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Agrobacterium Mediated Tumor and Hairy Root Formation from Different Explants of Lentils Derived from Young Seedlings

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ABSTRACT

Four lentil cultivars *(Lens culinaris* Medik) were treated with super virulent strain A281 (pTiBo 542) pBI121.1 of *Agrobacterium tumefaciens* and strain 15834 (pR 15834) of *A. rhizogenes* to evaluate their behavior towards tumor formation and rooting respectively. High frequency of tumor formation was observed from cotyledon node and with variable tumor formation on shoot meristems, which bore shoots in all cultivars. Rooting was only observed in cultivar Erzurum 89. *A. rhizogenes* was noted to induce roots in dark. Light was found to inhibit rooting in the treated explants. Histochemical GUS analysis showed variable results from tumors and shoots.

Key Words: *Agrobacterium tumefaciens; Agrobacterium rhizogenes*; *In vitro;* Tumors, Shooty tumors; Rooting

INTRODUCTİON

Lentil (*Lens culinaris* Medik.) is a pulse legume crop of wide importance all over the world because of high nutritional value. Many researchers have reported transformation of lentils and the other legumes such as *Lotus corniculatus* (Jensen *et al.,* 1986 & Petit *et al,* 1987), *Trifolium repens* (Díaz *et al.,* 1989), *Vigna aconitifilia* (Lee *et al.,* 1993), *Glycine max* (Cheon *et al.,* 1993); *Vicia hirsuta* (Quandt *et al.,* 1993), *L. japonicus* (Stiller *et al.,* 1997), *Trifolium pratense* (Díaz *et al.,* 2000), *Lens culinaris*, (Khawar *et al.,* 2001) and *M. truncatula* (Boisson-Dernier *et al.,* 2001). Kifle *et al.,* (1999) Warkentin & Mc Hughen, (1991); Khawar & Özcan, (2002) used oncogenic and non oncogenic strains of *Agrobacterium tumefaciens* via tumor induction, opine synthesis and southern analysis. A281 strains of *A.tumefaciens* (Hood *et al.,* 1986) and 15834 strain (pRG 15834) of *A. rhizogenes* (Petit, 1983) are widely used to study possibility of gene transfer in number of crop species.

Agrobacterium strains play an important role in the transformation process, as these are responsible for infectivity and aids in the gene transfer. An efficient and reproducible transformation protocol is important, since it could be used to insert foreign genes into species of interest. In order to investigate the effects of both of these strains on various explants of lentils. Present study investigated the behavior of both *Agrobacterium* strains towards four genotypes of lentil. Moreover, this paper reports induction of rooty and shooty tumors from excised shoot meristems of *in vitro* grown immature lentil explants. The study also compairs root formation ability of 15834 strain of *A. rhizogenes* and A281 strain of *A. tumefaciens.*

MATERİALS AND METHODS

Seeds of three Turkish lentil cv. Sultan, Erzurum 89, Akm 565 and one Pakistani cv. 93Cl003 were surface sterilised using commercial bleach (Axion–Turkey containing 5-6 % NaOCl) and germinated on agar solidified MS medium (Murashige & Skoog, 1962) containing 3 % sucrose. The pH of the medium was adjusted to 5.7 with 1N NaOH before autoclaving. All cultures were maintained under 16 h light/8 h dark potoperiod. Cotyledon node and shoot meristem explants obtained from one week old *ın vitro* grown plantlets were treated with A281 (pTiBo 542) pBI121.1 strain of *A. tumefaciens* for half hour; followed by transfer to MS co-cultivation medium for 1 d in growth chamber at 24 ± 2 ^{o}C. After co-cultivation the explants were transfered to the selection medium (MS medium) containg 500 mg L^{-1} Augumentin (SmithKline Beecham-Turkey). Untreated control was also planted.

