Wang[3]
[1]*Vietnam National University of Forestry*
[2] *Vietnam National University of Agriculture*
[3]*Jilin University, China*

SUMMARY

The isoflavones of soybean, is a class of secondary metabolite, which affects cell division, new cell formation, and may enhance the nutritional value and thus guarantee better health. The soybean transferred the *Gm*MYB12A gene, containing high levels of isoflavones, which could improve the valuable soybean productivity, thus, the significant micropropagation was studied. The result revealed that the half-seed method, with proper disinfection time for Jilin small granule soy was used in 15 hours with chlorine gas produced by pouring 100 ml of 4% sodium hypochlorite into a beaker and adding 4 ml of 12 N hydrochloric acid. In vitro regeneration of Jilin small granule soy was succeeded through multiplication axillary buds from half-seed soybean by using appropriate levels of some growth regulators supplemented in regeneration mediums such as B5 + 2 ml/L BAP for shoot multiplying; MS + 5 µg/L TRIA+ 1mg/L Zeatin + 0.5 mg/L GA3 + 0.1 mg/L IAA for shoot elongation and 1/2 MS + 1.25 mg/L IBA or 1/2 MS + 1.1 mg/L IAA for rooting. The report also determined the appropriate substrate as 1 the soil: 1 humus: 1 sawdust ratios for plantlets to grow in a greenhouse.

Keywords: *Gm*MYB12A, half-seed explants, Jilin small granule soy, multiply shoot, plant regeneration.

1. INTRODUCTION

Soybean (Glycin max (L.) Merrill) is a species of legume native to East Asia, widely grown for its edible bean which has numerous uses (Linhong et al., 2012). Soybean is one of the most important crops not only in Vietnam and China but also in other countries,, because it is an important food and oil crop. It has more than 5000 years of cultivation history in China (Zhang Z et al., 1999; Li et al., 2008; Zhang Y et al., 2013). Currently, the U.S and Brazil crush only about half of their annual production; whereas countries like Argentina, China and the European Union-25 essentially crush their entire annual supply. The U.S consumes 95% of its domestic production of soybean oil. Because of the emerging market for bio-diesel, a soybean oil production deficit was projected by 2020 (Bob G ., 2008).

A typical soybean seed consists of about 40% protein, 20% oil and 12% carbohydrate on a dry weight basis (Kijong et al., 2011). Moreover, it also acts as the main source of various isoflavone compounds which can protect the soybean from the pathogenic microorganisms, promote growth, and due to its bitter taste; the soybean is grazed less by herbivores. Isoflavones were considered to play diverse roles in plant-microbe interaction and also have great potential for human nutrition and health. Soybean isoflavones ranked a class of secondary metabolites of the phenylpropanoid pathway which exists throughout the whole plant system, a large body of research work had repeatedly proven that MYB transcription factors could regulate the biosynthesis of isoflavones in plants (Linhong et al., 2012; Xiao et al., 2013). Therefore, soy plant transferred GmMYB12A, which stimulate isoflavones, could play an important role in the body's health through contribution to disease resistance. Furthermore, it could optimize multiple shoot induction and plant regeneration of transferred soy for increasing soybean quality and yield and offers an important method for increasing agricultural productivity.

2. RESEARCH METHODOLOGY

2.1. Material

Mature Jilin small granule soy transferred GmMYB12A seeds were provided by Lab 703 - College of Plant Science, Jilin University - China.

2.2. Methods

2.2.1. Preparation of explants for culturing

T_1 progeny seeds were surface-sterilized, and placed in 100 x 15 mm petri plates with 150 seeds per plate. There were 3 - 4 plates arranged in a bell jar desiccator and the plates were opened and lids were set next to the plates within a fume hood in such a way that would leave enough space for a 250 ml beaker in the middle. The 250 ml beaker was filled with 100 ml of 4% sodium hypochlorite and an additional 4 ml of 12N HCl poured along the side of the beaker. The desiccator was closed immediately and let stand for 5, 10, 15, and 25 hours, respectively. Then after exposure to Cl^- gas, the petri plates were closed and brought to a laminar flow hood where they were left open for 30 minutes to remove the excess Cl^- gas.

