



GENETICS AND CYTOLOGY

INTERNATIONAL JOURNAL DEVOTED TO GENETICAL
AND CYTOLOGICAL SCIENCES

Published by
THE EGYPTIAN SOCIETY OF GENETICS

Volume 41

January 2012

No. 1

IMPROVEMENT OF GENETIC TRANSFORMATION AND PLANT REGENERATION VIA SUSPENSION CULTURES IN *Cucurbitaceae* FAMILY

Y. A. KHIDR^{1*} AND M. I. NASR²

1.Plant Biotechnology Dept., Genetic Eng. and Biotech. Res. Inst., Sadat City, Menofia University

2.Molecular Biology Dept., Genetic Eng. and Biotech. Res. Inst., Sadat City, Menofia University

*Corresponding author: yehia.khidr@yahoo.com

C*ucurbitaceae* is an important family of vegetables grown worldwide. It comprising of about 130 genera and more than 900 species of which only a few are cultivated (Jeffrey, 1962). It is dietary vegetable, which is primarily comprised species consumed as food worldwide. They are excellent fruit in nature that have composition of all the essential constituents *required* for good health to humans (Sikdar *et al.*, 2010; Rahman, 2003). Genetic engineering can improve crops by exploiting useful genes and engineering transgenic plants with desirable properties, such as disease resistance. Some re-

ports of genetic transformation of members of the family *Cucurbitaceae*, including *Cucumis melo* (Akasaka-Kennedy *et al.*, 2004), *Cucumis sativas* (Tabei *et al.*, 1998; He *et al.*, 2006; Vasudevan *et al.*, 2007), *Citrullus lanatus* (Akashi *et al.*, 2005; Choi *et al.*, 1994) and *Lagenaria siceraria* (Han *et al.*, 2005) are present. However, lack of an effective transformation method and transformation efficiency remains still variable and rather genotype dependent. To date, only two transformation methods have been reported (Tricoli *et al.*, 1995; Shah *et al.*, 2008). Tricoli *et al.* (1995) obtained transgenic

Cucurbita pepo with virus resistant gene, but the details of their method or the transformation efficiency remain unknown. Shah *et al.* (2008) obtained transgenic *Cucurbita pepo* via shoot organogenesis, but the efficiency of their method was only 0.7%. Several protocols were described in the literature for plant regeneration via indirect somatic embryogenesis (Jelaska, 1972; Debeaujon and Branchard, 1993) and recently, other groups have also succeeded in inducing shoot organogenesis in *Cucurbita pepo* (Ananthakrishnan *et al.*, 2003; Kathiravan *et al.*, 2006), *Cucurbita maxima* (Lee *et al.*, 2003) and *Cucurbita moschata* (Zhang *et al.*, 2008) using cotyledon explants. Nevertheless, specific conditions and protocols developed for a particular genotype are not necessarily reproducible for others. The objective of the present study was to develop a simple and efficient regeneration and *Agrobacterium*-mediated transformation system through suspension culture in cucumber and squash.

MATERIALS AND METHODS

Plant materials and sterilization procedure

Seeds of squash (*Cucurbita pepo* L., cv. Eskandrany) were provided by Horticulture Research Institute, Agriculture Research Center, Egypt and seeds of cucumber (*Cucumis sativus* cv, Faris) were obtained from Indo-American Hybrid seeds, Bangalore and India. Seeds of the both cultivars were surface-sterilized with 5% sodium hypochlorite (Clorox) for

20 min. and rinsed three times with sterilized distilled water. The seed coats were removed for squash; further sterilization method with 1% sodium hypochlorite for 10 min. was applied and rinsed three times with sterilized distilled water.

Preparation of explants

To prepare mature seed explants of squash and cucumber, the sterilized seeds (150 seeds) were cut transversely into three equal sections; one section consisted of the embryonic axis which was discarded and the two remaining sections contained the cotyledon. These two sections were cut into two longitudinal halves to produce 4 explants/seed with a total amount of 600 explants. To prepare cotyledon explants of squash and cucumber, 30 seeds were sterilized as described above and incubated on Murashige and Skoog (1962) medium in darkness at $25\pm 1^\circ\text{C}$. The cotyledons were removed from the seedlings after 10 days, divided into two longitudinal halves and five transversal sections to produce 20 explants/ plant with a total amount of 600 explants.

Induction of embryogenic calli

The explants of squash and cucumber were incubated in darkness at $25\pm 1^\circ\text{C}$ on MS medium including vitamins and containing 0.8% agar, 3% sucrose. The MS medium was supplemented with three concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D) at 1.0, 2.0 and 5.0 mg/L alone or in combination with kinetin at 0.0, 0.5, 1.0 and 2.0

mg/L. The pH was adjusted to 5.8 prior autoclaving at 121°C for 20 min. The 600 explants were separated on each medium with 50 explants per treatment, divided into five replicates onto 5 Petri dishes (10 explants/Petri), and subcultured every four weeks on a fresh medium for two months.

