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In Vitro Induction of Crown Galls by Agrobacterium tumefaciens Super Virulent Strain A281 (pTiBo 542) in Lentil (Lens culinaris Medik.)

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Research Note

In Vitro Induction of Crown Galls by *Agrobacterium tumefaciens* Super Virulent Strain A281 (pTiBo 542) in Lentil (*Lens culinaris* Medik.)

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Abstract: Twenty-one genotypes of lentils (*Lens culinaris* Medik.), obtained from different sources in Turkey and Pakistan, were included in the study. Seeds were germinated in MS medium for 6-10 days with a 16 h photoperiod at 24°C, from which leaf and stem explants were isolated for inoculation with super virulent strain A281 (pTiBo 542) :: pBI121.1 agropine/mannopine type of *Agrobacterium tumefaciens*. The binary plasmid pBI121.1 carried a GUS gene directed by CaMV 35S promoter in order to confirm transformation by GUS expression. Parameters recorded include percentage of tumour formation, tumour diameter, and tumour weight. Pul 11, Kışlık Yeşil 21, Akm 565 were best when leaf explants were used, whereas Kırmızı 51, Malazgirt, Akm 49 and Akm 196 were best when stem explants were used in tumour induction. Pul 11, Kırmızı 51, İll 62, Emre 20, Malazgirt, Akm 565, Akm 49, Akm 62, Akm 196, Akm 261, Akm 263, Akm 302 and Akm 362 formed tumours on both leaf and stem explants. Four cultivars, namely, Masoor 85, Masoor 93, Akm 247 and Akm 258, did not induce tumours on leaf explants, whereas six genotypes, namely, Sazak 91, Kayı 91, Masoor 85, Masoor 93 Akm 247 and Akm 260 had no tumours when stem explants were used. Tumour induction was also confirmed by histochemical GUS analysis.

Key Words: *Lens culinaris* Medik., lentils, *Agrobacterium tumefaciens*, tumour induction, GUS analysis

Süper Virülent A281 (pTiBo 542) *Agrobacterium tumefaciens* Hattı ile Mercimekte (*Lens culinaris* Medik.) *in vitro* Tümör Oluşumu

Özet: Çalışmada Türkiye ve Pakistan'dan temin edilen 21 farklı mercimek (*Lens culinaris* Medik.) genotipi kullanılmıştır. Tohumlar MS besiyerinde 16 saat fotoperiyot ve 24 °C sıcaklıkta çimlendirilmiştir. Ekimden 6-10 gün sonra, gelişen bitkilerden yaprak ve gövde eksplantları izole edilerek *Agrobacterium tumefaciens* A281 (pTiBo 542) :: pBI121.1 hattı ile inoküle edilmiştir. İkili (binary) plazmid pBI 121.1, gen aktarımı yapılan hücre ve dokuların belirlenmesini sağlayan ve CaMV 35S promotörü tarafından kontrol edilen GUS genini taşımaktadır. İnokülasyondan 6 hafta sonra tümor oluşum oranı, tümor çapı ve tümör ağırlıkları belirlenmiştir. Yaprak explantları kullanıldığında en yüksek tümör oluşumu Pul 11, Kışlık Yeşil 21 ve Akm 565; gövde eksplantları kullanıldığında ise, en yüksek tümör oluşumu Kırmızı 51, Malazgirt, Akm 49 ve Akm 196 genotiplerinden elde edilmiştir. Pul 11, Kırmızı 51, İll 62, Emre 20, Malazgirt, Akm 565, Akm 49, Akm 62, Akm 196, Akm 261, Akm 263, Akm 302 and Akm 362 genotipleri hem yaprak hem de gövde eksplantlarında tümör oluşturmuşlardır. Bununla birlikte, Masoor 85, Masoor 93, Akm 247 ve Akm 258 genotiplerinin yaprak eksplantlarından; Sazak 91, Kayı 91, Masoor 93, Akm 247 ve Akm 260 genotiplerinin de gövde eksplantlarından tümör elde edilememiştir. Ayrıca, tümör oluşumu histokimyasal GUS analizi ile doğrulanmıştır.