2.2.2. Media composition, preparation and establishment of cultures

The sterilized seeds were longitudinal cut along the hilum to separate the cotyledons, and subsequently the seed coat was removed. The embryonic axis found at the junctions of the hypocotyl and the cotyledon was excised to obtain the half-seed explants.

The explants were cultured in a shoot induction medium (SI; Gamborg's B5 basal salts with vitamins, 3% sucrose, and 0 - 2.5 mg/L BAP, pH 5.7) and incubated in a growth room at 25°C under an 18h photoperiod for two weeks.

Organogenesis shoots from the explants were trimmed and transferred to a shoot elongation medium (SE; MS basal salt with vitamins, 3% sucrose, 2.5 - 10 μM TRIA, 0.8 agar, pH 5.7). When the elongated shoot (3 - 4 cm in length) were survived , they were planted on root induction medium containing MS salts and vitamins, 3% sucrose, 0.8 agar, pH 5.7, filter sterilized 50 mg/L L-asparagine, 50 mg/L glutamine, and 0.5 - 1.5 mg/L indole butyric acid (IBA). After 2 - 4 weeks, rooted plantlets were rinsed with water to wash off the agar medium and then transplanted to soil in jiffy pots. The plantlets were planted at 24°C, 18 h photoperiod for 2 weeks, then transferred to the greenhouse.

2.2.3. Statistical analysis

The number of shoots per explant and the efficiency of regeneration were recorded and conducted to investigate differences in the above parameters.

3. RESULTS AND DISCUSSION

3.1. Effect of different sterilization times on seeds

Mature soybean seeds were disinfected by chlorine gas with different times (5, 10, 15, and 25 hours); then cultured for germination in 3 days. The results showed that the differences of sterilization times had a direct impact on germination and infection rates of cotyledons. The more sterilization time, the less rate infection. Specifically, the highest infection rate model was 10.67% with 5 hours sterilizing, and the figure gradually decreased, only 6.68% and 2.00% as sterilization time increased to 10, 15 hours, respectively. Specially, being sterilized in 25 hours, all samples were thoroughly cleaned. Moreover, sterilization time also effected the germination rate, which expressed the quality of the sample. It is observed that, the germination rates of seed were absolutely high, reaching up nearly 100% whenever seeds were treated in the sterilizing gas in a period of 5 hours, 10 hours or 15 hours. However, with 25 hours in the experimental sterilization period, the germination proportion of the sample was significantly lower (52.00%).

Overall, it was proven that the increase in sterilization time had inhibited seed germination as well as the contamination. The most suitable time for sterilizing Jilin small granule soy was 15 hours, shorter than that in the study treating the seeds of a number of other authors such as Paula et al. (2003); Paz MM et al. (2004); Margie M. Paz et al. (2006); Zhang et al. (2013).

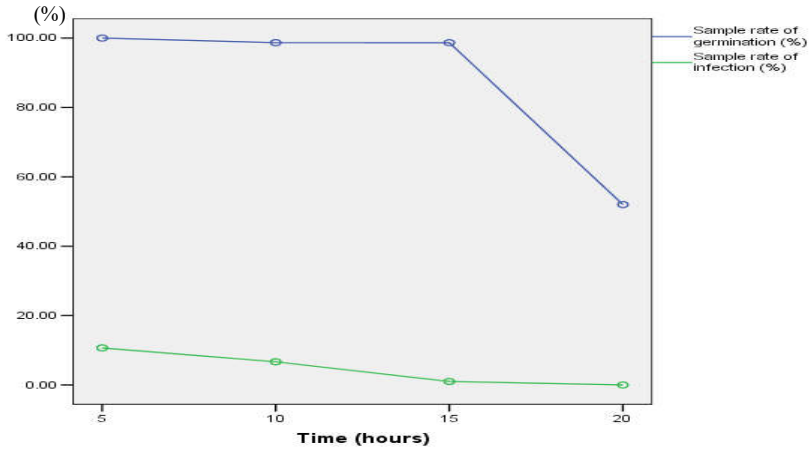


Figure 1. Effect of sterilization times on germination and contamination of samples

a B

Figure 2. Germination of seeds after sterilization in 15h (a) and in 25 h (b)