Suspension of embryogenic calli in liquid MS medium

Suspension cultures were initiated by shaking about 20 grams of friable squash or cucumber calli in liquid MS media. The shaker was adjusted at 70 rpm and incubated in the dark at 25°C in 500 mL Erlenmeyer flasks (5 g/flask) containing 100 mL of MS liquid medium with 1 mg/L 2,4-D. The flasks were covered with aluminum foil and the medium was replaced with fresh one every week for a month. At the same time, 20 grams of squash and cucumber each were sub-cultured on solid MS medium (5 g/Petri dish) with 1.0 mg/L 2,4-D to examine the multiplication and proliferation by weighing the embryogenic calli on the solid and in suspension culture medium every week for a month.

Agrobacterium preparation and transformation procedure

Agrobacterium strain (ATHV) was used in this study. The *Agrobacterium* harboring *pPNGus* as a binary plasmid carries *NPT-II* as a selectable marker gene and *Gus* as a reporter gene (Fig. 1). The *Agrobacterium* was streaked onto LB agar plates supplemented with 200 mg/L

spectinomycin (spec)/streptomycin (strep) each. The agar plates were incubated at 28°C for 2 days; a single colony was picked in liquid LB medium containing the same antibiotics concentration of spec/strep, and then incubated overnight at 28°C with shaking. The *Agrobacterium* culture was collected by centrifugation at 5000 rpm at room temperature for 10 min. The pellet was re-suspended in liquid MS medium, the optical density (OD) was measured with a spectrophotometer and adjusted to OD 0.8. Ten grams of squash embryogenic calli from the suspension culture and from the solid medium were mixed separately with *Agrobacterium* solution containing acetosyringone (AS) at zero and 100 µM for an hour and the same was performed for cucumber. Thereafter, cultures were transferred onto a solid MS medium containing 1.0 mg/L 2,4-D for three days. Subsequently, calli were transferred onto the same medium, in addition to 200 mg/L cefotaxime in order to inhibit *Agrobacterium* growth.

Histochemical analysis of β-glucuronidase activity

Histochemical *Gus* assay was measured three days after co-cultivation with *Agrobacterium* to estimate transient expression of the *Gus* gene. One gram of transformed calli derived from each treatment (suspension culture with or without 100 µM AS, and solid culture with or without 100 µM AS) of squash and cucumber cultures were washed with liquid MS medium. The cultures were incubated overnight at 37°C in *Gus* stain-

ing buffer (0.1 M sodium phosphate pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA and 0.1% Triton X-100) containing 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (X-gluc) substrate (Jefferson, 1987). The solution was replaced with 70% ethanol to remove chlorophyll before accounting the blue spots on the tissues under binocular microscope.

Selection of transformed calli

Three days after addition of the cefotaxime; embryogenic calli were transferred onto selection medium containing 1 mg/L 2,4-D, 1 mg/L kinetin, 300mg/L cefotaxime and 200 mg/L kanamycin (Khidr *et al.*, 2012) as a selection agent and subcultured onto a fresh selection medium every four weeks for two months. The cultures were incubated at $25\pm 1^\circ\text{C}$ in dark.

Conversion of somatic embryos and plantlet regeneration

The putative transformed cells and non-transformed one on the solid and suspension culture media (two grams each) were transferred to regeneration MS medium containing a combination of Benzylaminopurine (BAP) and 1-Naphthaleneacetic acid (NAA) at 1.0 and 0.1 mg/L, respectively. The cultures were incubated at $25\pm 1^\circ\text{C}$ in 16 hour light/8 hour dark. After six weeks, data were recorded on the number of developed plantlet on the regeneration medium.

DNA isolation and PCR analysis

Genomic DNA was extracted from transformed and non-transformed plantlets according to a method described by Edwards *et al.* (1991). The primers used for analysis of the genetically transformed plants were:

5'GAATGGTGATTACCGACG-AAA3'
and

5'CCAGTCGAGCATCTCTTCAGC3'

(Jefferson, 1987). The predicted sizes of the amplified DNA fragments were 574 bps for the *Gus* gene. PCR reactions were performed in a total volume of 25 μl as following: 12 pmol (0.25 μl) of two specific primers, 1 μl of a mixture of four deoxyribonucleoside triphosphates (dNTPs); 0.25 μl of Taq (Eppendorf) DNA polymerase, 2.5 μl of 10X Taq buffer and 20.75 μl of distilled water. The mixture was transferred to a 0.2 ml PCR tube. The mixtures were denatured at 94°C for 5 min followed by 30 cycles at 94°C (1 min), 55°C (1 min) and 72°C (2min). PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide using standard procedures (Sambrook *et al.*, 1989). Control DNA from non-transformed plants was used in the experiments to ensure that reagents were not contaminated.