Growing tender shoots of one week old seeedlings of 4 cultivars were cut below epicotyl and treateded with 15834 strain of *A. rhizogenes* (Lippincott *et al.,* 1973) by puncture inoculating the epicotyl 1.5 cm above the cut with fine pointed needle. They were placed in two ways on the growing medium such that the wounds touched the medium in one case and in other wounds remained above the surface of agar solidified medium. The procedure has successfully been adapted for several legumes by Díaz *et al.* (1989), Cheon *et al.* (1993), Lee (1993) and Quandt *et al.* (1993). Half of inuculated explants in each case were incubated in light (16 h light/8 h dark) and the other half in dark at 24 ± 2C$^{o.}$ Punctured controls without *A. rhizogenes* tretment were also planted under each condition.

Statistical analysis. Each treatment had 3 replicates

containing 5 explants and was repeated twice and evaluated by maintaining culture for 42 days for *A. tumefaciens* and two weeks for *A. rhizogenes* to allows appropriate growth of tumors and roots respectively. The recorded data were evaluated and analysed by one way analysis of variance and the differences between means were separated by Duncans test using SPSS for windows (version 11.0). Data given in the percentage were subjected to arcsine transformation (Snedecor & Cocharan, 1967) before statistical analysis.

Histochemical gus assay. Histochemical GUS assay were based on methods described by Jefforson *et al.* (1987) and Khawar and Özcan (2002). Putative transformed tumors, roots or shoots if any obtained from cotyledon node and shoot meristem explants were incubated at 37 ± 1 ^{o}C for 12 hours in 100 mM sodium phospate (pH =7.0), 10mM EDTA, 0.1 % Triton X 100 and 1 mM 5 bromo - 4 chloro 3 indolyl glucuronide (X-GLUC). Transformed tissues were detected by washing in 95% ethanol. Presence of GUS activity was indicated by blue staining of tissues.

RESULTS AND DİSCUSSİON

An efficient transformation protocol for introduction of novel genes is desirable to meet breeding objectives specific to short seasoned lentil (*Lens culinaris* Medik.) genotypes. No tumorogenic callus formation was observed on control explants. It was observed that MS growth medium lacking any external supply of plant growth regulators used for tumor development did not affect growth of tumors. Thus elaborate media was not required to rescue tumors which saved time for emperical tailoring of the appropriate media. An interaction was observed between cultivars and explant for frequency of tumor formation ($p<0.05$) and tumor diameter ($p<0.01$). Similarly an interaction was found between cultivars and explants for root formation ($p<0.05$) and secondary root formation ($p< 0.05$) number of roots, root length, number of secondary roots ($p< 0.01$). Treated explants resulted in induction of healthy and green tumorous growth after 10-12 days of inoculation. The results (Table I) indicated that although *A. tumefaciens* was highly virulent yet it had a little effect on 93CL003 and Erzurum 89 which failed to induce large tumors from shoot meristems. The effect of virulence of *A. tumefaciens* strain A281 have been shown earlier revealing that the strain carrying the plasmid pTiBo542 is able to induce large, early appearing tumors on a wide range of plants compared to other *A .tumefaciens* strains (Hood *et al.,* 1986 & Jin *et al.,* 1987). It was demonstrated that *A. tumefaciens* strain A281 is not only able to efficiently transform lentils but even that it is super virulent in the tested four genotypes. This is in line with the results of Cervera *et al.* (1998) having similar observation on woody plants (with strain A281). Hansen *et al.* (1994) suggested that extra copies of *virG* from pTiBo542 are responsible for stable transformation increase.