3.2. Effect BAP, TRIA on shooting

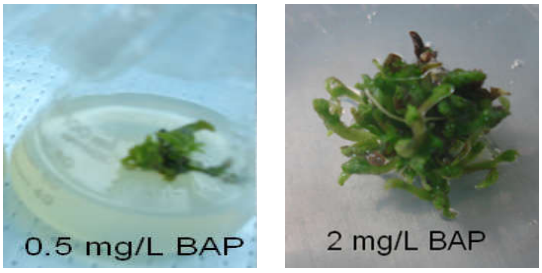
The majority of soybeans were regenerated by two main methods: multiple shoot regeneration and regeneration via somatic embryos. The factors affecting the ability to regenerate whole plants through somatic embryos had been studied in many different soybean varieties. However, these processes had some limitations such as difficulty collecting materials, long time recycling (at least 8 months), and a relatively complex process (Paula et al., 2003). In contrary, regeneration methods had many advantages with all the raw materials such as being less dependent on seasons, shortening the time needed for trees (3 months) and the disinfection of raw materials which could be controlled in simple ways. There were many recent published researches successfully using this method (Paz. MM et al., 2004; Liu H-K et al., 2004; Li et al., 2008). It is proven that 6-Benzylaminopurin is derived from adenine, substituted on the amino group in position 6. It was considered as a common model compound for one of the most important classes of plant hormones-cytokinins (Li et al., 2008; Natacha et al., 2013). Therefore, the development of a rapid, simple and sensitive method for the determination of 6-BAP was of great importance and interest.

In the multi-shoot regeneration method, growth hormone levels in a medium play an important role. Each plant has an appropriate threshold level at which it creates an optimum number of multiple shoots. The seed was split into two identical samples; each contained one cotyledon and germ axillary propagates half. After removal of the top growths, three to six vertical incisions were made at and around the cotyledon. The samples were then cultured on the mediums containing BAP concentrations from 0.5 mg/L to 2.5 mg/L (SIM2-SIM6) for shooting. The Table 1 presented results of effect of BAP on shoot formation.

Table 1. Effect of BAP on multiple shoot

Formula	BAP (mg/L)	Percentage of hypocotyls for multiple shoots ± SE	Number of shoots formed per hypocotyl ± SE
SIM1	0	11.05 ± 0.5	1.2 ± 0.3
SIM2	0.5	19.23 ± 1.6	3.2 ± 0.2
SIM3	1.0	71.67 ± 1.4	5.0 ± 0.5
SIM4	1.5	76.15 ± 1.3	5.5 ± 0.3
SIM5	2.0	82.31 ± 1.5	6.2 ± 0.5
SIM6	2.5	54.61 ± 1.6	5.1 ± 0.4

The sterilized seeds were germinated on a medium containing BAP at different concentrations from 0 to 2.5 mg/L BAP. The result (in Table 1, Figure 3) shows that BAP stimulated shooting from an implanted sample. When culturing in the medium supplemented with non BAP, almost all of the samples failed to create secondary shoots and roots appeared in the cotyledons. The highest rate of shoot generation was in the SIM5 formula at 82.31% and 6.2 shoots/clusters) while the lowest rate (19.23% and 3.2 shoots/cluster) occurred in SIM2 with 0.5 mg/L BAP. When BAP concentration was increased to 2.5 mg/l in SIM6, the growth rate of shoots decreased (54.61%), and the cotyledon developed black points. This result was entirely consistent with the theory that plant growth hormone stimulates growth at suitable levels but inhibits the samples at higher concentrations and is ultimately lethal at still higher concentrations.



A b

Figure 3. Shoot regeneration in medium with 0.5 mg/L BAP (a) and 2 mg/L BAP (b)

Furthermore, Triacontanol (TRIA) is considered as a naturally occurring plant growth promoter first identified in wax of alpha. The efficacy of TRIA on *in vitro* propagation of some plants. Pertaining to soybean, TRIA was able to restore the normal metabolic process, being developed for its micro-propagation, callus mediated regeneration, direct organogenesis and somatic embryogenesis, improving the *in vitro* shoot multiplication (Akitha MK et al., 2012).

