ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Regeneration and transformation efficiencies among five Egyptian clover cultivars (*Trifolium alexandrinum*)

Reda E. A. Moghaieb[1*], Amany M. A. Abdelazim[2], Sawsan S. Youssef[1], Sanaa A. M. Ibrahim[2], Basita A. Hussein[1]

1-Department of Genetics and Genetic Engineering Research Center, Cairo University, Giza, Egypt
2-Plant Protection Research Institute, Agriculture Research Center. Ministry of Agriculture, Dokki. Giza, Egypt.

Manuscript Info

Manuscript History:

Received: 15 May 2014
Final Accepted: 23 June 2014
Published Online: July 2014

Key words:
Agrobacterium, Egyptian clover, regeneration, transformation, gus gene expression

Corresponding Author

Reda E. A. Moghaieb

Abstract

The objective of the present investigation is to develop an efficient method for shoot and plant regeneration using five commercial Egyptian clover (*Trifolium alexandrinum*) cultivars grown under the Egyptian agricultural conditions. The regeneration efficiency from hypocotyls and petiole explants was examined. The data indicated that embryonic calli were formed within one week in the presence of 1.5 mgl^{-1} 2,4-D. Adventitious shoots emerged from the embryonic callus in the presence of 1.5 mgl^{-1} BA. The petiole explants show higher callus and shoot induction percentages compared with the hypocotyls in all cultivars tested. The cultivars showed a varied response to shoot regeneration. Regeneration frequency was high in the cultivar Serw 1 follwed by Helaly 22 and 19% respectively compared with the other cultivars tested. Petiole explants from the cultivars Serw 1 and Helaly were inoculated and co-cultivated with *Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector pBI-121 containing the β- glucurodinase (*gus*) gene under the genetic control of CaMV-35S promoter and nopalin-synthase terminator. The resulted putative transgenic plantlets were able to grow under knanamycin containing medium. The stable integration of the *gus* gene into the plant genomes was confirmed by dot blot and by PCR analysis using 35 S promoter specific primers. The transformation percentage of the clover cultivars Serw 1 and Helaly was 56.2 and 28 % respectively. The *gus* gene expression can be detected only in the transgenic plants. The developed protocol for regeneration of transgenic plants in the present study is repeatable and can be used to regenerate transgenic clover plants expressing the genes present in *A. tumifaciens* binary vectors.

INTRODUCTION

The genus *Trifolium* belongs to the family Leguminosae and includes many species, of which red clover (*Trifolium pratense* L.), white clover (*T. repen* L.), alsike clover (*T. hybridum* L.) and Egyptian clover (*Trifolium alexandrinum* L.) are the most important species. They are widely used as high quality forage (Zayed et al., 2012).

Berseem or Egyption clover (*Trifolium alexandrinum* L.) is the main annual winter forage leguminous crop in Egypt. Berseem forage is superior to grasses in protein and mineral contents so it has a high nutritional value for animal feed (Laghari et al., 2000). Also it contributes to soil fertility and improves its physical characteristics (Bakheit, 2013). Berseem represents an add value to Egyptian economic since the cycle of berseem capital equals tens billions American dollars (Tarrad and Zayed, 2009; Zayed et al, 2010 and Zayed et al, 2011).

Cell biologists strive to develop tools for producing genetic novelties through the regeneration of plants (from protoplasts, cells, tissues and organs). Genetic engineers develop methods and techniques for the isolation and successful insertion of genes controlling desirable traits into plants by transformation. The benefits of these techniques are to reduce the time needed to produce new genotypes from (in traditional methods) two to six years only with more genetic purification and lower costs (Zayed, 2013).

Unfortunately, a little work of biotechnology on Egyptian clover was published so far. The total number of publications on berseem during the nineties to 2012 is 1872 , amongst them only 199 papers published in the field of biotechnology representing only 10.6% (ENAL, 2012).