The T-DNA genes encode proteins which cause major alterations in the differentiation and development of the transformed plant cell and hence are responsible for neoplastic phenotype (Walden, 1993). It has been shown that the supervirulent phenotype strain A281 is ascribable to the *virG* gene and the 3' half of the *virB* operon of pTiBo542 (Jin *et al.,* 1987). Chen *et al.* (1991) suggested that the super virulence of this strain was attributable to the ability of the *virG* gene of pTiBo542, which activates *vir* gene transcription more strongly. Since pTiBo542, possess the 3' half of v*irB* in the commonly conserved region this might be important for the creation of supervirulance in the treated explants (Ogawa & Mii, 2001). Kovács and Pueppke (1994) supports the evolutionary mosaic concept of pTiBo542. It has been reported that the supervirulent phenotype of transconjugant strain A281 arises not only from *vir* genes located on pTiBo542, but also from the gene(s) encoded by either the chromosome or cryptic plasmid pAtC58 of strain C58 (Hood *et al.,* 1986, 1987).

The frequency of tumor formation from cotyledon node explants (66.67-93.33%) was higher compared to those obtained from shoot meristem (0 to 40.00%). Tumors from cotyledon node of cultivar Akm 565 and Erzurum 89 were larger with mean diameter of 1.2 cm and 1.1 cm respectively compared to the tumors from cultivars 93Cl003 and Sultan which were comparatively smaller with diameter of 0.77 and 0.64 cm respectively. Shoot meristem with smaller tumors were less stimulating to induction of tumors. The largest tumor was observed on cultivar Sultan (Fig. 1 ab, 0.50 cm) followed by Akm 565 (0.20 cm). Very small tumors were noted on shoot meristem tissues of cultivar 93Cl003 and Erzurum 89, which were not taken into consideration during scoring. The results are in agreement with those of Donaldson and Simmonds (2000), who had similar observation on explants of soybean. As such we observe that the response was plant-cultivar and explant dependent. By histochemical GUS-staining and polymerase chain reaction (PCR) of *gus* it became evident that crown gall tissues had 100% transformed cells, but *gus* was expressed in some of them only. Patchy staining, on tumors of cotyledon node and shoot meristem had specific areas where GUS activity was distinct, indicating that no diffusion of intermediates had occured in staining reaction. Moreover, this patchy and irregular distrubution of GUS-active cells often localized close to the tumor surface in putative transgenics and is in agreement with Plegt and Bino (1989) who observed a GUS activity dependent on the CaMV 35S promotor. The reporter gene expression was restricted towards central cells and other regions of tumors showing week or no staining indicated that regenerants were untransformed while blue spots indicated presence of transformed cells restricted to central regions of the tumors. We concluded that most cells of the tumors harbor *gus* gene and the non expression in some parts of the tumors may be due to the phenomenon of gene silecing (Rezmer *et al.,* 1999, Ray & Jha, 1999). *GUS* gene activity may be inhibited in some cells due to position effect at

transcriptional or post transcriptional level or as result of truncated transgene sequences as suggested by (Kohli *et al.,* 1999) resulting in chimeras. Another explanation to this could be that the proliferation of the untransformed tissues was induced by the hormones produced by the untransformed cells as suggested by Nakajima *et al.* (1981) and Akiyoshi *et al.* (1983) through the expression of specific oncogenes (Akiyoshi *et al.,* 1983; Schröder *et al.,* 1984). Organogenic process limited to the shoot meristems of the plant suggest that the high cytokinin levels due to the bacteria are compatible with organisation and proliferation of shoots from the shoot meristem or due to the proliferating cells themselves which produced cytokinins to maintain their own proliferation, as did tobacco BY2 cells (Redig *et al.,* 1996). However, production of cytokinins levels due to the bacteria were not compatible for shoot initiation from cotyledon node explants.