Anahtar Sözcükler: *Lens culinaris* Medik., mercimek, *Agrobacterium tumefaciens*, tümör oluşumu, GUS analizi

Introduction

The lentil is a pulse legume crop grown all over the world for its nutritional value. It is considered to be an under-exploited crop since very little research has been done so far to improve it compared with other pulse crops (Erskine, 1984). Consequently, almost all land races of lentil are characterized by poor yields, management problems and susceptibility to insect pests and fungal diseases (Erskine, 1984). The agronomic value of this crop could be improved by the use of recombinant DNA technologies. Biotechnological approaches have not been used for the improvement of lentil so far, except that Lurquin et al. (1998) found that longitudinally sliced embryonic axes from mature pea and lentil seeds, co-cultivated with *A. tumefaciens* carrying a GUS reporter gene in its T-DNA (transferred DNA),

provided a convenient means of evaluating the efficiency of gene transfer to tissues in different cultivars and co-cultivation conditions. The use of this technique has demonstrated wide variation in susceptibility to *Agrobacterium* between several commercial pea and lentil genotypes. The grain legumes have proven to be less amenable to transformation than most other dicotyledonous crop species. However, transformation reports for soybean (Hinchee et al., 1988; McCabe et al., 1988), moth bean (Eapen et al., 1987) and pea (Puonti-Kaerlas et al., 1990) have revealed that it is possible to produce transgenic plants in this important group of plant species.

Oncogenic strains of *Agrobacterium* can be used to determine whether T-DNA can be transferred prior to attempting the production of transgenic plants with disarmed strains. The induction of plant tumours, known as crown galls, by *Agrobacterium tumefaciens* originates from the transfer of T-DNA into the plant genome and its expression (Thomashow et al., 1980; Stachel et al., 1985). The T-DNA genes mainly code for enzymes involved in the synthesis of the phytohormones, auxin (indole –3 acetic acetamide-hydrolase, *iaaH* and tryptophan 2-monooxygenase, *iaaM*), cytokinin (isopentyl transferase, *ipt*) and opine metabolites (Willmitzer et al., 1982; Joos et al., 1983; Koncz et al., 1983). Tumours used to be regarded as chaotic cell aggregates, but Malsy et al. (1992) and Aloni et al. (1995) found that tumours consist of a sophisticated system of vascular bundles with phloem and xylem functionally connected to the vascular system of the host. The development of such strong vascularization originates in the bacterial T-DNA genes for phytohormone synthesis that are integrated into the plant genome. Differentiation of vascular bundles is known to be regulated by fine-tuned hormonal signals (Sachs, 1991; Aloni, 1995). The cytokinin content is generally higher in crown galls than in normal plant tissue as well (Weiler & Spainer, 1981); therefore, these tumours are an excellent model system for correlating phytohormonal regulation with structural and functional development, ranging from DNA over biochemical and physiological processes to the structural level.

De Cleene and De Ley (1976) reported that *Lens culinaris* Medik was susceptible to tumour induction by *Agrobacterium tumefaciens*. Karakaya and Özcan (1998) also reported the ability of tumours to form in two lentil varieties, namely Yeşil 21 and Pull 11 using A281 and A 136 NC strains of *A. tumefaciens in vivo*. Lurquin et al. (1998) found that the longitudinally slicing embryonic axes was a useful technique with wide variation in susceptibility to *Agrobacterium* among several commercial pea and lentil genotypes.

The current work was conducted to evaluate the susceptibility of 21 genotypes of lentil to the A281 (pTiBo 542) :: pBI121.1 strain of *A. tumefaciens* using leaf and stem explants *in vitro*.

Materials and Methods

Plant material

Twenty-one genotypes were used in this study. Seed from eleven lines, namely Akm 49, Akm 62, Akm 196, Akm 247, Akm 258, Akm 260, Akm 261, Akm 263, Akm 302, Akm 362, and Akm 565, and six varieties, Emre 20, Ill 62, Kayı 91, Malazgirt, Pul 11 and Sazak 91, was obtained from the Central Field Crops Research Institute, Ankara, Turkey; seed from the varieties Kışlık Yeşil 21 and Kırmızı 51 was obtained from the Department of Field Crops, Faculty of Agriculture, University of Ankara, Turkey; and seed from the varieties Masoor 85 and Masoor 93 was obtained from the National Agricultural Research Centre, Islamabad, Pakistan.

Seed germination and co-cultivation procedure

Seeds that were uniform in size and without cracks were surface sterilized using 100% commercial bleach (Axion) for 20 minutes with continuous stirring and subsequently rinsed five times with sterile distilled water. Seeds that had wrinkled seed coats and discoloration after treatment with commercial bleach were discarded. Sterilized seeds with healthy surfaces were germinated in Magenta vessels containing MSO medium (Murashige and Skoog, 1962).