Table 2. Effect of TRIA on shoot induction

Formula	TRIA (µg/L)	% Response	Mean no. of shoots/explant	Mean shoot length (cm)
SEM1	0.0	15	1.2 ± 0.8	2.0 ± 0.5
SEM2	1.0	72	2.0 ± 0.6	2.3 ± 0.8
SEM3	2.5	82	3.35 ± 0.2	2.5 ± 0.9
SEM4	5.0	85	4.5 ± 0.5	3.5 ± 1.2
SEM5	7.5	78	2.5 ± 0.6	3.1 ± 0.8
SEM6	10.0	57	2.3 ± 0.9	3.0 ± 0.7

In this experiment, multiple shoots were induced in nodal explants obtained from mature seeds on B5 supplemented with TRIA in various concentrations, 1; 2.5; 5; 7.5 and 10 µg/L of TRIA. The result revealed that the prolonged exposure of the culture to TRIA had an effect on explants. The highest shoot regeneration frequency was 85% in the SEM4 formula and the maximum number was 4.5 shoots per explant with a shoot length of 3.5 cm. It was also recorded in MS medium added 5 µg/L TRIA, whereas the lower number of nodes was found at 7.5 µg/L in the formula SEM5, SEM6 with a slightly reduced shoot length. Similarly to a large number of other studies established that comparatively lower concentrations of TRIA was needed for high frequency shoot regeneration (Akitha MK et al., 2012). It was also observed that higher concentrations of TRIA resulted in reduction of shoot length and heavy callusing (Natacha Soto et al., 2013; Haiyan Li et al., 2008).

The shoot length almost doubled with the addition of 5 µg/L TRIA along in case of half-seed explants, whereas the shoot tips exhibited a nearly threefold increase in large number shoots per explant, compared to the control and the samples cultured in medium with 1 µg/L TRIA (Figure 4).

a **b**

Figure 4. Explant with shoots in medium added 1 µg/L TRIA (a) and 5 µg/L TRIA (b)

Induction of multiple shoots from both shoot tip and cotyledonary leaf nodes in the presence of TRIA was evident in our study and similar observations noticed in other cultivars of soybean and also in other plants. In this research, cotyledonary node explants of soybean grown in media containing TRIA showed a maximum of 4.5 shoot buds per explant, which was similar to the results of MK Akitha (2012).

The effect of TRIA was evident in both the multiplication and elongation of soybean *in vitro* organogenesis. In the multiplication phase, maximum response was obtained in the medium containing 5 µg/L TRIA for cotyledonary node (Table 2, Figure 4). In contrast to multiplication rate, shoot elongation started a sharp decrease at 1 µg/L TRIA with a length of around 2 cm as compared to 4.5 cm in the controlled rate (Figure 4 a). The length of shoots of all cultivars decreased further as TRIA concentration increased. At 1 µg/L TRIA, Jilin small granule soy had very stunted shoots being below 3 cm, while the cultivars at 10 µg/L concentrations failed to develop shoots. It was also observed that TRIA suppressed multiple shoots (Figure 4 a, b). As compared to the control, multiple shoot suppression was easily seen at 5µg/L TRIA and above with very few shoot lengths of 3 cm.

Overall, multiple shoot induction increased from 0 to 5 µg/L range peaking at 5 - 7.5 µg/L TRIA so propagation to the effective length of the buds produce shoots increased from 0 - 5 µg/L, after the 7 µg/L TRIA threshold concentration decreasing the number of shoots.

3.3. Effect of IBA on rooting

In vitro rooting of isolated shoots was achieved best in a half-strength MS medium containing IBA. Properly rooted plants were

successfully hardened off and acclimatized in thermo cups containing sterile Soilrite. IBA was used to stimulate the roots of plants *in vitro* studies on soybean plants (Zin et al., 1998; Ren et al., 2006).