Overall staining, as well as patchy staining, was observed in areas with distinct GUS activity indicating that no diffusion of intermediates had occured in staining reaction. Rezmer *et al.* (1999) suggests that even if all cells are transformed, gus expression may be prevented by methylation of foreign genes. The claim is further supported by Rezmer *et al.* (1999) who found considerably higher proportion of GUS-active cells in leaf tumors of *Kalanchoe* indicating that the histochemical GUS assay does not enable the localization of all transformed cells, because the expresion of *gus* is disturbed in some areas. However Sacristán and Melchers (1977) and Ooms *et al.* (1982) does not support this concept of transformation and suggests that actual transformation occurs in only 1.2% of an *A. tumefaciens* (wild-type) induced tumors which can be regarded as transformed. In contrast, treatment of shoot meristem with A281 stimulated shoot formation from all explants with or without tumor formation. It was observed that the shoots obtained from the inoculated explants with A281 strain of *A. tumefaciens* harboring *uid A (gus)* gene gave rise to GUS positive shoots and that from roots were GUS negative. Rooting was observed on Erzurum 89 only with rooting frequency of 40% and mean roots of 1.73 (Fig. 1 cd). Cotyledon node explants did not favor the shoot or root formation, instead large tumors were observed on all genotypes (Fig. 1e). We found that A281 strain of *A. tumefaciens* has shooty potential which is in agreement with that of Azmi *et al.* (1997) in which they found shooty potential of strain of 82.139 associated with very high zeatin and ZR levels in tumors from on globulus hypocotyls of euclayptus (*Eucalyptus globulus*). They observed that shooty capacity of the tumor is not associated with overall increase in cytokinins but only with the presence of some small transformed areas highly provided with cytokinins. Furthermore they found that these areas do not correspond to the sites of bud formation, the regenerated buds being untransformed and provided with a moderate cytokinin signal. Furthermore, they correlated shoot-forming capacity to higher zeatin and zeatin riboside (ZR) levels in the shooty

Fig. 1. Effect of tumorgenic strain A281 of *A. tumefaciens* on cotyledon node and shoot meristem of lentils (a) initiating of shoot primordia and (b) shoots from tumors of shoot meristem of cultivar Sultan (c) development of shoots with negligible amount of tumors from shoot meristem (d) development of roots from shoot meristems of cultivar Erzurum 89 (e) tumors from cotyledon node explants of cultivar Sultan of lentils.

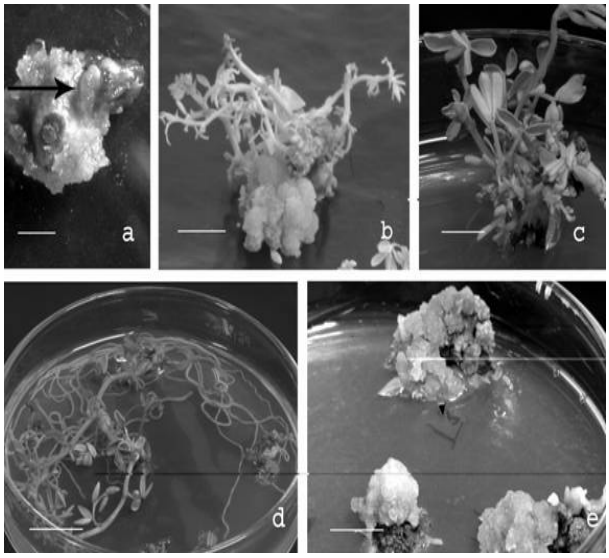
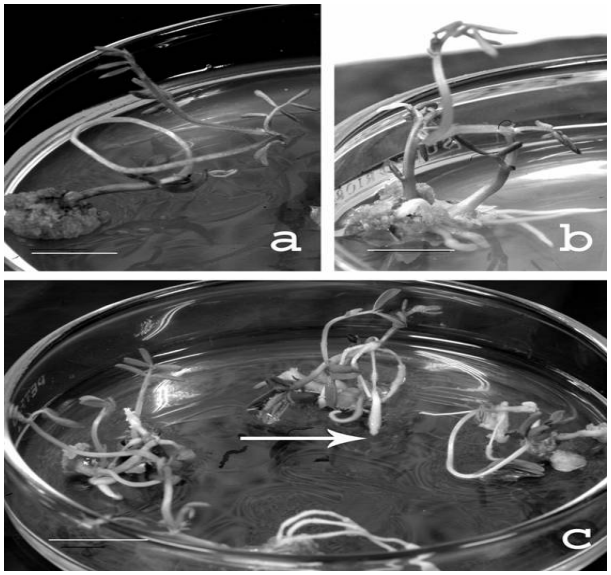