RESULTS AND DISCUSSION

Effect of 2,4-D and kinetin concentrations on callus induction

Cotyledons and mature seeds of squash (*Cucurbita pepo* L., cv. Eskandrany) and cucumber (*Cucumis*

sativus cv., Faris) were inoculated on MS medium with three 2,4-D concentrations or with nine combinations of kinetin to induce callus formation. Calli of the two genotypes emerged at the edges of the explants and became compact and friable at 8 weeks post inoculation. For the explants of cotyledon and mature seeds of squash; the percentage of callus induction was the highest with 1.0 mg/L 2,4-D (92% and 90%, respectively), followed by 2.0 mg/L 2,4-D (90% and 88%, respectively) as shown in Table (1). Increasing of 2,4-D concentration from 2.0 to 5.0 mg/L had no effect on callus formation in the both types of the explants. Furthermore, callus fresh weight was the greatest at 1.0 mg/L 2,4-D (3.97 g) followed by 2.0 mg/L 2,4-D (3.84 g) for cotyledon explants. In addition, the effect of these two concentrations of 1.0 and 2.0 mg/L 2,4-D on callus fresh weight in mature seed explants was likely similar giving 3.57 and 3.47 g, respectively. In contrast, kinetin had negative effect on the percentage of callus induction and on the callus fresh weight. This negative effect increased with increasing its concentration. For cucumber, the highest percentage of callus formation was induced with 1.0 and 2.0 mg/L 2,4-D (94% and 92%, respectively, Table 2) with cotyledon explants. Continuously, the same was observed for mature seed explants with the percentage of 90% and 92%, respectively, as shown in Table (2). Moreover, 1.0 mg/L 2,4-D gave the greatest value of callus fresh weight (3.68 and 3.35 g) for cotyledon and mature seed explants, respectively.

On the other hand, addition of kinetin at all concentrations decreased the percentage of callus induction in the presence of 2,4-D at 1.0 mg/L, whereas its effect was almost negligible with the other 2,4-D concentrations. Auxins are well known for the induction of embryogenesis alone or in combination particularly 2,4-D (Tabei *et al.*, 1991). Friable embryogenic calli was produced from zygotic embryos (53-56%) and cotyledons from seedlings (70%) of *Cucurbita moschata* cv. Sello de Oro cultured on callus induction medium (CIM) supplemented with 0.5 mg/L or 3.5 mg/L 2,4-D (Valdez-Melara *et al.*, 2009). Earlier reports of embryogenic callus induction in cotyledon and leaf disc explants in cucurbits like squashes and melons with 2,4-D (Noel *et al.*, 1992) are strengthening our results showing embryogenic callus induction in both explants using 2,4-D in cucumber. Our studies also in accordance with El-Absawy *et al.* (2012), who found that the maximum callus induction (94% and 92%) was observed in cotyledons and mature seeds, respectively, on MS medium supplemented with 1.0 mg/L 2,4-D. Ibrahim *et al.* (2009) reported that the highest percentage of callus induction was observed in MS medium supplemented with 0.5 or 1.0 mg/L 2, 4-D when using cotyledon or leaf explants. Usman *et al.* (2011) showed that Maximum callus induction 94.16% and 76% was observed in leaf disc explants on MS medium supplemented with 2 mg/L 2,4-D, NAA and BAP (1.5 mg/L, each), respectively. Cade *et al.* (1990) reported that the two best media for embryo initia-

tion were MS salts and vitamins containing either 1 or 2 mg 2,4-D/L and 0.5 mg kinetin/L. Pal *et al.* (2007) found that maximum morphogenic callus induction rate (86%) was observed from a hypocotyl explant by culturing in MS medium supplemented with 2.5 mg/L 2,4-D. Sul-tana *et al.* (2004) found that the highest percentage of callus induction was in MS medium supplemented with 2.5 mg/L 2,4-D. On the other hand, earlier reports of Noel *et al.* (1992) described no negative effects of cytokinin on somatic embryogenesis in squash in contrast of our results that cytokinin effect was inhibitory or negligible in callus induction on kinetin containing medium.

In general, auxin was effective in stimulation of callus formation and callus weight in the both genotypes, while kinetin often showed inhibited or had rarely negligible effect. From the results of our experiments, auxin (2,4-D) was the crucial growth regulator and played important role in callus induction in cucumber and squash.