In the experimental task, the ability to produce soybean roots was also conducted with different concentrations of IBA. Starting at 4 weeks after transferal of half-seed explants to SE medium, elongated shoots (≥ 3 cm) were excised and placed into a rooting medium (RM) containing MS salts and different concentrations of IBA (0.5 mg/L, 1 mg/L, 1.25 mg/L, 1.5 mg/L); and shock treatment at 1mg/L IBA. After 1 - 2 weeks, the roots were fully developed to 2 - 3 cm in length (Table 3, Figure 5). Efficient response for root number, root length, number of nodes, shoot length and fresh weight of shoots were evident during root induction phase in the presence of IBA after 4 weeks of culturing (Figure 5, Table 3). There were 75.63%, 89.87% and 100% improvement in sample rooting rate, number of pods, average number and quality of roots of micropropagation derived plants compared to the root quality and number and start date of the control, 0 mg/L IBA. Number of roots and root length considerably increased as shoot culturing in a medium with 0.5 to 1.5 mg/L IBA. The maximum shoot length and the highest number of nodes were recorded at 1.25 or 1.5 mg/L concentration of IBA. However, the root mass and quality roots were the best in the presence of the medium containing 1.25 mg/L IBA.

Table 3. Effect of IBA on rooting in vitro

Formula	IBA (mg/L)	Sample rate rooting (%)	The date start to roots (days)	Average roots (roots)	Quality roots
ĐC	0	11.12	10	1,5 ± 0.9	+
RM1a	0.50	75.63	7	5.5 ± 0.5	+
RM2a	1.00	89.87	6	7.0 ± 0.5	++
RM3a	1.25	100	5	8.5 ± 0.8	+++
RM4a	1.50	89.36	5	8.3 ± 1.2	++
RM5a	Shock 1mg/mL	90.78	5	7.5 ± 0.9	+++

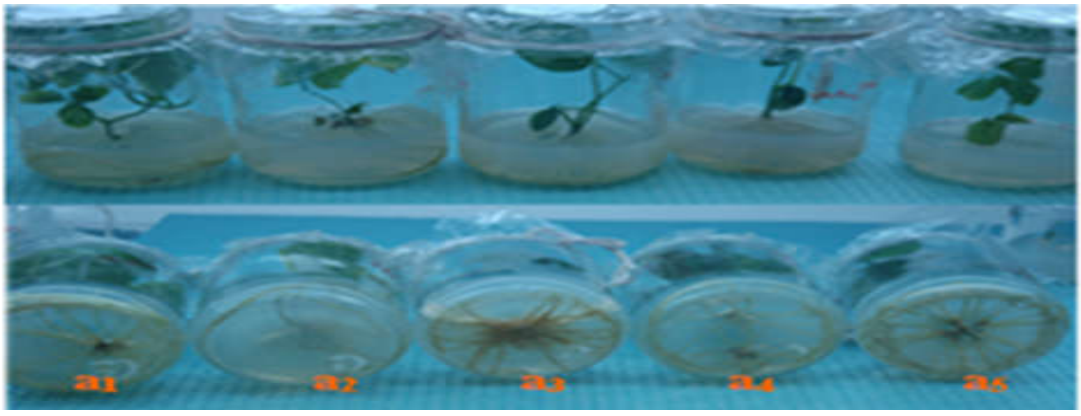


Figure 5. Shoot in medium added different of IBA

(a_2-1 mg/L; a_3-1.25 mg/L; a_4-1.5 mg/L IBA and a_5- shocked treatment in 1.0 mg/L IBA.)

3.4. Hardening of plantlets

After 30 days of culturing shoots on a rooting medium, it resulted in the sufficient rooting of shoots; the plantlets were transplanted to plastic pots containing humus; soil and sawdust for their hardening. For the first ten days, the plantlets were kept in a poly-house. To maintain the appropriate humidity level (80%), plants were thoroughly watered with the help of a manual sprinkler every 2 hours; the temperature of the poly-house was maintained at 25°C with a humidity level of

nearly 80%. Plantlets that were transferred to the plastic pots in the poly-house showed a good percentage of survival at 85% (Table 4). After keeping plantlets for an initial 15 days in the poly-house the plantlets were transferred to a shade house under less humidity and temperature controlled conditions and indirect sunlight. In the shade house plants were watered in the morning and the evening. Among the surviving plants, some showed symptoms of leap tip necrosis during shade house conditions. However, it did not hamper the overall growth of the plants. The plants with these symptoms were also growing well.

