*J. Agric. Sci. Mansoura Univ., 30 (9): 5201 - 5208, 2005*

# TISSUE CULTURE ORGANOGENSISES IN SOYBEAN

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## ABSTRACT

The aim of this study was to initiate and maintain callus organogenesis cultures of soybean and evaluate the response of seven soybean genotypes in callus formation. The genotypes were selected on the basis of their reaction to cotton leaf worm infection. This investigation was conducted in the field at Giza research station in 2003 and 2004seasons. Immature embryos (with 0.5-10 mm long) were excised from immature seeds and used as ex-plant. The basal Murashige and Skoog (MS) medium with addition of 4 times of micronutrients, Proline (1381 mg/l), NAA (0.0372 mg/l) and BAP (2.996 mg/l) gave complete satisfaction for callus production. Formation of shoots and roots were succeeded and rooted plantlets were transplanted to pots in green house. The results indicted that the genotype L86K-73 was the best in response to tissue culture technique in soybean. Callus growth rate was positively and significantly correlated with each of number of shoots/callus and number of roots. Therefore, both characters could be used to predict succeeding of callus growth in soybean.

## INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is important oil and poultry feed crop in Egypt. The crop has been mainly grown in the Nile valley and the Delta since 1972. The cultivated area has declined from 100,000 feddan in 1991 to less than 38,000 feddan in 2004 (Anonymous, 2004). The reduction in the area is due to high production cost and lower net return comparing with corn, the main competitor summer crop to soybean. The crop production faces several constraints. Insect infestation during vegetative growth stage by feeding insects such as cottons leaf worm (*Spodopetra littoralis*, Boisd.) and green cotton leaf worm (*Spodopetra exigua* Hubner) usually causes dramatic yield losses (Lutfallah *et al.*, 1998). The seed yield reduction in soybean due to infection by cotton leaf worm in Egypt ranged from 36.6% to 42.7% (Lutfallah *et al.*, 2003). Therefore, insecticides are extensively used in soybean fields, which rising production cost, causing accumulation of undesirable residues and increasing environmental pollution (Awadallah *et al.*, 1990). The use of host-plant resistance is necessarily to solve most of those problems. Despite several cotton leaf worm-resistant soybean varieties have been released such as Giza21,Giza35,Giza83 and Giza111 (Anonymous, 2005), the farmers still prefer to grow susceptible varieties such as Clark, Giza 82 and Giza 22. Hence, insect infection still exists in soybean fields and insecticides still widely used.

Advances in biotechnology could facilitate the development of insect-resistant soybean cultivars by means of gene transformation. Successful use of gene transfer requires that the gene for insect resistant is identified, isolated and then reconstructed for expression in relevant organ of the new host. In addition, gene transfer procedures and appropriate tissue culture methods must be developed for each target species to regenerate fertile, transgenic plants (Schroeder *et al.*, 2000).

The first transgenic plants with resistance to insects contained genes for insecticidal proteins called δ-endotoxins from the soil microorganisms, *Bacillus thuringiensis* (Bt). Bt protected cotton, potato, and corn were introduced to the market place in 1996 (Schroeder *et al.*, 2000). In soybean, the induction of indirect organogenesis from immature embryos was first demonstrated by Christianson *et al.* (1983) and Tiliton and Russell (1984). They succeeded to regenerate plants from callus, drove from immature embryos. The obtaining callus organogenesis or somatic embryogenesis was depending on the composition of the medium (Barawale *et al.*, 1986 and Yang *et al.*, 1990). Organogenesis resulted when embryos were plated on Murashige and Skoog (MS) medium, which contained high concentration of micronutrients. This technique has been used successfully to perform callus and plant regeneration from callus in soybean (Coppens and Dewitte, 1990; Komatsuda, 1995; Chandra and Pental, 2003 and Tripathi and Tiwari, 2003). Despite the fact that biotechnology offers good option for genetic enhancement of crop plants, little *in vitro* work has been done in soybean in Egypt.

Prior to gene transfer, responding soybean genotypes to organogenesis in tissue culture technique needed to identify. The aim of this study was to initiate and maintain callus organogenesis cultures of soybean. The differences among soybean genotypes in callus formation were also evaluated.

## MATERIALS AND METHODS

Seven exotic and Egyptian soybean genotypes selected on the basis of their reaction to cotton leaf worm infection. The seeds these genotypes were obtained from Food Legume Research Program, Field Crops Research Institute, ARC, Giza, Egypt (Table 1). The genotypes were grown in the field at Giza research station in 25$^{th}$ May 2003 and 2004.