Proliferation of embryogenic calli in liquid and solid MS media

Establishment of suspension culture was achieved by transferring 5 g of embryogenic calli into liquid MS medium for 4 weeks. Small, globular embryos were observed after 3-4 weeks on initiation of suspension culture medium containing 2,4-D, (Fig. 2B). In squash as shown in Fig. (3A), the growth and pro-

liferation of embryogenic callus were faster in liquid MS medium than those observed in the solid one. The callus weight increased from 0.6 to 0.7 g with a percentage of 16.67% at end of the first week. This increase in the callus weight continued higher in the liquid than in the solid medium at the end of the second, third and fourth week from 1.4 to 2.5 g, 3.1 to 5.3 g and 5.8 to 10.55 g with a percentage of 78.57, 70.79 and 81.19%, respectively. In cucumber as shown in Fig. (3B), calli weights in liquid MS media were higher than those obtained in the MS solid media. At the end of the first, second, third and fourth week, callus weight increased from 0.64 to 0.9 g, 1.5 to 3.4 g, 3.5 to 7.6 g and 6.3 to 15.1 g with a percentage of 40.63, 126.67, 123.53 and 139.68%, respectively. The growth curve (callus weight) in the liquid and solid media indicated that the proliferation of embryogenic callus was initially slow during the first week in both genotypes. The slow proliferation in the first week may be due to that the embryogenic calli were collected from different media having different plant hormones with different concentrations and transferred to a new one. At the end of the fourth week of incubation, the embryogenic calli incubated in liquid medium showed approximately 2 fold increase over the initial inoculum (5 g) in squash and nearly three fold increase in cucumber and that incubated in solid medium showed approximately one fold increase in both genotypes. Overall, culture of callus in liquid allowed for rapid increase in callus fresh weight. This increase varied between the

both genotypes which was faster and higher in cucumber than that obtained in squash. Raharjo and Punja (1992) reported that suspension cultures offer several advantages over plant tissue growth on solid media. Since the cells and developing embryos are evenly exposed to nutrients and growth regulators in the liquid medium, the effects of any gradients can be eliminated, allowing more synchronous control over plantlet development. Second, the cell clusters and embryos usually separate from each other in the medium, allowing easy handling and recovery. Third, the number of potential plantlets which can be obtained from a single culture can be large if the conditions are optimized, thereby achieving exponential increases in plant numbers. The use of liquid medium has great promise in studies of embryogenesis, as it theoretically permits the isolation of embryogenic cells in a controlled condition. Additionally, liquid culture opened the possibility of mass production of somatic embryos in plant tissue culture (Levin *et al.*, 1989). Cucumber and squash embryogenesis in liquid had rarely been attempted, Chee and Tricoli (1988) found embryo conversion was problematic, and circumvented this problem by washing and reculturing in liquid medium.

Transient expression of Gus gene in embryogenic calli

Expression of *Gus* gene was evaluated in the precultured embryogenic calli (1 g) in the liquid and on solid media with

or without acetosyringone for squash and cucumber. Results in Fig. (4A and B) and Fig. (2F) indicated that the number of blue spots observed in precultured embryogenic calli was higher than those obtained in the embryogenic calli derived from solid medium. For squash, the number of blue spots increased in the precultured embryogenic calli in the liquid than in solid medium from 16 to 22.5 with a percentage of 40.63% and from 21 to 34.25 with a percentage of 63.31%. Along the same way, acetosyringone (0.1 mM) also enhanced transformation frequency in all cases. The number of blue spots increased from 16 to 21 with a percentage of 31.25% and from 22.5 to 34.25 with a percentage of 52.22% in the precultured embryogenic calli on solid and in liquid medium, respectively. In cucumber, the number of blue spots increased in the precultured embryogenic calli in the liquid than in solid media from 19.75 to 30.75 with a percentage of 55.70 % and from 29.25 to 40.50 with a percentage of 38.46%. In the same manner, acetosyringone (0.1 mM) also promoted transformation frequency in all situations. The number of blue spots increased from 30.75 to 40.5 with a percentage of 31.71% and from 19.75 to 29.25 with a percentage of 48.10% in the precultured embryogenic calli on solid and in liquid medium, respectively. In general, our results indicated that the number of blue spots was higher in the precultured embryogenic calli in liquid medium than those derived from solid medium for the both genotypes. This may be attributed to the increased number of somatic embryos in the

