AGROBACTERIUM-MEDIATED TRANSFORMATION OF TWO TOMATO CULTIVARS (*LYCOPERSICON ESCULENTUM* MILL.) CV. SANDRA AND ROCKY

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ABSTRACT

An efficient protocol for *Agrobacterium*-mediated transformation of tomato cultivars Sandra and Rocky was conducted to examine the possibility of producing transgenic tomato plants cultivars harbouring the *nptII* gene, conferring kanamycin resistance. To achieve this aim, tomato cotyledon explants were transformed using EHA105 *Agrobacterium tumefaciens* strain harboring the binary vectors pBI121 which contains Gus gene, and neomycin phosphotransferase II (*nptII)* as selectable marker gene under the control of a *CaMV35S* promoter and nopaline synthase (*nos*) Terminator. Transformant detection was carried out in three distinct ways. First antibiotic selection, Kanamycin at a concentration of100 mgl^{-1} found to be efficient for this purpose. Second histochemical GUS assay revealed the presence of blue colored zones in a number of shoots and leaves for both *in vitro* and the greenhouse-grown transgenic plants. Third PCR analysis indicated positive result by showing the fragment for *nptII* gene in tested transformants, while was absent in non-transgenic control (wild type). On the other hand, the results showed that Sandra cultivar was more efficient for regeneration and subsequently transformation frequency than Rocky cultivar, which record 26.66% of transformation frequency compared with 11.57% in Rocky cultivar.

Keywords: Tomato, binary vectors, transformation frequency, *nptII*, GUS assay.
***Part of PhD Dissertation at Zakho University of the 1st author.**

مجلة العلوم الزراعية العراقية -2021 :52 (3):745-755 دانيال وآخرون

استعمال تقانة الاكروبكتريوم لأنتاج نباتات الطماطة المعدلة وراثيا في صنفي ساندرا وروكي

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المستخلص

جريت الدراسةلاختبار امكانية انتاج نباتات الطماطة المعدلة وراثيا في صنفي (ساندرا وروكي) تأوي المورثة nptII التي تمنح صفة المقاومة للمضاد الحيوي كانامايسين. لتحقيق هذا الهدف تم اجراء التحول الوراثي لنبات الطماطة عن طريق الاوراق الفلقية باستعمال السلالة البكتيرية EHA105 التي تأوي الناقل الثنائي pBI121 الذي يحمل المورثتين GUS و نيوميسين فسفوترانسفيراز nptII تحت سيطرة البادئ CaMV35S والناهي nos. حيث تم الكشف عن عملية التحول الوراثي بثلاث طرق ، اختبار المضادات الحيوية حيث وجد ان المضاد الحيوي الكانامايسين بتركيز100ملغرام/ لتر كان فعالا في الكشف عن عملية التحول الوراثي ، ثانيا الكشف النسيجي الكيميائي GUS الذي كشف عن وجود مناطق زرقاء مخضرة اللون في عدد من البراعم والاوراق للنباتات المعدلة وراثيا في المختبر وفي البيت الزجاجي و ثالثا تحليل التفاعل التسلسلي البوليمرازي PCR. اظهرت نتائج ايجابية من خلال اظهار حزم للمورثة nptII في النباتات المعدلة وراثيا ، بينما لم تظهر مثل تلك الحزم في المجموعة الضابطة (النباتات غير معدلة وراثيا) النوع البري. وقد اوضحت النتائج ان صنف ساندرا كان اكثر كفاءة في عملية التمايز والتحور الوراثي ، اذ بلغت نسبة التحول الوراثي فيه 26,66% مقارنة مع صنف روكي حيث بلغت نسبتها 11,57% .

كلمات مفتاحية: طماطم ، نواقل ثنائية ، تردد التحويل ، nptII ، مقايسة GUS.