The studies on Egyptian clover based on direct and indirect somatic embryogenesis. Sultan (1990) reported that the leaf is the best explant for callus production with the addition of trace amounts of 2, 4-D (2, 4-Dichlorophenoxyacetic acid. Barakat (1990) succeeded to obtain shoot from the cultivars Sakha 4 and Giza 10 using MS medium containing 2.0 mg l^{-1} NAA and 0.5 mg l^{-1} BAP. As well as, he established cell suspension cultures from hypocotyl and cotyledons derived callus. In addition to, Abogadallah and Quick (2010) reported new and fast procedures for regeneration of Egyptian clover that are applicable to the regeneration of various genotypes (Meskawi-ahaly, Sakha3 and Sakha4). Shoots were regenerated from intact and wounded cotyledons as well as hypocotyls of Mescawi-ahaly on naphthalene acetic acid/benzyl adenine (NAA/BA) and naphthalene acetic acid/thidiazuron (NAA/TDZ) media.

The tissue culture system is divided in two main methods direct and indirect somatic embryogenesis or organogenesis, the first method is utilized by Aly et al. (1994) who established somatic embryogenesis tissue culture system and succeeded to regenerate shoots from Egyptian clover. On the other hand Bhowal et al. (2011) established a protocol for direct organogenesis for berseem by culturing the explants (shoot tip, cotyledonary node without cotyledons, cotyledon and hypo-cotyledon) on different media like MS or L2. Shoot tip was found to be the best responsive explants for direct organogenesis. Subsequently, it was found that 75% of explants produced shoots on L2 media supplemented with BAP (1 mg l^{-1}) and IAA (0.1 mg l^{-1}). Direct organogenesis from the shoot tip gives a pure line which can be utilized in genetic improvement of this crop plant. In this respect, Masoud and Hamta (2008) studied the potential uses of somaclonal variation in improvement of berseem clover.

Agrobacterium mediated gene delivery system is the method of choice for regenerating transgenic plants in many leguminous species. Tanaka et al., (2001) could regenerate a fertile transgenic berseem plants from hairy root cultures. Abogadallah et al., (2011) in order to improve drought and salt tolerance in Egyptian clover they could obtain transgenic plants expressing the Arabidopsis HARDY gene that belongs to the stress-related AP2/ERF (APETALA2/ethylene responsive element binding factors) super-family of transcription factors.

The objectives of the present study were to compare the endogenous capacity among five clover cultivars for callus induction and regeneration ability, and to utilize this regeneration system for improving the efficiency of transgenic clover plant production.

Materials and Methods

Plant material:

Five Egyptian clover cultivars (*Trifolium alexandrinum*) namely Serw 1, Helaly, Sakha 4, Gimmeza 1 and Fahel were provided by Field Crops Research Institute, Agricultural Research Center- Ministry of Agriculture-Egypt.

Callus induction and plant regeneration:

Seeds were surface sterilized in 70 % ethanol for 1 min., followed by immersion in 50% commercial Clorox for 15 min.; then rinsed three times in sterilized distilled water. Sterilized seeds were germinated in MS medium (Murashige and Skoog, 1962) containing 3% sucrose and 0.8% agar. Petri dishes were maintained at 25 °C.

The endogenous capacity for callus formation and shoot regeneration among five clover cultivars was compared. For callus induction, the hypocotyle and petiole explants prepared from 3 days old seedlings were dissected and cultured on MS medium supplemented with different concentrations of 2,4-D (0.5 , 1 ,1.5 and 2 $mg1^{-1}$). The culture was kept at 25 °C under complete dark condition. One week later the calli induction frequency were recorded for the five cultivars under different levels of 2,4-D.

In order to induce shoot formation, the embryonic calli obtained were transferred to shoot induction medium consists of MS medium supplemented with BA at graded levels (1.5, 3 and 4.5 $mg1^{-1)}$. Each plate contained 10 embryonic calli and all the treatments were performed with 10 replications. The plates were sealed with parafilm and incubated at 25°C under a 16/8 hrs light/dark photoperiodic regime (1000-Lux). The explants were sub-cultured weekly on corresponding medium freshly prepared. Data were collected on the following traits: callus induction and the shoot induction frequencies (CIF and SIF) which were calculated as follows:

CIF= number of calli-producing explants / total number of explants in the culture X 100

SIF = number of shoots-producing explants/ total number of explants in the culture X 100

Elongation and Acclimatization:

Mature embryos were selected for germination based on their size. Plantlets (4.0 cm in height) were transferred to half strength MS medium contains 0.5 $mg1^{-1}$ NAA for rooting. The plantlets which showed a well developed root system were transferred to sterilized soil in 15 cm plastic pots and irrigated with one tenth MS solution in a humid chamber at 30°C, under a 16/8 hrs day/night cycle. After acclimatization the plants were grown under the greenhouse conditions. The plant regeneration frequency (PRF) was calculated based on: PRF = number of plants producing shoots and roots / total number of shoots initiated in the culture x 100.