liquid medium than that in the solid medium. These embryogenic calli have frequently been the target tissue for transformation (Ribas *et al.*, 2011). In the same sequence, Nhut *et al.* (2006) found that the number of somatic embryos derived from embryogenic calli cultured in liquid medium, especially at a volume of 20 mL, was shown to be more than in solidified medium as 170 rather than 28. Regarding the effect of acetosyringone, it was effective and increased the expression of *Gus* gene in squash and cucumber. It plays a major role in the natural infection of plants by *Agrobacterium tumefaciens* and is known to induce the virulence genes of the Ti-plasmid that initiates the transfer of the T-DNA region into the plant genome (Wei, 2001). A cocultivation medium supplemented with 0.1 mM of acetosyringone was found to produce higher transformation rate in the embryogenic calli in squash and cucumber after three days. The effect of acetosyringone on transformation efficiency was well documented in watermelon (Suratman *et al.*, 2010). The transient *Gus* expression was lower when acetosyringone was omitted from the cocultivation medium. This was confirmed in previous research that low frequency of explants showed *Gus* positive effect was obtained due to the absence of the acetosyringone in cocultivation medium (Shrawat *et al.*, 2007). In any transformation study, acetosyringone has been routinely used because it is a potent *vir* gene inducer and can enhance the transformation efficiency (Saharan *et al.*, 2004; Wenck *et al.*, 1999). Furthermore,

the higher expression of *Gus* gene in cucumber than in squash, probably refer to the different genotypes.

Plant regeneration from putative transformed and non-transformed plant cells

The putative transformed cells were selected on kanamycin containing medium. Subsequently, about 2 g of the developed somatic embryos were transferred to the regeneration medium (Fig. 2C, D and E). For squash, the results in Fig. (5A) showed that the higher main values (22 and 7.25) of regeneration rate were obtained with somatic embryos, which were precultured in liquid MS medium, in the non-transformed and transformed cells, respectively. Whereas, the lower main values (7.5 and 3.5) were obtained with somatic embryos, which were continued on the solid medium, in the non-transformed and transformed cells, respectively. In cucumber, the results in Fig. (5B) revealed that the higher main values (30 and 10.25) of regeneration rate were obtained with somatic embryos, which were precultured in liquid MS medium, in the non-transformed and transformed cells, respectively. Whereas, the lower main values (11 and 5.5) were obtained with somatic embryos, which were continued on the solid medium, in the non-transformed and transformed cells, respectively. Generally, the regeneration frequency was higher in the non-transformed and transformed cells (each individual) with cells derived from precultured calli in the liquid MS medium than those continued in the solid medium

in the both genotypes. It was reported that embryogenesis using liquid medium from initial culture in sunflower (Bergervoet *et al.*, 1989) and melon (Kageyama *et al.*, 1991) enhanced regeneration frequency compared to the previous reports which employed a solid medium during all culture phases. Raharjo and Punja (1992) reported that suspension cultures offer several advantages over plant tissue growth on solid media. Since the cells and developing embryos are evenly exposed to nutrients and growth regulators in the liquid medium, the effects of any gradients can be eliminated, allowing more synchronous control over plantlet development. Second, the cell clusters and embryos usually separate from each other in the medium, allowing easy handling and recovery. Third, the number of potential plantlets which can be obtained from a single culture can be large if the conditions are optimized, thereby achieving exponential increases in plant numbers. In addition to a high number of regenerates, somatic embryogenesis is more attractive than organogenesis as a plant regeneration system due to the low frequency of chimeras and limited level of somaclonal variation (Van *et al.*, 2008).

Molecular analysis of putative transgenic plantlets by PCR

To verify gene integration, genomic DNA was extracted from putative transformed, non-transformed plants as negative control and the plasmid DNA was used as positive control as shown in Fig. (6). PCR analysis was performed from some putative transgenic plants, 5

samples for squash and 4 for cucumber. In all tested transformants one band corresponding to the expected *Gus* fragment of 570 bps was detected. No band was detected in the case of non-transformed plant (negative control DNA) in squash and cucumber. According to the results of *Gus* assay and selection of *NPT-II* plants on kanamycin containing medium and PCR analysis, we conclude that the *Gus*, *NPT-II* genes were introduced into these transformed plants.