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INTRODUCTION

Transformation systems refers to the capacity for introducing foreign genes into plants. Plant transformation is an important genetic engineering tool for introducing foreign genes into the genomes of plant species and refers to the transfer and incorporation of engineered genes or plasmids into the plant genome to generate genetically modified plants also known as transgenic plants (7). It is not only an essential biotechnological approach for in-depth research of plant growth, but also a promising tool for modern agriculture, specifically for the introduction and selection of desired crop traits. (22). Plant transformation is now a core research tool in plant biotechnology, it is a practical tool for transgenic plant development that is done by several techniques for transfer of isolated genetic materials into a viable host cell. (17). *Agrobacterium tumefaciens* is a soil phytopathogen that naturally infects plant wound sites and causes crown gall disease via delivery of transferred T-DNA from bacterial cells into host plant cells through its Ti plasmid. *Agrobacterium* mediated transformation is the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants in diverse fields of biological and biotechnological research (35). Selectable marker genes are required for the plant genetic engineering processes to simplify the identification of transgenic plants; however, it is more desirable to obtain transgenic plants without selection markers (8).*Neomycin phosphotransferase II (NPTII)* is the most widely used selectable marker gene for plant transformations. It confers transgenic plants resistance to the antibiotic, kanamycin. (3). Reporter gene is a type of marker genes used to quantify the gene expression. (19) The □-glucuronidase (GUS) enzyme is reporter gene that has been well documented to provide desirable characteristics as a marker gene in transformed plant and resulting color is clearly visible (16). Many researches and reports showed a great deal of interesting to develop tomato crop by introducing value agronomic traits by either plant tissue culture or genetic transformation (28). Successful application of biotechnology in plant improvement depends upon the type of explants and availability of efficient plant regeneration protocol to clone new plants (11, 6, 1, 18 and 14) these factors could limits the plant genetic transformation (10). Tomato production in Iraq is a challenge due to the lack of water resources, the high salt concentration of some irrigation water, lack of suitable pesticides and chemical fertilizers. Therefore, the crop production can be enhanced by improving agricultural species through genetic manipulation. This study was aimed to production of transgenic tomato plant from Sandra and Rocky cultivar harboring *nptII* and Gus genes that confirmed the potential to add a foreign gene for these two cultivars.

MATERIALS AND METHODS

This investigation was carried out at Plant Biotechnology Resource and Outreach Center, Horticulture Department, College of Agriculture, Michigan State University, United States of America during the period from September 2018 to September 2019. Seeds of two cultivars of tomato (*Lycopersicon esculentum* Mill.) plant used in this study Sandra and Rocky were obtained from Agriculture Research Station, Duhok Province, Kurdistan Region of Iraq.

Seed surface sterilization

Seeds were surface sterilized with 70% ethanol for 1 minute followed by immersion in 2.5% (v/v) sodium hypochlorite solution containing 0.1% Tween-20 with continuous stirring. Seeds were then rinsed five times with sterile distilled water and drying, germinated in half-strength MS medium (23) + B5 vitamins, 1.5% sucrose, pH adjusted at 5.6, and solidified with 0.6 % (W/V) Bacto agar. The seeds were grown at 25 ±2 ◦C and 70% relative humidity for 8 days under light density of 30-40 µE m^{-2} S^{-1}, with 8 h dark and 16 h light of photoperiod.

Kanamycin sensitivity test

Tomato regeneration media with different concentrations of kanamycin (0, 25, 50, 100 and 200 mgl^{-1}) were used to test the sensitivity of cotyledon explants. Cotyledon leaves explant of one week old were cultured in selection medium containing respective concentrations of kanamycin, in addition to control treatment without antibiotic. Each experiment was carried out with three

replicates .After four weeks of incubation, the regeneration percentage and number of shoots /explants were recorded.

***Agrobacterium* strain and plasmid vectors**

A. tumefaciens strains EHA 105 harboring pBISN1 plasmids (provided by Dr. Song, Michigan State University (MSU), were used containing the neomycin phosphotransferase II (nptII) gene conferring kanamycin (km) resistance and an intron-interrupted β-glucuronidase (GUS) gene (gus A) within the left and right border region (Fig.1). The portable intron was cloned from the potato ST –LSI gene and allows expression of GUS only in transformed plant cells and not bacterial cells (26). The gus A gene was driven by the chimeric promoter $(Aocs)_3$ AmasPmas (27).