**Table 1: Origin and main characteristics of the seven-tested soybean genotypes.**

| Name | Origin | Pedigree | Characteristic |
|---|---|---|---|
| L86K-73 | USA | L73-4673 X L73-0132 | Maturity group no. I, white flower color. |
| Corsoy-79 | USA | Corsoy X Lee 68 | Maturity group no. II, white flower color. |
| Forrest | USA | Dyer X Bragg | Maturity group no. V, white flower color. |
| Hutcheson | USA | - | Maturity group no. VI, purple flower color. |
| Lakoto, | USA | Selection | Maturity group no. II, purple flower color. |
| Giza 21 | Egypt | Crowford X Celect | Maturity group no. IV, purple flower color. |
| Giza 83 | Egypt | Selection from MB80-133 | Maturity group no. II, purple flower color. |

Sowing was done at a crop density of 33 plants/$m^2$.in 3.0 meter long ridges, 60 cm. apart and 4 ridges per plot. Fertilizers at 30 kg $P_2O_5$/feddan and 15 kg N/feddan were added to the soil prior for cultivation. All agronomic practices were applied as recommended. In early pod initiation stage, 40 young pods were collected from the plants of each genotype and moved to the laboratory immediately.

Immature seeds were taken out from young pods and then surface sterilized by immersion in a solution containing 30% commercial bleach Clorox with a drop of Tween 80 (polyethylene sorbitan monooleate) for 20 min. The immature embryos (with 0.5-10 mm long) were excised from the seeds by taking the seed coat off and then cutting next to the hilum. The immature embryos were immersed in an organogenesis medium (MR), which prepared according to Murshige and Skoog (1962) and Gamborg *et al.* (1968) and presented in Table 2.

The immature embryos were incubated under complete darkness at 25°C±2 for 4 weeks

**Table2. Composition of nutrient media for callus initiation (OR) and shooting formation (MSR) as described by Murashige and Skoog (1962) and Gamborg *et al.* (1968).**

| Components | OR (Mg/l) | MSR (Mg/l) |
|---|---|---|
| **Macronutrients** | | |
| $NH_4NO_3$ | 1650.00 | 1650.00 |
| $KNO_3$ | 19000.00 | 19000.00 |
| $MgSO_4.7H_2O$ | 370.00 | 370.00 |
| $KH_2PO_4$ | 170.00 | 170.00 |
| $CaCl_2.2\ H_2O$ | 440.00 | 440.00 |
| **Micronutrients** | | |
| KI | 4X 0.830 | 0.830 |
| $H_3Bo_3$ | 4X 6.200 | 6.200 |
| $MnSO_4.H_2O$ | 4X 22.300 | 22.300 |
| $ZnSO_4.7H_2O$ | 4X 10.600 | 10.600 |
| $NaMaO_4.2H_2O$ | 4X 0.250 | 0.250 |
| $CoCl_2.6H_2O$ | 4X 0.025 | 0.025 |
| $Na_2$ EDTA | 4X 37.250 | 37.250 |
| $FeSO_4.7H_2O$ | 4X 27.850 | 27.850 |
| **Vitamins B5** | | |
| Nicotinic acids | 1.00 | 1.00 |
| Thiamin-HCl | 10.00 | 10.00 |
| Pyridoxine-HCl | 1.00 | 1.00 |
| Myoinsitol | 100.00 | 100.00 |
| **Amino acids** | | |
| Proline | 1381.00 | 1381.00 |
| **Hormones** | | |
| NAA | 0.0372 | - |
| BAP | 2.996 | 0.383 |
| IBA | - | 0.0406 |
| **Additions** | | |
| Thiamin-HCl | 1.687 | - |
| Nicotinic acids | 3.693 | - |
| Sucrose | 30000.00 | 30000.00 |
| Agar | 8000.00 | 8000.00 |
| pH | 5.8 | 5.8 |

After 4 weeks the percentage of callus induction was calculated as (number of explants performed calli/total number of used explants) x 100, and callus growth rate was measured as callus weight (g). To perform shoots, callus were put on MSR medium (Table 2) at 25°C at day and 18°C at night with 16 h day (light source was from cool white fluorescent lamps 80 um photons $m^{-2}$ $s^{-1}$). The calli that did not perform shoots during 3 weeks were sub-cultured to new MSR medium. This procedure was repeated every 3 weeks till callus perform shoots. The callus that performed 1.0cm. long shoots were transformed to glass tubes containing the hormone-free MS medium (Murashige and skoog, 1962) for rooting formation. When reasonable number of roots are grown, usually after 3-4 weeks, the plantlets were removed from glass tubes in to 10.0cm. diameter- plastic pots filled with fumigated soil mixture of peat and sand with a ratio of 3:1. The Pots were placed in the green house at Giza research station and were covered with polyethylene bags. To maintain optimum air humidity surround plants, pots. Irrigation was done with Hogland solution (0.25) (Hogland and Arnon, 1950).