Fig. 2. Effect of rhizogenic 15834 strain of *A. rhizogenes* on rooting (a) direct touch of cut ends with MS medium resulted in (hairy root) tumors without development of roots (b) or suppressed roots roots arising from the tumorous mass (c) transgenic roots revealed no differences in growth and development when compared to non transgenic roots morphologically, except nodules at tips.



tumors of *E. globulus* micropropagated plantlets.

This suggests that effect of *A. tumefaciens* was inhibitory towards rooting and stimulating towards shoot formation from this explant in general. The observed roots in Erzurum 89 were normal and healthy and bore both primary and secondary roots. Mean number (1.73) of primary roots with mean length (5.54 cm) for primary roots and 2.53 secondary roots with mean cm length of 0.5 cm showed that they were vigorous and developing. Tumors from shoot meristems of 2 out of 4 genotypes continued to produce new shoot buds after for 2 weeks, after which the gall calli lost potential of shoot regeneration. It seemed that shooty callus due to altered endogenous ratio of auxin and cytokinin favored shoot induction uptill a certain time. No morphological difference in the developing shoots was recorded and this was in contrast to Spencer (1990), who found the shooty teratomas differed phenotypically from normal untransformed shoots. Brasileiro *et al.* (1991), Flenning *et al.* (1996) and Braun and Woody (1976) also reports induction of shooty tumors differentiating normal or teratological shoots with strains 82.139 or T 37 and suggest that these may have arisen from semi or non transformed cells of tumor due to over production of cytokinin or auxin. De Greeve (1982) found that inactivation of either *tms1, tms2 or tmr* (onc genes) resulted in an attentuation of the oncogenicity of the mutated Ti plasmid. *Agrobacterium* strains carrying such mediated plasmids induce crown galls with an altered hormone balance resulting in generation of "shooty" mutant tumor due to *tms 1 or tms 2* which are inactivated. Draper *et al*. (1988) adds that majority of these shoots are non transformed as most arise from non transformed cells "cross fed" with cytokinins secreted from adjacent tumor cells. Our results partially support Saborio *et al.* (1999) who found that co-culture of micro shoots with *Agrobacterium tumefaciens* (A281 × 200) enhanced rooting. We are not clear if the effect was a result of the genetic transformation of the plants, or a modification of the rhizosphere caused due to Strain A281 × 200 mediated transformation. As such rooting may have occured independently as a result of co-culture of microshoots with *A. tumefaciens*. Augmentin at the rate of 500 mg L^{-1} in place of commonly used cefotaxime as bacteriostatic agent proved good as no bacterial outgrowth were observed on cultures throughout the period of experimentation. Bacterial outgrowths (due to *A. tumefaciens*) hinders transformation efficiency of explants and this in turn hinders growth and development of tumors. We observed continuous proliferation of tumor tissues able to grow in the bacteriostatic media (Fig. 1e), which suggested that tumor cells were able to synthesise plant growth regulators resulting in neo plastic growth of the tissues that can be used for selection of transformed tissues. Khawar and Özcan (2002) and Khawar *et al.* (2003) using augmentin as bacteriostatic agent observed similar results in lentil and *Salvia sclarea* and *S. pratense*. *Agrobacterium* mediated tumors proliferate autonomously in the absence of the phytohormones (auxins and cytokinins) that are needed for growth of normal plant cells (Braun, 1958), because of this property, *İn vitro* culture crown gall cells grow and form a callus even when the growth stimulating phytohormones are absent from the culture medium (Hooykaas & Schilperoort, 1992).