Table 4. Effect of substrates on the efficiency of soybean plants

Formula	Ingredients culture	Survival rate (%)	Quality seedlings
G1	Humus	86.57	+
G2	1 humus : 1 the soil	91.68	++
G3	Mixed crop; 1 humus: 1 the soil: 1 sawdust	98.86	+++

Note: (+++) good plant growth, body fat, early new leaves; (++) body, which is small; (+) slow-growing plant, yellow leaves.

a **b** **C**

Figure 6. Soybean growth on humus substrates (a), humus and soil (b), humus, soil and sawdust (c)

Survival rate is the most important criteria in evaluating the suitability of the substrate. Results in table 4 show the survival rate ranged from 86.57% to 98.86%, the lowest to highest valuations were G1 and G2. When the roots in *in vitro* are capable of adapting to the external medium to survive they begin to form new roots, thus restoring the plant which will then produce new leaves. This is reflected in the quality seedlings bring to the plants. Based on seedling quality, the suitability of the substrate is again confirmed G3. Seedlings can be valued over time with G3 which had new leaves appearing soon,. The value of G1 plants on the recovery level was low, due to slow-growth, yellow leaves, and a tendency for stems not to extend. It was demonstrated that the addition of sawdust to the soil helped porous scaffolds and breathable value while retaining essential moisture. When the medium was only sawdust, the valuation of the moisturizing ability dropped while G3 was a mixture of manufacturing plant availability with a high hit rate and additional fertilizer had created a certain degree of compression of the roots especially when watering, fertilizers make the young roots dehydrate which lead to wilting and dying, so the in-ability of roots to recover and low survival rates dropped to its lowest valuable in G1 studies 86.57% (Figure 6).

Based on the results obtained from Table 4 and Figure 6, value G3 (1 humus: 1 the soil: 1 sawdust) was chosen as feedstock to make soybean *in vitro* to the natural medium.

4. CONCLUSION

In vitro regeneration of Jilin small granule soy was successfully multiplied with auxiliary buds from half-seed soybean. It was evident in almost all the seeds that were sterilized in 15 hours with chlorine gas being clean and germinating with a high percentage. Being cultured in different mediums added appropriate levels of some growth regulators, the seeds had multiplied shoots in mediums added 2 ml/L BAP, and elongated shoots in mediums added 5 μg/L TRIA and stimulated roots in medium added 1.25 mg/L IBA. In the greenhouse, the plantlet carrying roots had grown in appropriate substrate with the most suitable ratio of 1 soil: 1 humus: 1 sawdust.

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NHÂN IN VITRO DÒNG ĐẬU TƯƠNG JILIN SMALL GRANULE CHUYỂN GEN *Gm*MYB12A

Nguyễn Văn Phong[1], Bùi Thị Thu Hương[2], QingYu Wang[3]
[1] Trường Đại học Lâm nghiệp
[2] Học viện Nông nghiệp
[3]Trường Đại học Cát Lâm, Trung Quốc

TÓM TẮT

Isoflavone của đậu tương là một lớp các chất chuyển hóa thứ cấp, ảnh hưởng tới phân chia tế bào, và có thể làm tăng giá trị dinh dưỡng do đó đảm bảo sức khoẻ của con người. Gen *Gm*MYB12A có thể nâng cao hàm lượng isoflavone, đã được chuyển vào đậu tương Jilin small granule, Trung Quốc. Nhằm nhân nhanh những dòng đậu tương chuyển gen này phương pháp nhân nhanh sử dụng hạt giống nửa hạt đã được nghiên cứu. Thời gian khử trùng thích hợp cho chúng là 15 giờ với khí clo bằng cách đổ 100 ml 4% sodium hypochlorite vào một cốc thủy tinh và thêm 4 ml 12 N hydrochloric acid. Sự tái sinh cây in vitro đã thành công khi nuôi cấy chúng trong môi trường B5 + 2 ml/L BAP để nhân chồi; MS + 5 µg/L TRIA + 1mg/L Zeatin + 0,5 mg/L GA3 + 0,1 mg/L IAA cho sự kéo dài chồi và 1/2 MS + 1,25 mg/L IBA hoặc 1/2MS + 1,1 mg/L IAA để ra rễ. Trong nghiên cứu này, cây con đã xác định là sinh trưởng thích hợp trong giá thể trong nhà kính là đất, mùn cưa, trấu hun với tỷ lệ 1:1:1.
Từ khoá: *GmMYB12A*, half-seed explants, Jilin small granule soy, tái sinh thực vật, tạo đa chồi.

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