To measure the response of soybean genotypes to callus induction the following characters were measured and calculated in 4 replicates: Number of shoots/callus, Percentage of plantlets performed roots [(number of plantlets/total number of shoots) x 100], number of root/plantlet, length of root cm. and diameter of root mm.. Analysis of variance was made for each character and the simple correlation among all characters was calculated (Gomez and Gomez, 1984).

## RESULTS AND DISCUSSION

Seven exotic and Egyptian soybean genotypes were used in this study. Several types of explants have been used in previously reported plant-regeneration studies on soybean, but immature embryos have been successfully cultured to produce plants (Christianson *et al.*, 1983). Thus, cultures in this study were initiated from immature embryos at early developmental poding growth stage with length from 0.5 to 10 mm. Sterilized immature embryos obtained from each of the tested soybean genotypes were cultured on OR organogenesis medium. During the first week explants were enlarged, but no calli have been observed. At the end of the second week callus began to initiate on OR medium, which consisted of 4 times of micronutrients, Proline (1381 mg/l), NAA (0.0372 mg/l) and BAP (2.996 mg/l). The calli obtained were vigorously growing, fragile and had greenish color (Fig.1A). The obtained calli were subculture onto MSR media, which consisted Proline (1381 mg/l), BAP (0.383 mg/l) and IBA (0.0406 mg/l). Within 4 weeks of culturing, shoots were produced (Fig. 1B). The newly formed shoots growing on the shooting media were translated to rooting medium when shoot length reached 3-5 cm. Roots were grown well (Fig. 1C) in MS medium with hormone free. These results are in accordance with those of Ghanem (1995) on *hyoscyamus*, how found that free hormone-MS medium was the best among five rooting media and gave the best root formation. After 3-4 weeks of root formation, the plantlets were grown enough to be transferred to pots for adaptation (Fig. 1D).

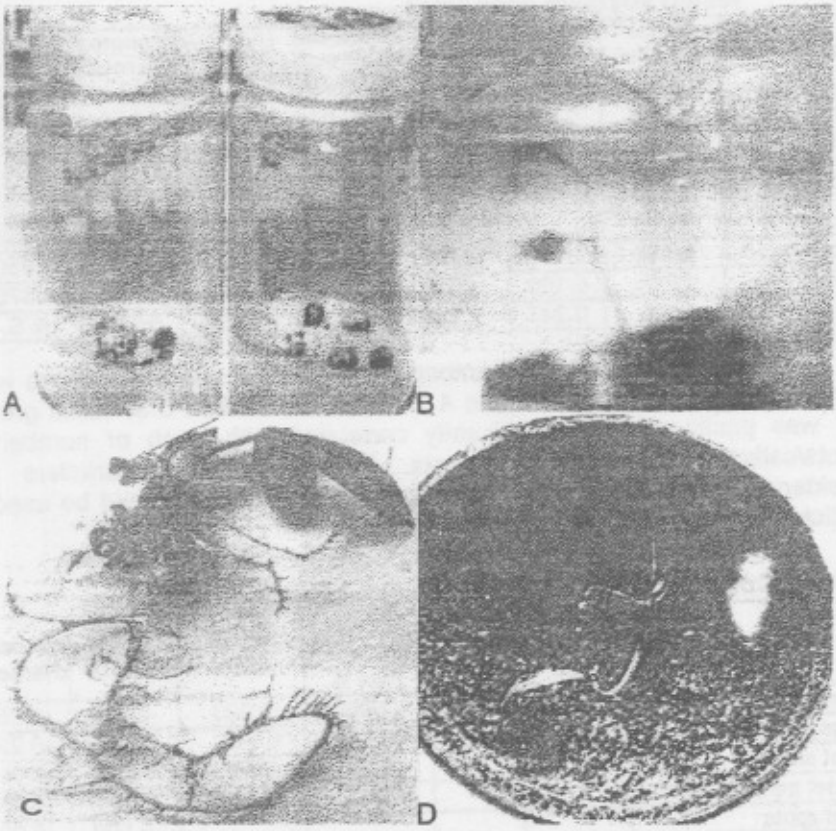


**Figure1: Illustration of Callus induction(A), Shooting stage(B), Rooting stage(C), and Adaptation (D)**

The performance of callus and plantlet characters for all tested genotypes are presented in Table3. Callus induction frequencies among genotypes were different and ranged from 63% for Corsoy-79 to 79% for L86K-73, but with no significant differences among genotypes Table, 3. This result indicated that all tested genotypes had almost equal responses to callus induction with culture method used in this study. The callus growth rate ranged widely among genotypes. The genotype L86K-73 gave the highest growth rate value of 1.18 g followed by Corsoy-79 with 0.93 g Table, 3. The genotype L86K-73 performed also the highest number of shoots/callus (16.25), while all other genotypes had markedly lower number of shoots/callus, which ranged from 3.75 to 9.75. No significant differences observed among genotypes for percentage of plantlets performed roots and diameter of roots. The genotype L86K-73 had also the longest root of 14.25 cm and laid among the best three genotypes performed the highest number of roots. These findings indicted that the genotype L86K-73 was the best in response to tissue culture technique in soybean.