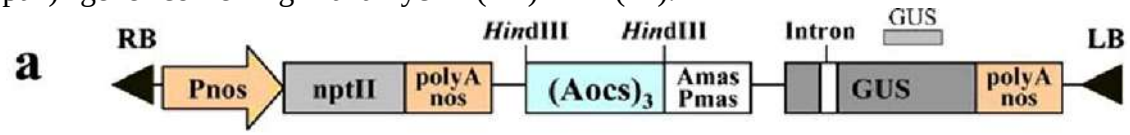

Figure 1. Schematic diagram of the T-DNA region of binary vector pBI121

Transformation procedure

Cotyledons explant derived from one week old of tomato cv. Sandra and Rocky were immersed for 10 min in a suspension of *A. tumefaciens* that harbored the pBI121 binary vector. Then were transfer on the MS medium plus B5 vitamins (TCIM), which contain 1.0mgl^{-1} BAP, 0.5 mgl^{-1}NAA, the explants were kept in darkness at 25±2˚C for 4 days. After co-cultivation, the explants washed four to five times in liquid tomato callus induction medium (TCIM) during the last wash, 1gl^{-1} of the timentin was added to remove the excess *Agrobacterium* cells, and explants then were blotted dry on sterile filter paper. After washing, the inoculated explants were cultured on tomato regeneration and selection media TRM (MS medium plus B5 vitamins contained 0.5 mgl^{-1} IAA, 1mgl^{-1} zetain riboside in addition to 50 mgl^{-1} kanamycin, 250 mgl^{-1} timentin and 250mgl^{-1} cefotaxime for two weeks at 25±2˚C in the dark, followed by photoperiod of 16 h light and 8 h dark at 30 µE m^{-2} S^{-1}. After 3 weeks of incubation the kanamycin concentration was increased to 100 mgl^{-1} in the regeneration and selection medium, after 6 weeks of selection, the number of explants that regenerated shoots for both cultivar was counted and the explants with multiple shoots were transferred to Magenta GA7 boxes, containing 50 ml fresh selection medium (TRM) contained 100 mgl^{-1} kanamycin, 250 mgl^{-1} timentin, and 250 mgl^{-1} cefotaxime. After 3 weeks in culture, regenerated shoot explants were excised and cultured on TRM containing the same concentration of antibiotic. Shoots of 2-3 cm. in length were excised from the regenerated explant and rooted on MS medium free of growth regulators augmented by 100 mgl^{-1} kanamycin. The rooted shoots (plantlets) were transplanted to 4 inch plastic pots containing water-soaked Suremix Perlite planting medium (Michigan Grower Products Inc., Galesburg, MI) and covered with plastic bags to maintain high humidity at 25 ^{o}C under 16/8 h light/dark photoperiod cycle. The plantlets were hardened by gradually removing the plastic bags over 2 weeks. The acclimated plants were repotted into (17.8 (H) x 18.3 (D) cm.) pots and grown in greenhouse.

Histochemical assay

Histochemical Gus assays for transformed and non-transformed plant were conducted for Sandra and Rocky cultivars to recognize histologically if transformed explants treated with pBI121 were positive for the *GUS* gene. Tested plants were regenerated from cotyledons leaves in TRM medium supplemented with100 mgl^{-1} kanamycin according to (8). Transient GUS expression assays were carried out after co-cultivation and regeneration. Stable GUS expression assays were performed on T0 explants, *in vitro* and greenhouse grown plants, pieces of plant leaves for each line were put into 1.5 ml eppendorf tubes and stained in a 2 mM X-Gluc (5-bromo-4-chloro-3-indoly-D-glucuronide, cyclohexylammonium salt) phosphate buffer. The tube was closed and incubated in the dark at 37 ◦C overnight to allow the blue color which is the characteristic expression of GUS (glucuronidase) to develop, the staining solution was removed after staining, the chlorophyll was removed by soaking in 95% ethanol for 2 hours and 70% ethanol for 48

hours at 37 ◦C. The transgenic leaves appeared blue and the non–transgenic leaves were white.