After 6-10 days of culture, leaf and stem explants were excised from *in vitro* growing seedlings and immersed for 30 minutes in a 1:50 (1 x 10^8 cells/ml) dilution of overnight grown *A. tumefaciens* strain A281 (pTiBo 542) :: pBI121.1. After inoculation, explants were transferred to MSO medium in glass petri dishes (100 x10 mm) and co-cultivated for two days in a culture room. Following co-cultivation, explants were transferred to fresh MSO medium containing 500 mg/l augmentin. Tumour formation on explants was monitored closely for

several weeks. After 6 weeks, the number of explants producing tumours, tumour diameter and tumour weight were scored. Each treatment had 3 replicates containing 10 explants. Significance was determined by analysis of variance (ANOVA) and the differences between the means were compared by Tukey's b test using SPSS for Windows. Data given in percentages were subjected to arcsine (√X) transformation (Snedecor & Cochran, 1967) before statistical analysis.

Growth media and culture conditions

The medium (MSO) used for seed germination and co-cultivation consisted of Murashige and Skoog's (MS) mineral salts and vitamins (Murashige & Skoog, 1962), 3% sucrose and 0.8% agar (Sigma agar type A). The pH of medium was adjusted to 5.6 with 1N NaOH or 1N HCl before autoclaving at 121°C, 1.4 kg/cm^2 for 20 min. After a co-cultivation period, 500 mg/l augmentin was added to MSO medium for elimination of *Agrobacterium*. All cultures were incubated at 24°C under cool white fluorescent light (35 µmol photons m^{-2} s^{-1}) with a 16-h photoperiod.

Histochemical GUS Assay

Histochemical GUS assays were based on methods described by Jefferson (1987) and Özcan et al. (1993). For histochemical staining, plant tissue explants were incubated at 37°C for 4 h to overnight in 100 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.1% Triton X-100 and 1 mM 5 bromo 4-chloro-3- indolyl glucuronide (X-GLUC). The tissue was then rinsed in 70% ethanol and the presence of GUS enzyme activity was indicated by staining in the tissue.

Results and Discussion

No tumour formation was observed in the control explants that were not inoculated with *Agrobacterium* cells. Tumour formation was visible after 10-12 days of inoculation; however, scoring was done after six weeks (Fig. 1). Analysis of variance (ANOVA) shows variety x explants interaction at 0.01 level of significance for each of percentage of tumour induction, tumour diameter and tumour weight. Tukey's b test results distinguishing genotypes for leaf and stem explants for each of the aforementioned categories are given in Table 1.

Table 1. Effect of *A. tumefaciens* for tumour formation on stem and leaf explants of different lentil genotypes.

Genotypes	Percentage of tumour induction %		Tumour diameter (cm)		Tumour weight (g)	
	Leaf	Stem	Leaf	Stem	Leaf	Stem
PUL 11	82.50[1] a[2]	27.5 efg	0,62 bc	0,53 abcde	0.67 b	0.14 de
KIŞ. YEŞIL 21	82.50 a	32.5 def	0.74 ab	0.41 bcdef	0.89 a	0.30 cd
KIRMIZI 51	10.00 e	65.0 ab	0.62 bc	0.63 ab	0.20 cde	0.87 a
ILL 62	42.50 b	27.5 efgh	0.57 bc	0.19 fgh	0.37 c	0.12 de
EMRE 20	5.00 ef	7.5 jk	0.45 cd	0.10 gh	0.05 f	0.01 e
MALAZGİRT	7.50 ef	52.5 bc	0.27 de	0.70 a	0.02 f	0.49 b
AKM 565	77.50 a	7.5 jk	0.88 a	0.18 fgh	0.69 b	0.02 e
SAZAK 91	35.00b c	0 k	0.97 a	0 h	0.36 f	0 e
KAYI 91	5.00 ef	0 k	0.16 ef	0 h	0.019 f	0 e
MASOOR 85	0 f	0 k	0 f	0 h	0 f	0 e
MASOOR 93	0 f	0 k	0 f	0 h	0 f	0 e
AKM 49	35.00 bc	50.0 bcd	0.54 bc	0. 68 ab	0.24 f	1.03 a
AKM 62	7.50 ef	17.5 fgh	0.35 cde	0.28 efg	0.09 f	0.08 e
AKM 196	10.00 de	62.5 a	0.21 def	0.24 efgh	0.05 f	0.44 bc
AKM 247	0 f	0 k	0 f	0 h	0 f	0 e
AKM 258	0 f	12.5 hij	0 f	0. 57 abc	0 f	0.06 e
AKM 260	7.50 ef	0 k	0.36 cde	0 h	0.02 cd	0 e
AKM 261	20.00 cd	15.0 ghi	0.90 a	0.55 abcd	0.23 f	0.13 de
AKM 263	7.50 e	25.0 efg	0.55 bc	0.67 ab	0.06 def	0.17 de
AKM 302	12.50 e	12.5 hij	0.22 def	0.33 cdefg	0.07 def	0.04 e
AKM 362	37.50 bc	37.5 cde	0.42 cde	0.30 defg	0.02 cd	0.35 bc