Agrobactrium mediated gene transfer in clover:

Bacterial strain and plasmid used: The *A. tumifaciens* strain LBA4404 harboring the Ti-binary vector pBI-121(Jefferson et al.1987) (Fig. 1) was used in the present study.

Co-cultivation

Agrobacterium tumefaciens strain LBA4404 cells harboring the binary Ti vector pBI101 were grown overnight in 30 ml of LB medium containing 50 μg/ml kanamycin sulfate, and then collected by centrifugation at 1120 x g for 5 min. The pellet was resuspended in MS medium containing 100 μM acetosyringon.

The petioles explants prepared from 3 day old seedlings of two clover cultivars (Serw 1 and Helaly), were immersed in the bacterial suspension for 10 min. The explants were then blotted with sterilized filter paper and placed on a co-cultivation medium consisting of MS medium with 100 μM acetosyringon and supplemented with 1.5 $mg1^{-1}$ 2,4-D. After co-cultivation for 3 d, the explants were transferred to a selection medium consisting of MS medium containing 500 $mg1^{-1}$ cefotaxime and supplemented with 1.5 $mg1^{-1}$ 2,4-D. Six days later the callus were transferred onto shoot induction medium supplemented with 1.5 $mg1^{-1}$ BA in addition to 50 $mg1^{-1}$ kanamycin. The shoots obtained were transferred to root induction medium with 0.5 $mg1^{-1}$ NAA , 1.5 $mg1^{-1}$ BA and 50 $mg1^{-1}$ kanamycin. The plates were sealed with parafilm and incubated at 25°C under a 16/8 hrs light/dark photoperiodic regime (1000-Lux). The explants were sub-cultured weekly into corresponding medium freshly prepared.

PCR analysis: DNA samples were isolated from both of the transformed and non- transformed plantlets according to the method described by Rogers and Bendich (1985). In order to confirm the stable integration of the T-DNA into the plant genome, the putative transgenic plantlets were analyzed by PCR using 35 S-promoter specific primers. The sequences of the forward and reverse primers for the 35 S- promoter detection were 5'AAAGGAAGGTGGCTCCTACAAAT-3' and 5'-CCTAGTAA AGTAAACCTCTCC3', respectively. The reaction mixture (20µl) contained 10 ng DNA, 200 µM dNTPs, 1 µl of each primer , 0.5 units of Red Hot Taq polymerase (AB gene House, UK) and 10-X Taq polymerase buffer (AB gene House, UK). Samples were heated to 94°C for 5 min. then subjected to 35 cycles of 1 min at 94°C; 1 min at 56°C and 1 min at 72 °C. The PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide.

Dot bolt analysis: Total genomic DNA was isolated from both transformed and non-transformed plants according to a method described previously by Rogers and Bendich (1985), Then denatured for 10 min., and spotted to a nylon membrane followed standard protocols. Labeling of the probes (gus-pBI 121), hybridization and detection was carried out using the Biotin Chromogenic Detection kit #K0661, #K0662 according to instructions provided by the manufacturer (Ferments Life Sciences, USA).

Histochemical GUS assay: The expression of β- glucurodinase (GUS) activity in transgenic clover plants was carried out according to the method described by Jefferson et al., (1987). Leaf tissue and callus were incubated in a reaction buffer containing 12.5 mM $K_3Fe(CN)_6$, 12.5 mM $K_4Fe(CN)_6$, 20% methanol; 1% Triton X-100 and 38.3 mM 5- bromo-4-chloro-3-indolyl glucuronide as a substrate for the enzyme. The tissue and callus were incubated in staining solution at 37 °C for 24 h and developed blue spots were recorded.