Polymerase chain reaction (PCR)

Total genomic DNA was isolated from young leaves of greenhouse-grown plants using a CTAB (Cetyltrimethylammonium bromide) protocol described by (24). For PCR analysis, the primers corresponding of the nptII coding region 5-GAGGCTATTCGGCTATGACTG-3 and 5-ATCGGGAGCGGGGATACCGTA-3 were used for analysis of the transgenes. The reaction conditions were 94 ◦C for 2 min for initial denaturation, 25 cycles of 94 ◦C for 45 s for second denaturation, 58 ◦C for 1.5 min for primers annealing and 72 ◦C for 2 min for extension, with a final extension at 72 ◦C for 10 min. Products were separated on 1% agarose gel containing ethidium bromide and visualized under UV light.

RESULTS AND DISCUSSION

Kanamycin sensitivity test

Kanamycin is phytotoxic and diversely affects the regeneration ability in plant tissue cultures, inhibitor for the regeneration of untransformed plantlets and is widely used as selective agents in plant transformation process **(**31, 34 and 12). Different concentrations of kanamycin (25, 50, 100 and 200 mgl^{-1}) were tested in this study for both cultivars Sandra and Rocky added to the regeneration media which was compared with control (free in kanamycin). It was found that there are no survival plantlets (%) in tomato regeneration using different concentration of kanamycin after four weeks of cultured (Table 1).

Table 1. kanamycin sensitivity test for Sandra and rocky cotyledons leaves after four weeks of cultured

Kanamycin concentration mgl^{-1}	Regeneration %		Shoots number/ explants	
	Sandra	Rocky	Sandra	Rocky
0	97.0 a ±3.3	90.0 a ±5.8	4.7 a ±0.3	4.0 a ± 0.3
25	0.0 b ± 0.0	0.0 b ± 0.0	0.0 b ± 0.0	0.0 b ± 0.0
50	0.0 b ±0.0	0.0 b ± 0.0	0.0 b ±0.0	0.0 b ± 0.0
100	0.0 b ±0.0	0.0 b ±0.0	0.0 b ±0.0	0.0 b ± 0.0
200	0.0 b ±0.0	0.0 b ±0.0	0.0 b ±0.0	0.0 b ± 0.0

Different letters within columns represent significant differences according to Duncan's multiple range tests at 5% level.

The explants started to gain yellow color at 100 mgl^{-1} kanamycin and turn to brown and finally died at 200 mgl^{-1} kanamycin (Figure 2). So, according to this result the optimum kanamycin concentration was found to be 100 mgl^{-1}as lethal level for the selection and recognize the transformed shoots. Thus, the shoots surviving in this selection pressure for more than 30 days will be considered as putative transformed. (33, 25 and 9) used the same concentration of kanamycin 100 mgl^{-1} for selection of transformed tomato plant regeneration from cotyledon leaves. Lower concentrations 50 mgl^{-1} were used in different studies (10 and 21). A high concentration of kanamycin 200 mgl^{-1} was reported by (20, 5, 15 and 30) for positive selection of the transformed tomato tissue.