[1] Each value is the mean of 3 replications with 10 explants
[2] Values within a column followed by different letters are significantly different at 0.01 level of significance using Tukey's b test

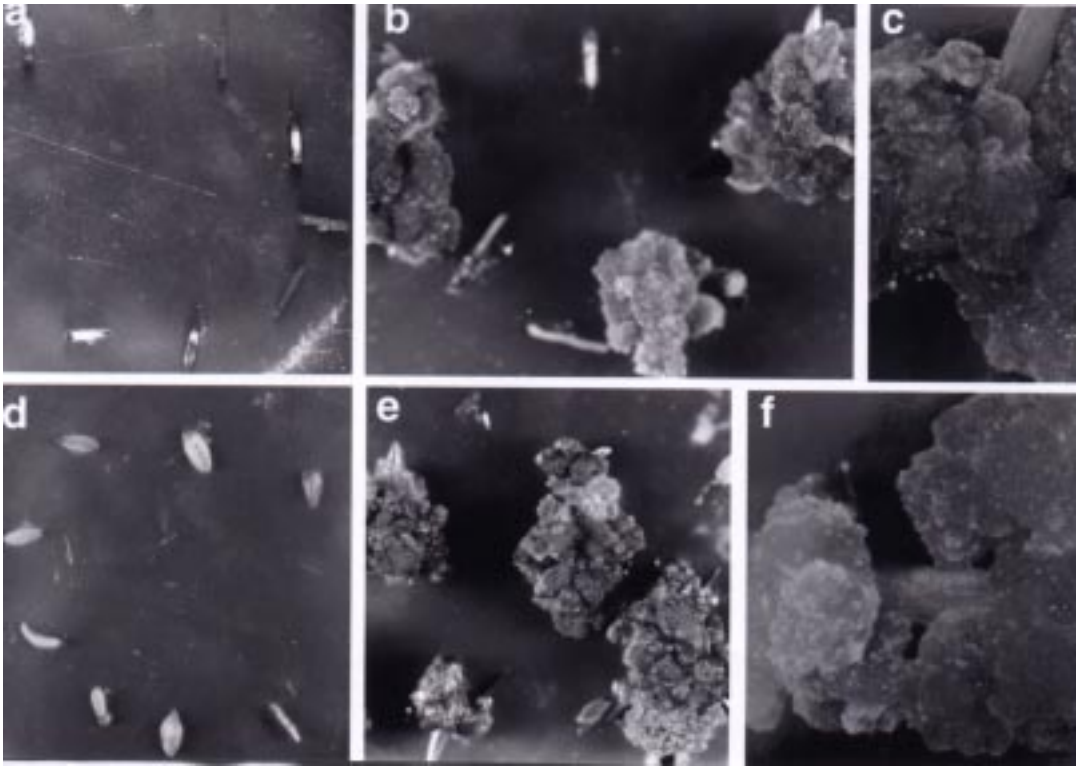

Figure 1. *In vitro* tumour induction caused by wild-strain *A. tumefaciens* A281 (pTiBo 542) :: pBI121.1 in lentil genotype Pul 11 after 6 weeks of inoculation. (**a**) Non-inoculated stem explants. (**b,c**) Stem explants inoculated with A281 (pTiBo 542) :: pBI121.1 (**d**) Non-inoculated leaf explants. (**e,f**) Leaf explants inoculated with A281 (pTiBo 542) :: pBI121.1.

Percentage of tumour induction

The results indicate that most cultivars were susceptible to *A. tumefaciens* when leaf explants were used. Four genotypes, namely Masoor 85, Masoor 93, Akm 247, and Akm 258 did not produce tumour on leaf explants, whereas six genotypes, namely Sazak 91, Kayı 91, Masoor 85, Masoor 93, Akm 247 and Akm 260, had no tumours when stem explants were used. Nevertheless, three varieties, Masoor 85, Masoor 93 and Akm 247, had no tumours with either of the explants (Table 1).