Presence of normal roots on shoot meristems of Erzurum 89 and shoots on all explants of the under study cultivars suggested that it is possible to develop physiologically and morphologically normal plants or parts there of from oncogenic cells with in the crown galls. Regeneration from non oncogenic cells in the majority of crown galls is normally represented by hormones secreted by the oncogenic cells.

Transformation of lentils with *agrobacterium rhizogenes.* It is possible that plant genotype and manipulation of culture environment and media may affect the rates of both cell growth and under the influence of *A. rhizogenes*. The treated explants were controlled periodically both under light and dark conditions. Profuse hairy root initials protruded from the puncture inoculated explants placed in the dark from cultivar Erzurum 89 and negligible rhizogenes was observed on cultivar Sultan (Table II). The results showed that cultivar Erzurum 89 had 53.33% frequency of root formation, with 5 roots/inoculated explant having root length of 7.17 cm. Secondary roots were also observed on primary roots in high frequency (46.67%) with 5 secondary roots per primary root. No rooting was observed on cultivars 93Cl003 and Akm 565. Whereas, the cultivar Sultan showed extremely low rhizogenes (6.67%) with mean number of 0.67 roots per explant.

Morphological variability was noted down between puncture inoculated and directly inoculated explants. The growth medium and the inoculation procedure had a significant effect on hairy root induction. Direct touch of cut ends with MS medium resulted in (hairy root) tumors without development of roots (Fig. 2a) or suppressed roots arising from the tumorous mass (Fig. 2b). It is assumed that in this case, excessive auxins secreted by Ri plasmid on touch with the regeneration media resulted in stimulation of tumors in place of roots from non oncogenic cells present at the wound site. Such observations were also recorded by Nguyen *et al.* (1992) suggesting that morphologial differentiation is due to increased sucrose concentration and osmotic stress. Moreover, these explants gave a vitrified look and were non responsive towards growth. No hairy roots tumors were recorded on treated explants when the wounds remained above the MS medium contained in agar. No roots or rhizogenic tumors were recorded from control explants as well. Morphologically transgenic roots revealed no differences in growth and development when compared to non transgenic roots except nodules at tips (Fig. 2c) in aggreement with Díaz *et al.* (1989) and Vincent *et al.* (2000). Difference of growth patterns on explants treated

Table I. A281 Strain of *A. tumefaciens* mediated tumor and root formation on 4 cultivars of lentil

Cultivars	Frequency of Tumor Formation*		Tumor Diameter **	
	Cotyledon node	Shoot meristem	Cotyledon node	Shoot meristem
Sultan	93.33 a	40.00 a	0.64 b	0.50 a
93CLOO3	66.67 b	6.67 bc	0.77 ab	0.00 a[1]
Erzurum 89	86.67 ab	0.00[1] c	1.10 a	0.00 a[1]
Akm 565	73.33 ab	20.00 ab	1.20 a	0.20 a
Cultivars	**Frequency of root formation ****		**Number of roots****	
	Cotyledon node	**Shoot meristem**	**Cotyledon node**	**Shoot meristem**
Sultan	0.00	0.00 b	0.00	0.00 b
93CLOO3	0.00	0.00 b	0.00	0.00 b
Erzurum 89	0.00	40.00 a	0.00	1.73 a
Akm 565	0.00	0.00 b	0.00	0.00 b
Cultivars	**Root length ****		**Secondary root ****	
	Cotyledon node	**Shoot meristem**	**Cotyledon node**	**Shoot meristem**
Sultan	0.00	0.00 b	0.00	0.00 b
93CLOO3	0.00	0.00 b	0.00	0.00 b
Erzurum 89	0.00	5.54 a	0.00	2.53 a
Akm 565	0.00	0.00 b	0.00	0.00 b
Cultivars	**Secondary root length****			
	Cotyledon node	**Shoot meristem**		
Sultan	0.00	0.00 b		
93CLOO3	0.00	0.00 b		
Erzurum 89	0.00	0.50 a		
Akm 565	0.00	0.00 b		

The tumors were very small, therefore they were not counted.
**[1]Values with in a column followed by different letters are significantly different at 0.01 level using Duncan's Multiple Range Test.
*Values with in a column followed by different letters are significantly different at 0.05 level using Duncan's Multiple Range Test.