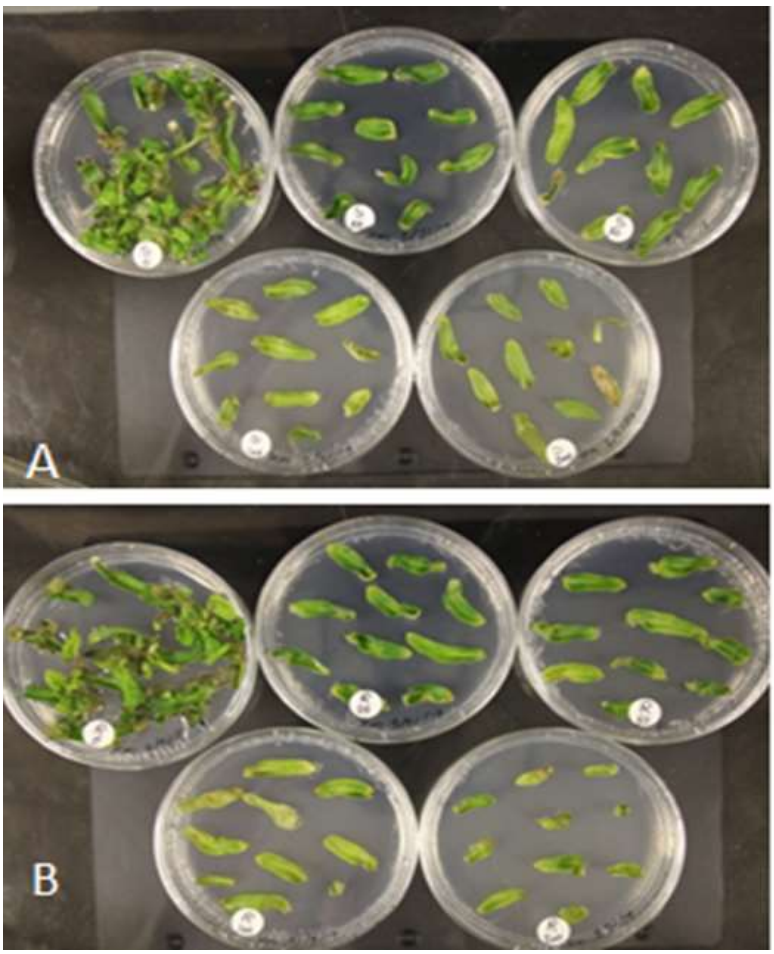

Figure 2: kanamycin sensitivity for cotyledon explant cultured in TRM with (0,25,50,100 and 200) mgl-1 kanamycin started from left to the right after 4 weeks, A in Sandra cultivar and B in Rocky cultivar.

Stability of Gus gene expression

Histochemical GUS assay revealed the presence of blue colored zones in a number of shoots and leaves that remained survived in kanamycin selection. Cotyledonary leaf explants of both cultivars of tomato (Sandra and Rocky) were found to be compatible with *Agrobacterium tumefaciens* strain EHA 105. In most of the cases the entire cut surface of the explants was found to be blue following transient GUS assay. GUS activity was detected in the tissues of T0 transformants. Blue color was observed in the tissues of both in vitro-cultured (Figure 3, A, B) and the greenhouse-grown T0 transgenic plants, but not in non-transgenic tissues (Figure 3, C, D, E, F). Although a number of randomly selected shoots from the selection medium (100 mgl^{-1} kanamycin) showed a positive GUS expression. Not all survived shoots on the selection pressure show positive GUS expression. A number of studies reported same phenomenon in sunflower and in potato plant (38 and 29). These observations indicate that the expression of the GUS gene activity in the regenerated shoots was not direct correlated with kanamycin resistance. The lack of GUS expression in kanamycin resistant shoots may be due to the methylation of the gene that can alter gene expression or loss of GUS gene resulting from rearrangement of the coding sequence (4).