The highest tumour induction percentage (82.5%) was observed for Pul 11 (Fig. 1) and Kışlık 21, followed closely by Akm 565 (77.5%), statistically falling in to the same group in leaf explants. Twelve genotypes had a range of 0-10%. Two genotypes had a range of 11-20% and 4 had a range of 21-50%, whereas for stem explants the highest value observed was 65% for Kırmızı 51, followed closely by Akm 196 (62.5%), statistically falling in to the same group, and Malazgirt (52.5%) and Akm 49 (50%) falling into group b. For stem explants, 8 cultivars had a range of 0-10%, 4 cultivars had a range of 11-20%, and 5 had a range 21-50%. Overall analysis reveals leaf explants to be more favourable for tumour induction in genotypes Pull 11, Kışlık Yeşil 21 and Akm 565 (Table 1).

Tumour diameter

Analysis of variance for tumour diameter again shows that the diameter of tumours from leaf explants was higher compared to that from stem explants. The highest value, of 0.97 cm, was obtained from Sazak 91, followed closely by Akm 261 (0.90 cm) and Akm 565 (0.88 cm) and Kışlık 21 (0.74 cm), statistically falling in to the same group for leaf explants. In contrast, the highest value, of 0.70 cm, was obtained for Malazgirt, followed by Kırmızı 51 (0.63 cm), Pul 11 (0.53 cm), Akm 49 (0,68 cm), Akm 258 (0.57 cm) and Akm 263 (0.67 cm) for stem explants. All other varieties had a lower range, of 0-0.33 cm. Again the results obtained are better for leaf explants, suggesting that they are more susceptible to *A. tumefaciens*, giving bigger tumours (Table 1 and Fig. 1).

Tumour weight

Tumour weight is in accordance with the previously mentioned phenomenon, yet the highest value was 1.03 grams for Akm 49 and 0.87 g for Kırmızı 51 in stem explants, as compared to leaf explants, where the highest value was 0.89 cm with Kışlık 21. We have more variation with better values in leaf explants than in stem explants (Table 1).

For histochemical GUS assay, tumours of both stem and leaf explants in respective genotypes were incubated in X-GLUC at 37°C. After 6 hours of incubation, tumours stained blue, which confirmed the transfer and expression of the GUS gene in plant cells (data not shown).

A. tumefaciens is being used for the transfer of genes to many crop plants, but has host range limitation. In this research, the susceptibility of 21 lentil genotypes was assessed for use in developing transgenic lentil plants in the future. Response by different genotypes can vary according to the genotype and explants used. The results are consistent with strain and genotype differences reported previously for other legumes, like soybean (Owens & Cress, 1985, Byne et al., 1987), pea (Özcan, 1995), chickpea (Islam et al., 1994) and lentil (Warkentin & McHughen, 1991, Karakaya and Özcan, 1998). Rezmer et al. (1999) found that regularly growing stem and leaf tumours from the A281 strain showed irregular GUS-staining patterns in different plant species: *Ricinus communis* L., *Cucurbita maxima* L., *Vicia faba* L. and *Kalanchoe daigremontiana* Aym.-Hamet & Terr. Ogawa et al. (2000) observed that strain CNI5 induced tumours at a higher frequency and in a larger area of explants in most tested plant species, especially in chrysanthemum cultivars, than the octopine-type strain C58C1 (pTiB6S3). Similarly, Azmi et al. (2001) found that the expression of T-DNA was restricted to small areas located deep in tumours in *Eucalyptus globules* Labill. after treatment with an oncogenic strain of *A. tumefaciens*. Our results for the susceptibility of *A. tumefaciens* to various genotypes confirm previous studies in legumes which state that the susceptibility of Pul 11, Kışlık Yeşil 21, and Akm 565 were best when leaf explants were used and Kırmızı 51, Malazgirt, Akm 49 and Akm 196 were best when stem explants were used. However, Pul 11, Kırmızı 51, İll 62, Emre 20, Malazgirt, Akm 565, Akm 49, Akm 62, Akm 196, Akm 261, Akm 263, Akm 302 and Akm 362 formed tumours both on leaf and stem explants in tumour induction. These results suggest that transgenic lentil cultivars could be produced via non-oncogenic *A. tumefaciens* strains in lentil provided that there is an efficient system of developing adventitious shoots in lentils through leaf or stem explants.

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