SUMMARY

An improved plant regeneration and *Agrobacterium*-mediated genetic transformation system was established for squash (*Cucurbita pepo* L.) and cucumber (*Cucumis sativus* L.). The effect of 2,4-D and kinetin on callus formation, the effect of liquid and solid MS medium on embryogenic callus proliferation, expression of *Gus* gene beside its influence by acetosyringone, and regeneration of transformed and non-transformed plant cells were investigated. Cotyledon explants and 2,4-D of 1 mg/L were found to be the best for induction of callus formation giving the highest percentage; 92% and 94% in squash and cucumber, respectively. Transfer of induced callus on liquid MS medium containing 1 mg/L 2,4-D for 4 weeks greatly increased the proliferation of embryogenic callus with an increased percentage of 81.9% and 139.7% in squash and cucumber, respectively. Preculture of embryogenic callus on the liquid MS medium prior to inoculation and cocultivation with *Agrobacterium* enhanced the genetic transformation effi-

ciency with an increased percentage of 40.6% and 55.7% in the number of blue spots in the absent of acetosyringone in squash and cucumber, respectively, and with an increased percentage of 63.1% and 38.5% in the number of blue spots in the presence of 0.1 mM acetosyringone in squash and cucumber, respectively. Higher percentage of non-transformed regenerates (193.3% and 172.7%) and transformed regenerates (107.1% and 86.4%) were obtained from the embryogenic calli, which were previously cultured on the liquid MS medium in squash and cucumber, respectively. Molecular evidence of transgenic plants was confirmed by polymerase chain reaction (PCR) analysis and demonstrated the effectiveness of the transformation procedure.

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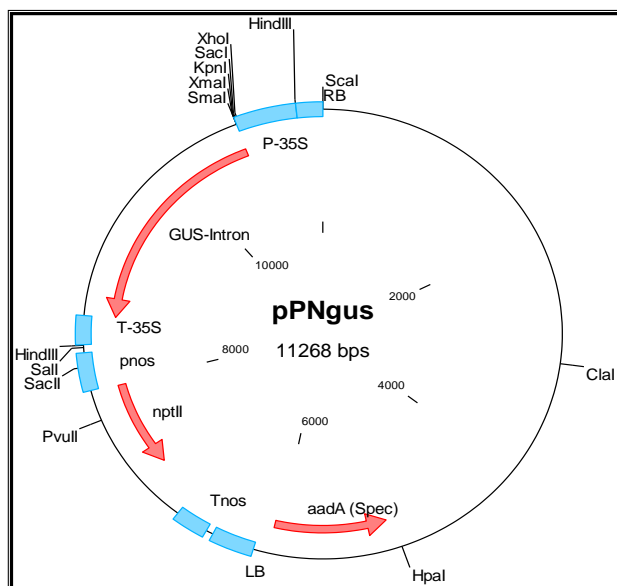
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Table (1): Effect of 2,4-D and kinetin concentrations on induction of callus using cotyledon and mature seed explants of squash.

Cytokinin and auxin conc.		Cotyledon explants			Mature seed explants		
2,4-D (mg/L)	Kinetin (mg/L)	% of Explants induced callus	Fresh weight After 60 days (g)	Callus type	% of Explants induced callus	Fresh weight After 60 days (g)	Callus type
1.0	0.0	92	3.97	compact	90	3.57	compact
	0.5	86	2.41	compact	84	2.96	compact
	1.0	80	2.19	friable	80	2.88	friable
	2.0	66	2.03	friable	64	2.79	friable
2.0	0.0	90	3.84	compact	88	3.47	compact
	0.5	84	3.44	compact	82	2.58	compact
	1.0	82	2.83	compact	76	2.44	compact
	2.0	62	2.65	friable	62	2.26	friable
5.0	0.0	90	3.80	compact	88	3.09	compact
	0.5	88	2.37	compact	84	2.47	friable
	1.0	88	2.13	friable	84	2.36	friable
	2.0	84	1.76	friable	84	2.15	friable

Table (2): Effect of 2,4-D and kinetin concentrations on induction of callus using cotyledon and mature seed explants of cucumber.

Cytokinin and auxin conc.		Cotyledon explants			Mature seed explants		
2,4-D (mg/L)	Kinetin (mg/L)	% of Explants induced callus	Fresh weight After 60 days (g)	Callus type	% of Explants induced callus	Fresh weight After 60 days (g)	Callus type
1.0	0.0	94	3.68	compact	90	3.35	compact
	0.5	90	3.44	compact	82	3.28	compact
	1.0	84	3.37	friable	80	2.49	friable
	2.0	60	2.59	friable	60	2.41	compact
2.0	0.0	92	3.53	compact	92	3.04	compact
	0.5	88	3.41	compact	84	2.75	compact
	1.0	88	3.06	compact	82	2.39	compact
	2.0	80	2.43	friable	64	2.32	friable
5.0	0.0	90	3.41	compact	82	2.88	friable
	0.5	90	3.33	compact	80	2.71	friable
	1.0	90	2.92	friable	80	2.07	friable
	2.0	90	2.14	friable	76	2.04	friable

Fig. (1): Schematic representation of plasmid *pPNGus*.