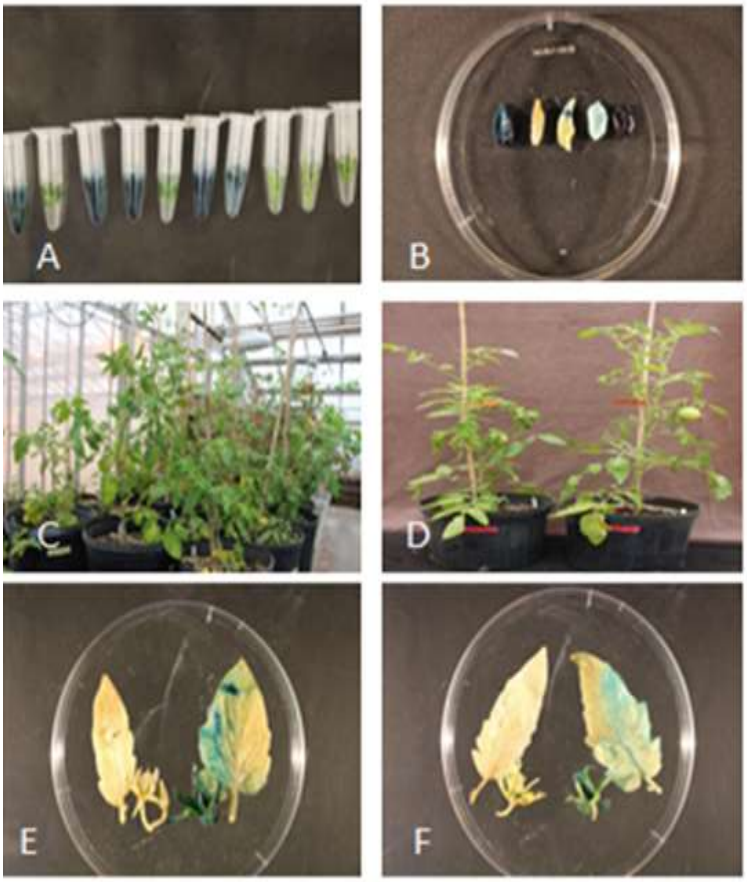

Figure 3: To histochemical Gus assays of transformed and non-transformed L esculentum tissues in vitro cultured.
A Sandra cultivar, B Rocky cultivar. C and E cultivar in the greenhouse. D and F Rocky cultivar in the greenhouse.

Polymerase Chain Reaction (PCR) as a molecular analyses of putative transformants

The results were initially confirmed by polymerase chain reaction via DNA extraction for putative tomato leaves. This test was carrying out using the primer for amplify nucleotide segment of *nptII*. The PCR analysis of the putative transgenic plants were carried out to confirm the presence of *nptII* genes that give kanamycin resistance from genomic DNA. The results clarified that nine putative transformed plants from Sandra cultivar and six putative transform plants from Rocky cultivar were assayed through PCR amplification by using specific primers for transgene. PCR analysis indicated positive result by showing the fragment for *nptII* gene in tested transformants compared to the positive control (plasmid control) (Figure 4), while was absent in non-transgenic control (wild type). The results indicate that the criterion based on the production of *in vitro* kanamycin-resistant shoots is reliable in determining genetic transformation frequencies.

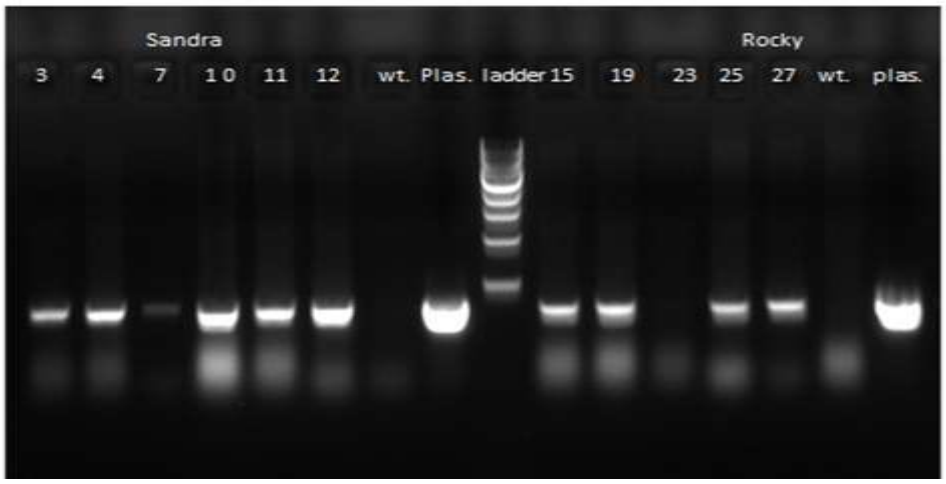

Figure 4. Amplification of *nptII* gene segment in Sandra and Rocky tomato cultivars using *nptII* primers

This result illustrate that the *Agrobacterium* strain holding (EHA105/ pBI121) plasmid with *nptII* gene in T-DNA which confirm kanamycin resistance has been inserted into transgenic cells genome. On the other hand, the main step for gene detection during practical stage is expose the putative transgenic plantlets to selection potential to prove gene insertion into plant cell genome. Thus, the plantlets were exposed to low concentration of kanamycin (50 mgl^{-1}) in the initial selection media (Figure 5, A and B). Then, the selection pressure was increased to (100 mgl^{-1}) in subsequent-sub cultures (Figure 5, C and D).