Results and Discussion

Berseem or Egyption clover (*Trifolium alexandrinum* L.) is the main annual winter forage leguminous crop in Egypt, it has a high nutritional value for animal feed due to its higher contents of protein and mineral (Laghari et al., 2000). One the main problem of berseem production is the infestation with cotton leaf worm (*Spodoptera littoralis*), This insect attacks the clover plant during early winter (September, October) when the seedlings appear above the ground. The full-grown larvae pupate in soil where moths emerge during next May and June resulting in the major infestation of cotton and maize (Tawfik et al., 1979, Salama et al. 1999). The strategies to protect cotton plant form this pest is to break its life stages on berseem by the production of transgenic insect resistance plants. The main restriction for transgenic plants production is the establishment of the regeneration system of the target plant. In this regards several attempts had been made to improve clover plants through tissue culture using different types of explants. Barakat (1990) could induce callus from hypocotyl explants in some *Trifolium alexandrinum* cultivars

(Sakha 3, Sakha 4, Giza 6 and Giza 10) using MS medium supplemented with 2 .0 $mg1^{-1}$ NAA and 0.5 $mg1^{-1}$ BAP). Masoud and Hamata (2008) produced callus from petiole and inter node on MS media having 1 $mg1^{-1}$ 2,4-D and 0.2 $mg1^{-1}$ Kinitin also 2 $mg1^{-1}$ 2,4 D and $mg1^{-1}$ Kinitin but only the calli that produced on MS media having 0.5 $mg1^{-1}$ NAA+ 0.5 $mg1^{-1}$ kinitin, could regenerate into whole plants.

In the present study the callus induction and regeneration capacity of the hypocotyl and petiole explants that have been isolated from five Egyptian clover cultivars was compared. Both of the hypocotyl and petiole explants exhibited an initial swelling followed by callus formation within one week of incubation on MS medium supplemented with 1.5 $mg1^{-1}$ 2,4- D. It was noted that callus proliferation started from cut ends of the explants (Fig.2). The cultivars differ for their callus induction ability reflecting their genetic differences. Serw-1 records the highest callus induction percentage while the cultivar Sakha-4 shows the lowest value (Fig. 3).

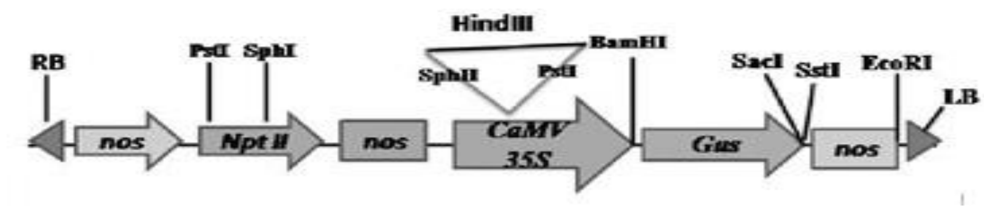

Fig. 1: Schematic representation of the *pBI-121 - gus* plasmid harboring the *gus* gene under the CAMV 35-SP and the nos terminator used in clover transformation. (Jefferson et al. 1987)

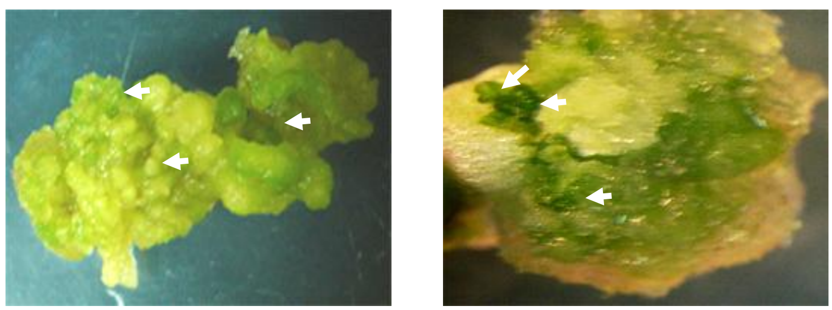

Fig. 2 Embryogenic calli showing different developmental stages of somatic embryogenesis indicated by arrows