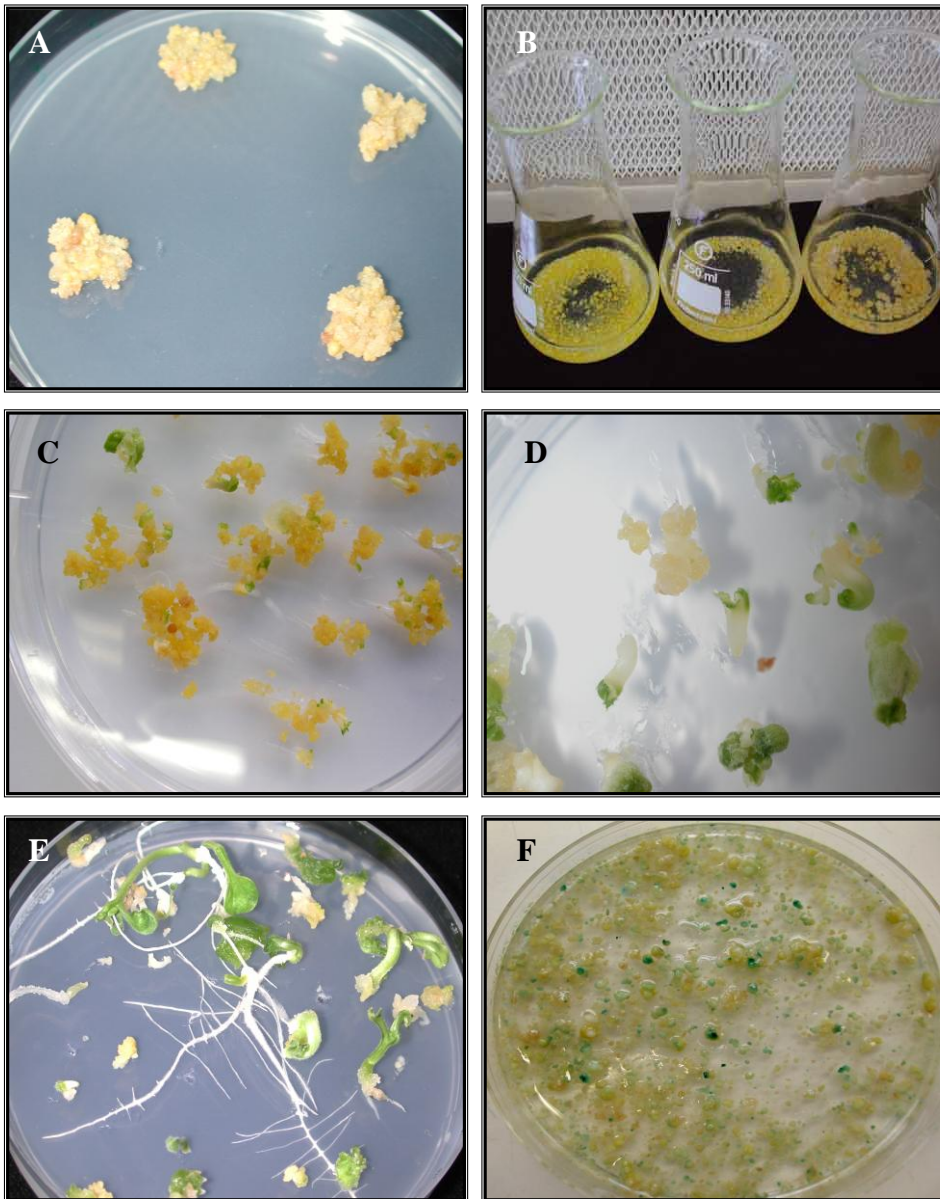


Fig. (2): Development of embryogenic callus and plantlets regeneration. (A), formation of embryogenic calli after 8-9 weeks. (B), establishment of embryogenic cell suspension culture after 4 weeks formation of globular somatic embryos (C), (D) and (E), conversion of somatic embryos and plantlets regeneration after exposing to light/dark for 2-3 weeks. (F), transient *Gus* gene expression in embryogenic calli.

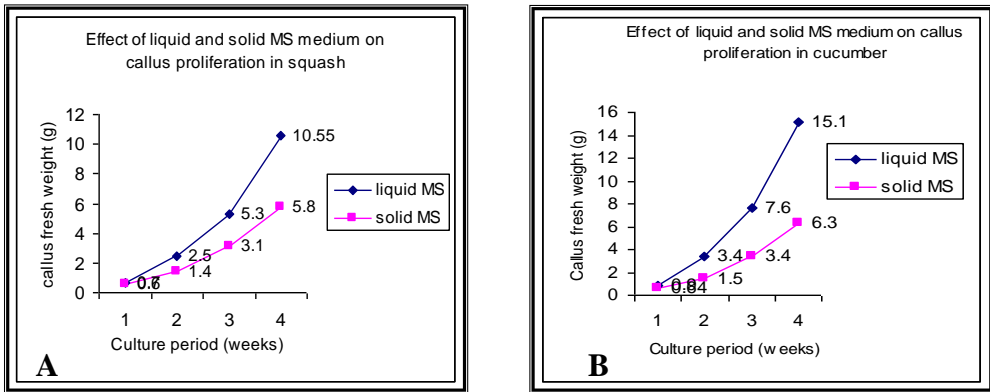


Fig. (3): Growth curve showing callus fresh weight (g) in liquid medium (◆) and solid medium (■), (A): squash and (B): cucumber.