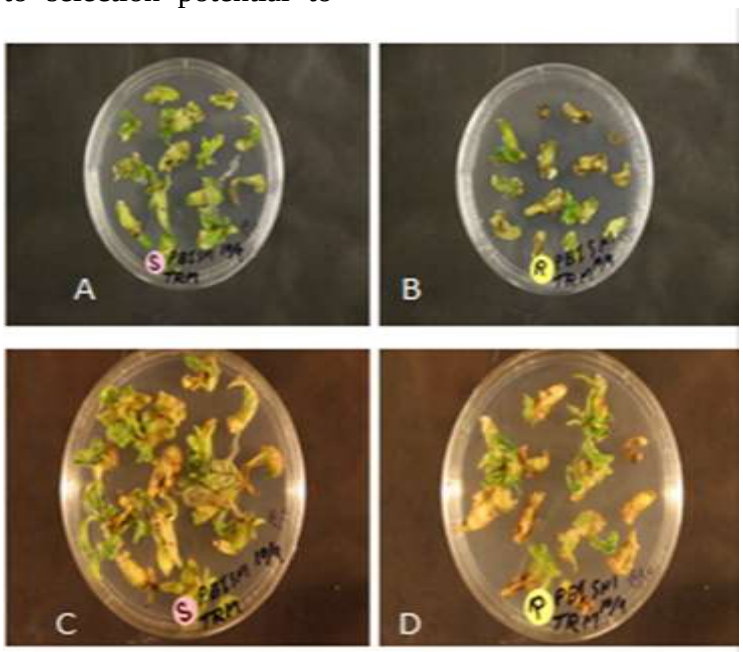

Figure 5: A. tu mefaciens mediated transformation of cotyledon leave explants of L esculentum.

A Sandra cultivar and B Rocky cultivar in TRM supplemented with 50 mgl-1 kanamycin after four weeks, C and D Sandra and Rocky cultivars in selection medium content 100 mgl^{-1} kanamycin after six weeks.

To evaluate the transformation efficiency, table (2) indicated that total of 100 cotyledonary leaves from Sandra and 157 one of Rocky cotyledonary leaves one week old (Figure 6, A and B) were co-cultivated with Agrosuspension (Figure 6, C and D).

Table 2. Sandra and Rocky cultivar response to kanamycin resistance 100mgl^{-1} and Gus gene in tomato transformation plants after 6 weeks

	Total number of explants	Number of explant producing kanamycin resistance shoots	Total number of shoots /explant	Transformation frequency %
Sandra	**100**	**80**	**3.71**	**26.66**
Rocky	**157**	**54**	**2.27**	**11.57**

The efficiency of gene transfer was evaluated via histochemical analysis of transients during the stability of *gus*-gene expression and determination of kanamycin resistant through the molecular analysis. Initially, kanamycin resistance was used for selection of transient gene expression. Transformation trials revealed great variations in the transformation frequency depending on the tomato cultivars (Sandra or Rocky). The results showed that Sandra cultivar was more efficient for regeneration and subsequently transformation frequency than Rocky cultivar. Around 80 regenerates were produced which record 26.66% of transformation frequency when compared with Rocky cultivar that 54 explants were regenerated and the transformation frequency reached 11.57%. Morever, the high number of shoots / explant 3.71 was recorded in Sandra cultivar (Figure 6, E) versus 2.27 shoots / explant in Rocky cultivar. *A. tumefaciens* strain EHA105 has been used for a successful transformation of tomato plants and efficient gene delivery is essential for genetic transformation (36, 37 and 2). This results has agreed with that reports of transformation efficiency of tomato plant which depends upon many factors such as the plant genotype (32 and 13). This result strongly verifies the successful transformation of two tomato cultivars Sandra and Rocky which is recorded for the first time in Iraq. Survived shoots that remained alive in 100 mgl^{-1} kanamycin (Figure 6, F) were cultured on MS supplemented with 100 mgl^{-1} kanamycin and incubated for 10 days for root induction (Figure 6, G). All normally rooted plants were planted in the soil and transferred to the greenhouse after acclimatization (Figure 6, H