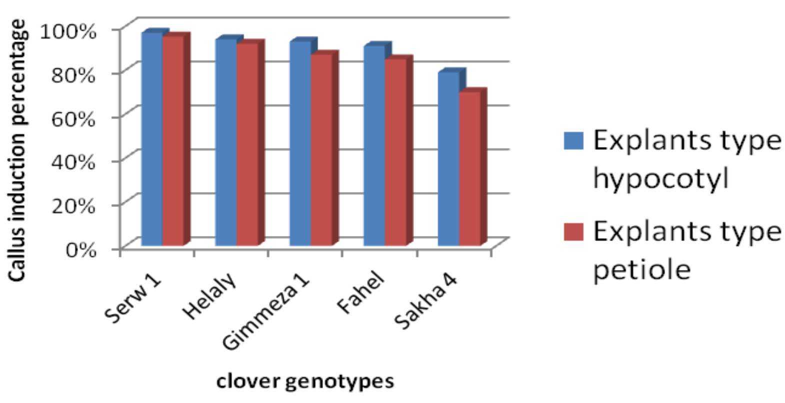

Fig. 3 Callus induction frequencies among the five clover genotypes

The embryonic calli from the five clover cultivars were transferred into shoot induction media consists of MS supplemented with different concentration of BA (1.5, 3 and 4.5 mg l^{-1}). The regeneration frequency varied among the cultivars, the estimated regeneration percentages of the tested clover cultivars varied from 0 % to 21 % for the hypocotyl and from 5 to 22 for the petiole. The data indicates that Serw 1 and Helaly show higher regeneration percentages 22 % and 19% respectively, whereas the Sakha 4 recorded 0% when hypocotyl is used and 5% for petiole The 1.5 mg1^{-1} BA proved to be the best condition for achiving higher shoot regeneration percentages, also the data indicate that petiole explants show high shoot induction frequency than those of the hypocotyls (Figure 4 A and B). Different results were reported by Abogadallah and Quick (2010) whom reported that, shoots were regenerated from intact and wounded cotyledons as well as hypocotyls of Mekcawi-ahaly on naphthalene acetic acid/benzyl adenine (NAA/BA) and naphthalene acetic acid/thidiazuron (NAA/TDZ) media. According to their data the highest shoot regeneration frequencies were obtained from intact cotyledons cultured on NAA/BA (0.05 mg l^{-1} NAA combined with 2.0 mg l^{-1} BA) and NAA/TDZ (0.05 mg l^{-1} NAA combined with 1.0 mg l^{-1} TDZ) media (66.2 and 43.1% respectively).

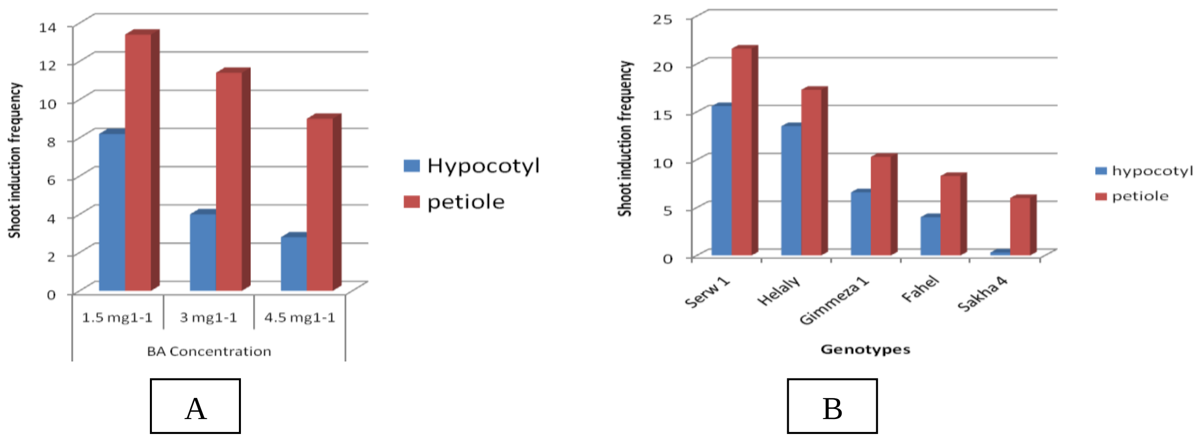

Fig. 4 Illustrate the differences in shoot induction frequency among two explants type (hypocoyl and petiole) under different BA concentration (A) and (B) show the differences in the shoot induction frequency among the five clover genotypes

Rooting of the regenerated shoots was induced on 0.5 mg1^{-1}NAA, which disagree with Abogadallah and Quick (2010) whom induced roots on indole butyric acid (IBA: 0.24 mg l^{-1}) or NAA (2.0 mg l^{-1}) media where IBA medium supported significantly higher frequencies of rooting.