**Table 3. Mean performances of callus and plantlet characters for seven tested soybean genotypes.**

| Genotypes | Callus induction (%) | Callus growth rate | No. of Shoot/ callus | Plantlet performed roots (%) | No. of roots | Length of root (cm.) | Diameter of root (mm) |
|---|---|---|---|---|---|---|---|
| L86K-73 | 79.00 | 1.180 | 16.25 | 20.633 | 6.00 | 14.25 | 2.625 |
| Corsoy-79 | 63.00 | 0.930 | 8.75 | 26.806 | 7.00 | 11.75 | 1.625 |
| Forrest | 66.00 | 0.135 | 7.00 | 22.173 | 4.50 | 13.00 | 2.300 |
| Hutcheson | 67.00 | 0.086 | 3.75 | 36.250 | 3.00 | 11.25 | 2.525 |
| Lakota | 69.00 | 0.278 | 4.50 | 23.750 | 4.50 | 7.75 | 2.825 |
| Giza21 | 68.00 | 0.118 | 9.75 | 21.023 | 7.00 | 6.75 | 1.950 |
| Giza83 | 69.00 | 0.100 | 8.75 | 28.819 | 4.00 | 12.50 | 2.375 |
| L.S.D 5% | NS | 0.247 | 2.209 | NS | 2.254 | 5.164 | N.S. |

Correlation coefficients among pairs of all studied characters were calculated and presented in Table 4. The results showed that callus growth rate was positively and significantly correlated with each of number of shoots/callus and number of roots. Therefore, both characters are considering important indicator for callus growth rate, and could be used to predict succeeding of callus growth.

**Table 4. Correlation coefficients among all studied characters.**

| Character | Callus growth rate | No. of shoot | plantlet performed root % | No. of roots | Root length | Root diameter |
|---|---|---|---|---|---|---|
| Callus induction % | 0.158 | 0.157 | 0.088 | 0.033 | -0.112 | -0.050 |
| Callus growth rate | | 0.605** | -0.083 | 0.467** | 0.320 | -0.220 |
| No. of shoots | | | -0.381 | 0.253 | 0.228 | -0.026 |
| Plantlet performed root | | | | 0.335 | 0.191 | -0.259 |
| No. of roots | | | | | -0.093 | -0.226 |
| Root length | | | | | | 0.157 |

** Significant at 0.1 level of probability.

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# تكوين الأعضاء النباتية بواسطة زراعة الأنسجة في فول الصويا

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تستهدف هذه الدراسة إلى تكوين أعضاء نباتية من الكالوس لسبعة أصناف مـــن فـــول الصويا وهم L 86 k – 7 3 ،كورسى-٧٩، فورست، هيتشسون ، لاكوته ،جيـــزة٢١وجيـــزة٨٣ وتقيم مدى استجابة هذه الأصناف لتكوين الكالوس، وقد تم اختيار هذه الأصناف بناء على درجة مقاومتها للإصابة بدودة ورق القطن، وقد إجريت هذه التجارب في مزرعة محطة بحوث الجيزة ومعامل قسم بحوث دراسة الخلية في موسمى٢٠٠٣ و ٢٠٠٤. وقد تم استخدام الجنين غير الناضج البالغ طوله من ٠,٥الى ١٠مم والذي تم الحصول عليه من البذور غير الناضجة لعمل الكالوس، ، وتم إزالة محور الجنين واستخدام البادئ النباتي علي بيئة المورشيج وسكووج بتركيز ٤ أضعاف لأمـــلاح العناصـــر الصـــغرة وتركيـــز هرمـــوني٠,٠٣٧٢مليجـــرام/لنـــر NAA و ٢,٩٩٦ مليجرام/لترBAP وحمض أميني بر ولين بتركيز ١٣٨١مليجرام/ لتر لإنتاج الكالوس، وقـــد تـــم الحصول على أفرع خضريه وتكوين جذور بنجاح، ثم تم عمل أقلمة للنباتات الناتجة حيث زرعت في قصارى بقطر ١٥سم ونقلت إلي الصوبة النباتية، وقد أوضحت الدراسة أن صنف فول الصويا L 86 k – 7 3 كان أفضل الأصناف استجابة لزراعة الأنسجة، كما أظهرت النتائج أيضـــا أن معدل نمو الكالوس يرتبط ارتباطا موجبا مع عدد الأفرع الخضرية وعدد الجذور.