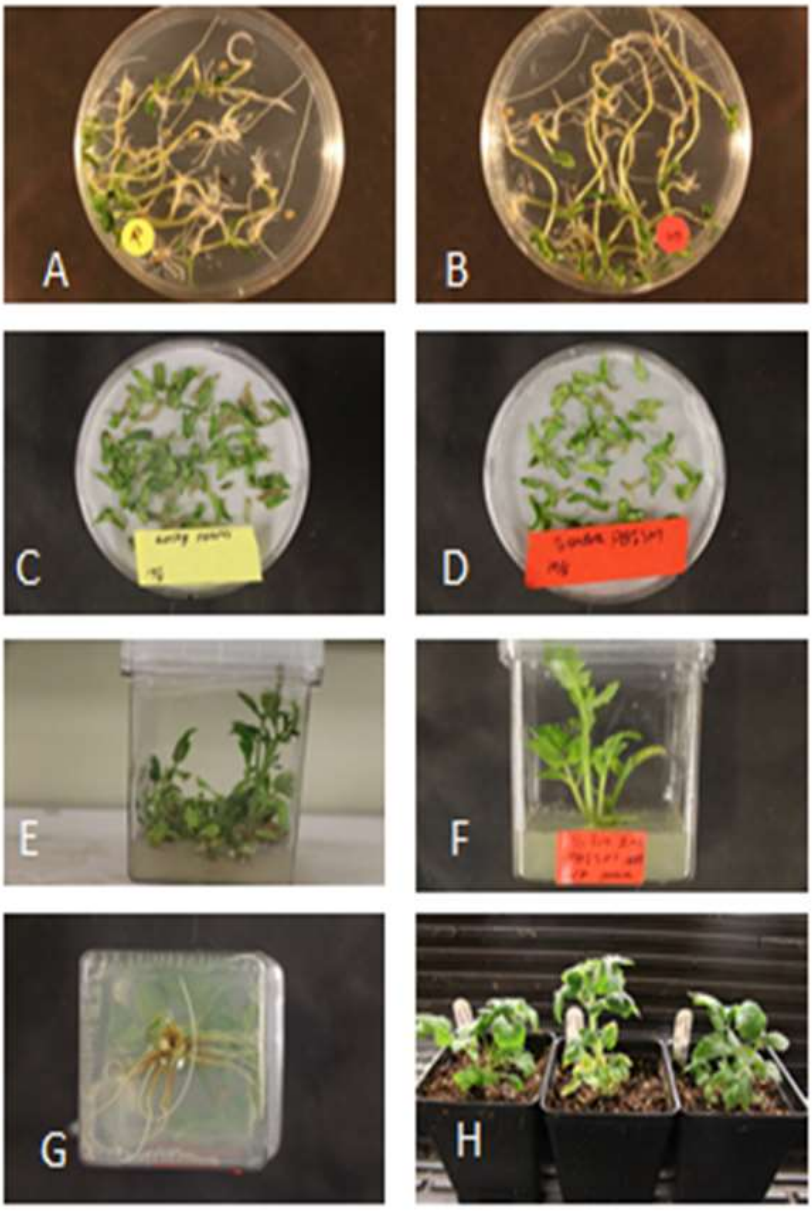

Figure 6. A. 7 days old seedlings of Rocky tomato cultivar, B. 7 days old seedlings of Sandra tomato cultivar, C. cotyledon explants from Rocky cultivar in co- cultivation medium after four days, D. cotyledon explants from Sandra cultivar in co- cultivation medium after four days, E. plant regeneration in selection medium after 8 weeks, F plant regeneration in selection medium, G rooting plantlets in MS medium content 100 mgl-1 kanamycin, H growth of transgenic plant in the greenhouse.

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