The most common method used for the transformation of clover is *via* Agrobacterium mediated gene transfer. Both *A. tumefaciens* and *A. rhizogenes* have been used for genetic transformation (Tanaka et al. 2001). The petiole explants from the two clover cultivars (Serw 1 and Helaly) that showed higher regeneration capacity were co-cultivated with *A. tumefaciens* LBA4404 harboring the Ti binary vector pBI-121 for 3 days. After co-cultivation, the infected explants were placed on MS medium with low selection pressure. Under these conditions, the ends of the petioles gradually initiated the formation of callus. During the process of selection, the successfully transformed explants continued to grow vigorously to produce calli, whereas the untransformed ones failed to form callus and eventually bleached and became necrotic within 3 weeks. Fig.5 illustrated the different developmental stages of transformed plantlets.

Shoots were usually regenerated within 4 to 6 weeks on the MS medium after co-cultivation. During the selection culture, sub-culturing the explants with a change of fresh medium containing 50 mg l^{-1}kanamycin greatly reduced the number of escapes. The transformation percentages for the two clover cultivars after infection and selection were 56.2 and 28 % for Serw 1 and Helaly respectively (Table 1).

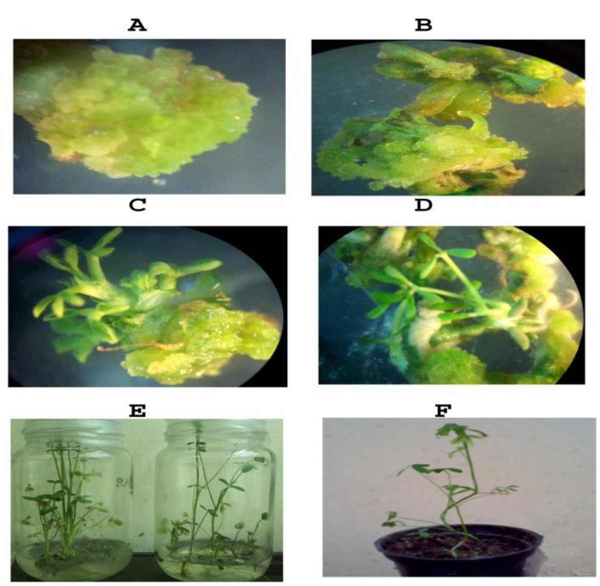

Fig. 5: The recovery of fertile *gus* expressing Egyptian clover plantlets, A and B: callus induction, C and D; shoot initiation and selection, E and F; elongation and acclimatization.

Table 1. Regeneration frequency of *gus* expressing plants derived from the hypocotyls explants of the two clover cultivars infected with *A. tumifaciens* LBA4404.

Genotype	No. of explants	No. of shoots	No. of regenerated plants	GUS positive plants	Molecular conformation using PCR 35S-primer	Transformation %
Serw 1	150	84	32	18	18	56.2
Helaly	150	60	25	7	7	28

In order to confirm the stable integration of the T-DNA into the genome of the regenerated plantlets, T_0 plants were subjected to PCR analysis using 35 S- promoter specific primers. All regenerated plants examined showed a clear band corresponding to the relevant sequence size of the 35 S- promoter (250 bp) (Fig.6). For further confirmation dot blot analysis was performed. The Dot blot analysis indicate that 7 out of 13 plants analyzed could hybridize specifically with the probe prepared from the gus-pBI-121 plasmid (Fig. 7).

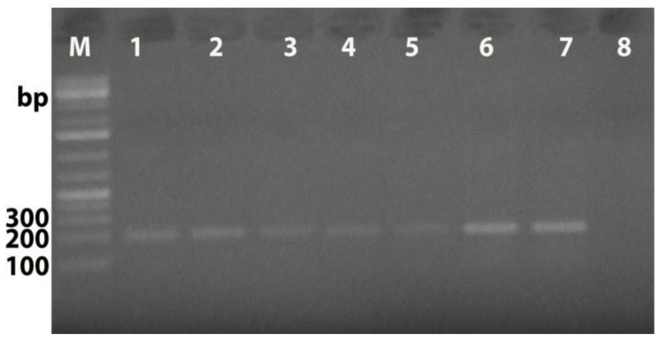

Fig. 6 : PCR analysis confirming stable integration of the 35 S- promoter into clover genome. M: DNA ladder100 bp, 1-7 transgenic clover DNA samples, 8: negative control.