Table II. 5834 strain of A. rhizogenes mediated hairy root formation on 4 cultivars of lentil

Cultivars	Frequency of Tumor Formation*	Number of roots**	Root length **(cm)	Frequency of Secondary root formation*	Number of Secondary root **
Sultan	6.67 b	0.67 b	1.00 b	6.67 b	0.67 b
Erzurum 89	53.33 a	5.00 a	7.17 a	46.67a	5.00 a
93CL003	0.00 b	0.00 b	0.00 b	0.00 b	0.00 b
Akm 565	0.00 b	0.00 b	0.00 b	0.00 b	0.00b

**Values with in a column followed by different letters are significantly different at 0.01 level using Duncan's Multiple Range Test.
*Values with in a column followed by different letters are significantly different at 0.05 level using Duncan's Multiple Range Test.

differently appears more due to treatment effect. Altinkut *et al.* (1997) found wounded callus from 15 day old seedlings resulted into hairy root formation in frequency of 10.4% from MB10, 8.1% from Canitez 87, and 5.37% on red chickpea callus derived plantlets; while transformation rates were lower for seedlings with rooting frequency of 4.4, 3.3 and 3.17% respectively. *In vitro* response of other genotypes for *A. rhizogenes* mediated transformation for root initiation of other genotypes was inefficient. High salt media such as LS (Linsmaier & Skoog, 1965) or MS (Murashige & Skoog, 1962) favors hairy root formation in some plants (Giri & Narasu, 2000). We supose that the bacterial concentration and the method of placement played an important role for the production of transformed roots or rhizogenic tumors. Similar results were obtained on California poppy and opium poppy seedlings by Park and Facchini (2000). They observed rooting in the former and no rooting in the later case using 15834 and C58C1 strains of *A. rhizogenes*. Whereas, Surya *et al.* (1992) using *A. rhizogenes strain* R1000 reported smaller hairy roots and green coloured tumors on one week seedlings of chickpea cultivars C235 and H75-35. They found that pBI121 infected plants produced fast growing pure white tumor free roots, which were longer than pBI333 infected plagiotropic slow growing roots characterised by numerous light green tumors. Roots attained length of 4-5 cm in 42 days time.

Roots developed from Erzurum 89 were thicker as compared to roots from Sultan. This confirms previous reports (Tepfer, 1984) that the type of palnt cells and conditions of the plant cells before or during transformations importantly affects transformation efficiency. Although *A. rhizogenes* is infectious for a considerable pecentage of dicotyledonous species yet failure in transformation has been freqeuntly reported (De Cleene & De Ley, 1981; Tepfer, 1984; Porter, 1991; Menze & Mollers 1999), which is in confirmation with our results. It is well known that the molecular mechanism of hairy root induction is influenced by several bacterial- and plant- related factors, capable of modulating the transformation efficiency (Gaudin *et al.,* 1994; Owens & Cress, 1985) and rooting in case of Erzurum 89 and non rooting in others should not be taken as surprise.

This study presents data that could be used to draw conclusions pertaining to stable transformation. This is particularly important when the objective is recovery of transgenic plants. The results indicated that under studied cotyledon node and shoot meristem of the lentils are susceptible to tumor formation and transformation but the susceptiblity of explants to *A. tumefaciens* vary according to the genotype and the explant. The results could be very helpful in evaluating these explants while working with non oncogenic strains carrying desired characteristics. It was concluded that cotyledon nodes were superior to shoot meristem and could be used in preference to shoot meristems in transformation studies with non-oncogenic strains of *A. tumefaciens*. The results further showed higher transformation frequency with *Agrobacterium*, making possible the recovery of transgenic plants in lentils.

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