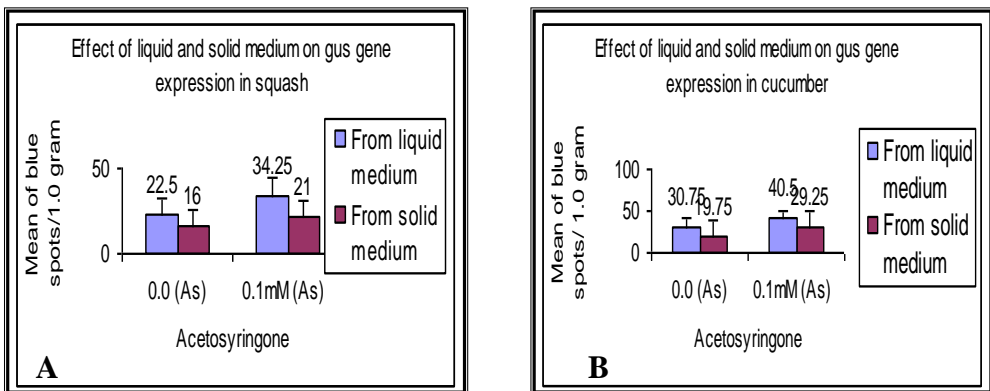


Fig. (4): Effect of liquid and solid preculture MS media on the average of *Gus* gene expression (blue spots) in squash (A), and in cucumber (B) embryogenic calli.

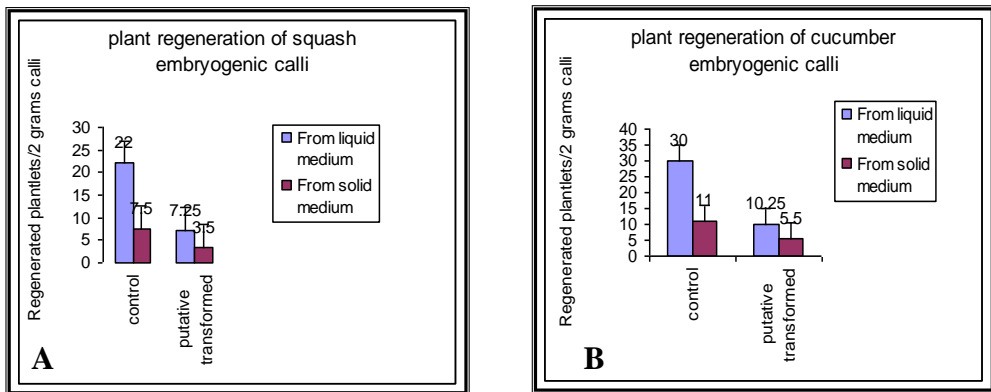


Fig. (5): Effect of liquid and solid preculture media on the average of regenerated plantlets in squash (A) and cucumber (B) embryogenic calli.

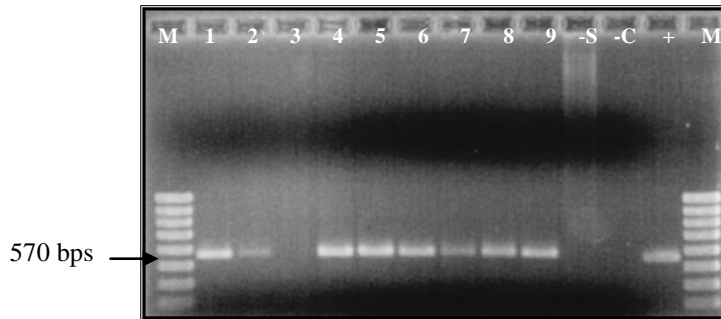


Fig. (6): PCR screening of some regenerates with *Gus* primers. Transformed regenerates show amplification of fragment about 570 bp in size. M: 100 bp DNA ladder; 1-5: transformed regenerates of squash; 6-9: transformed regenerates of cucumber; -s: negative control for squash; -c: negative control for cucumber; +: positive control.