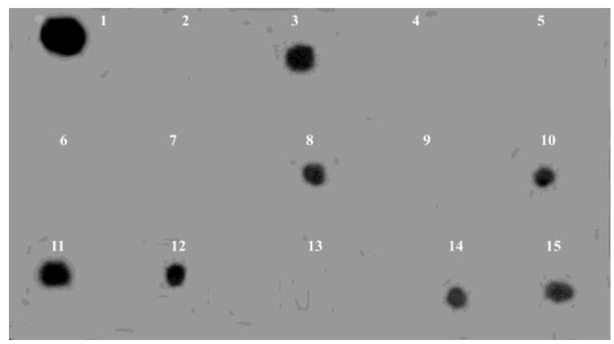

Fig. 7 : Dot blot analysis of transgenic plants. 1: Positive control, 2: Negative control and 3-15 show some transformed plants.

The *gus* gene expression could be detected only in the transgenic plants while the non-transgenic did not show any *gus* activity (Fig. 8).

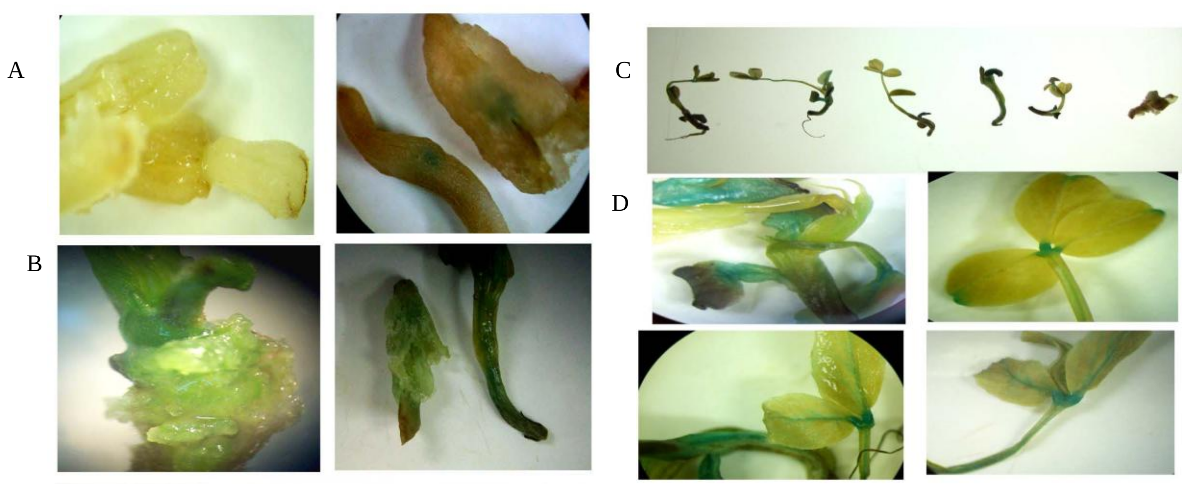

Fig. 8: The histochemical gus assay showing the expression of *gus* gene in the transgenic plants, A: expression in callus compared to control (up-left), B: in shoot initiation, C: expression in seedling, D: expression in the leaves and stem.

Based on the data of the present study we can conclude that the reported regeneration system is repeatable and can be easily used to regenerate transgenic Egyptian clover plants expressing the genes present in the Agrobacterium binary vector T-DNA. Using this regeneration and transformation protocol we can achieve our main goal which is the production of insect resistant transgenic Egyptian clover plants.

References

Abogadallah, G. M.; Nada, R. M.; Malinowski, and Quick R. (2011). Overexpression of HARDY, an AP2/ERF gene from Arabidopsis, improves drought and salt tolerance by reducing transpiration and sodium uptake in transgenic Trifolium alexandrinum L.Planta., 233 (6): 1265-1276.

Abogadallah, G. M. and Quick, W.P. (2010). Fast Versatile Re-generation of *Trifolium alexandrinum* L., Plant Cell, Tissue and Organ Culture., 100: 1, 39-48.

Aly, M.A.M., Khalil, M.F.M., Moghieb, R.E.A., Yousif, S.S and El-Sharkawy, A.M.A. (1994).Somatic embryogenesis in berseem (*Trifolium alexandrinum* L.).The influence genetic background. Egypt J. Genetic and Cytology., 23;151- 161.

Bakeit R.B. (2013). Egyptian clover (*Trifolium alexandrinium* L.) breeding in Egypt. Asian Journal of Crop Sci., 5(4): 325-337.

Barakat, M.N.(1990). *In vitro* cultures of tissues, cells and protoplasts *Trifolium alexandrinium* L. (Egyptian clover). Euphytica; 48 (2): 103-110.

Bhowal M., Cherian K.J. and Das L. (2011). Direct organogenesis in fodder crop *Trifolium alexandruim* L., J. Environ. Res. Develop., 5(4): 892-897.

ENAL [Egyptian National Agriculture Library](2012). Journals and books @OVID and CAB data base.

Jefferson RA, Kavanagh TA, Bevan MW (1987). GUS fusion. β glucuronidase as a sensitive and versatile gene fusion marker in high plants. EMBO J., 6(13): 3901-3907.

Laghari, H. H., channa, A.D., solngi, A.A. and soomro, S. A. (2000). Comparative digestibility of different cuts of berseem (*Trifolium alexandrinium* L.) in sheep. Pak. J. Biol. Sci., 3:1938-1939.

Masoud, S.and Hamata, A.(2008). Cytogenetic analysis of somaclonal variation in regenerate plants of Berseem clover (*Trifolium alexandrium* L.). Caryologia., 61(4):392-396.

Murashige T. and Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol., 15:473–497.

Rogers,S.O. and Bendich, A.J. (1985). Extraction of DNA from milligram amounts of fresh herbarium and mummified plant tissues. Plant Mol. Biol., 5: 69-76.

Salama H. S., F. N. Zaki, S. A. Salem, and Foda M.S. (1999) Utilization of *Bacillus thuringiensis* to control *Spodoptera littoralis* in Egyptian clover fields. J. Pest Science., 72: 21-23

Sultan, F. (1990). MSc. Thesis, Department of Agronomy, Faculty of Agriculture, Ain Shams University.

Tanaka N.; Fujikawa, Y. Aly, M.A.M.; Saneoka, H.; Fujita K. and Yamashita, I. (2001). Proliferation and *rol* gene expression in hairy root lines of Egyptian clover. Plant Cell, Tissue and Organ Culture., 66: 175–182.

Tarrad M.M. and Zayed E.M. (2009). Morphological, biochemical and molecular characterization of Egyptian clover (*Trifolium alexandrinum* L.) varieties. Range Mgmt. and Agroforestry., 30 (2) : 115-121.

Tawfik, M. F.; El-Sheif, S. I.; and Heneidy, A. H., (1979): Insect fauna of Egyptian clover fields in Giza region, Egypt. Bull. Soc. Ent. Egypt., 60: 171-178.

Zayed E.M., Soliman, M. I.; Ramadan, G. A. and Tarrad, M. M. (2010). Molecular characterization of two cultivars of Egyptian clover (*Trifolium alexandrinum* L.). Range Mgmt. and Agroforestry., 31 (2) : 140-143.

Zayed E.M.; Metwali, E. M. R.; Khafaga, A. F. and Azab, M. M. (2011). Field performance of commercial Egyptian clover (*Trifolium alexandrinum* L.) cultivars under high temperature condition. Range Mgmt. and Agroforestry 32 (2) : 87-91.

Zayed, M.E.; Metwali, E.M.R.; Gad-Allaha, N.O. and Shoaib, R.M. (2012). Comparison cytological and biochemical studies among four Egyptian clover (*Trifolium alexandrinum* L.) cultivars referring to cutting type. Australian Journal of Basic and Applied Sciences, 6(3): 622-629.

Zayed E. M. (2013). Application of biotechnology on Egyptian clover [(Berseem) (*Trifolium Alexanderium* L.)]. International Journal of Agricultural Science and Research (IJASR), 3: 99-120.