

## Multiple shoot formation in lentil (*Lens culinaris*) seeds

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**Abstract** A protocol based on seed culture was developed for efficient *in vitro* propagation of lentil (*Lens culinaris* Medik). Benzyladenine (BA), thidiazuron (TDZ), and kinetin all induced multiple shoot formation. In terms of the number of long shoots (>2.0 cm) produced per seed, BA and TDZ at optimum concentrations (0.2–0.4 and 0.1 mg/litre, respectively) had similar efficiency, whereas kinetin produced less shoots. Murashige and Skoog (MS) salt composition was better than that of Gamborge (B5) for shoot induction. Increasing calcium (Ca) concentration was necessary to overcome shoot-tip necrosis. For shoot elongation, fresh medium of the same composition of shoot induction medium could be used for stumps from medium with low BA (<0.8 mg/litre) or TDZ (<0.4 mg/litre). Otherwise, MS medium supplemented with 0.5 mg/litre gibberellic acid (GA<sub>3</sub>) or 0.05 mg/litre  $\alpha$ -naphthaleneacetic acid (NAA) was appropriate.

Multiple shoots were induced from 10 inbred of four species. Best rooting was achieved using MS medium supplemented with 1.5 mg/litre NAA. Using this rooting medium more than 50% shoots from the 10 inbreds rooted. From 80 to 100% plantlets of the 10 inbreds survived after being transferred to potting mixture in glass house.

**Keywords** cytokinins; micropropagation; seed culture; lentil; *Lens culinaris*

### INTRODUCTION

*In vitro* culture of lentil (*Lens culinaris* Medik) has proved difficult. In the last 20 years, techniques have been progressively improved, starting with Bajaj & Dhanju (1979) who first reported *in vitro* lentil regeneration from meristem tips. Later Williams & McHughen (1986) described a protocol for the regeneration of lentils from callus cells using hypocotyls and epicotyls as explants. Saxena & King (1987) obtained whole plants from callus induced from embryonic axes, while Polanco et al. (1988) reported multiple shoot formation from shoot tip, first node, and first pair of leaves in media supplemented with benzyladenine (BA) or BA and  $\alpha$ -naphthaleneacetic acid (NAA). Using seed culture, Malik & Rashid (1989) obtained multiple shoot formation from cotyledonary nodes on a medium with BA. Singh & Raghuvansi (1989) reported that plants could be regenerated directly from nodal segment and shoot tip explants as well as from callus. On a medium containing kinetin, nodal segments and shoot tip explants produced multiple shoots without intervening by callus or root formation. Malik & Saxena (1992) reported multiple shoot formation by culturing seeds on Murashige & Skoog (MS) medium supplemented with thidiazuron (TDZ). Warkentin & McHughen (1993) reported multiple shoot formation from cotyledonary nodes using BA. These studies showed that cytokinins induced multiple shoot formation from different types of explants.

Warkentin & McHughen (1993) obtained multiple shoots from cotyledonary nodes. Halbach et al. (1998) reported that the best shoot formation was from bisected embryos, and that keeping the cotyledons attached to the embryo-axis was beneficial. Ahmad et al. (1997) developed a protocol for the micropropagation of lentil and hybrids based on single node culture. Ye et al. (2000) further improved the single node culture protocol.

The narrow genetic base of cultivated lentil calls for the transfer of useful genes from other sources. However, the low success rate of artificial crossing has greatly hampered this process. Embryo culture has been used to rescue interspecific hybrids between *L. culinaris* and *L. orientalis* (Cohen et al. 1984; Ladizinsky et al. 1985), and *in vitro* multiplication of F<sub>1</sub> hybrids has been used as a way of enlarging hybrid populations (Ahmad et al. 1997; Ye et al. 2000). However, a promising option to transfer useful genes by genetic transformation has not yet been successful. The lack of an efficient *in vitro* regeneration system may be one of the reasons. Therefore, the development of an efficient regeneration procedure for lentil is required.

The objective of this work was the development of an efficient regeneration procedure for lentil based on seed culture.

## MATERIALS AND METHODS

### Plant materials and surface sterilisation

For all experiments except for the testing of genotypic differences, seed of 'Titore' was used. The seed was surface sterilised by rinsing in 95% ethanol for 30 s, then soaking in a 1.25% (w/v) sodium hypochlorite containing a few drops of the surfactant Tween 20 with continuously stirring for 20 min, followed by 5–6 washes in sterile deionised water.

### Multiple shoot induction

#### Medium and BA concentration

MS medium (Murashige & Skoog 1962), MS medium with a doubled content of CaCl<sub>2</sub> (750 mg/litre) (MSC), B5 medium (Gamborg et al. 1968), and B5 medium +750 mg/litre of CaCl<sub>2</sub> (B5C) were tested in combinations with four concentrations of BA (0.2, 0.4, 0.6, and 0.8 mg/litre). After 3 weeks of culture the average number of shoot/seed, the number of long shoot/seed, and the shoot-tip necrosis was collected.

#### Kinetin and TDZ concentrations

MS supplemented with different concentrations of kinetin (0.2, 0.4, 0.6, 0.8, 1.0, or 1.5 mg/litre) or TDZ

**Table 1** Effects of medium and benzyladenine (BA) concentration on multiple shoot formation of lentil (*Lens culinaris*) seeds (data collected after 3 weeks of culture). (MS = Murashige & Skoog (1962); MSC = MS + 440 mg/litre CaCl<sub>2</sub>; B5 = Gamborge et al. (1968); B5C = B5 + 750 mg/litre CaCl<sub>2</sub>.) (NS = not significant at  $\alpha = 0.05$ .)

	Average no. of shoots/seed				Average no. of long shoots/seed (>2.0 cm)			
	BA (mg/litre)				BA (mg/litre)			
Medium	0.2	0.4	0.6	0.8	0.2	0.4	0.6	0.8
MS	7.4	13.7	11.6	13.2	4.1	4.9	3.8	3.4
MSC	9.7	12.5	13.6	12.1	5.5	4.4	3.6	3.8
B5	6.0	7.5	8.3	8.7	4.0	2.8	3.4	3.6
B5C	7.5	9.0	9.7	11.5	4.4	4.4	4.1	3.5
ANOVA								
Source	d.f.	Average no. of shoots/seed			Average no. of long shoots/seed (>2.0 cm)			
		Mean square	<i>F</i>		Mean square	<i>F</i>		
Medium	3	322.18	78.35	( <i>P</i> < 0.001)	11.70	12.32	( <i>P</i> < 0.001)	
MS versus B5	1	265.31	51.10	( <i>P</i> < 0.001)	6.34	6.67	( <i>P</i> < 0.001)	
MS versus MSC	1	13.65	3.01	NS	1.55	1.63	NS	
B5 versus B5C	1	83.53	18.94	( <i>P</i> < 0.001)	3.81	4.01	( <i>P</i> < 0.001)	
BA	3	225.36	54.80	( <i>P</i> < 0.001)	13.29	13.99	( <i>P</i> < 0.001)	
Medium*BA	9	25.50	6.20	( <i>P</i> < 0.001)	6.15	6.48	( <i>P</i> < 0.001)	
Error	224	4.41	–		0.95	–		

(0.1, 0.2, 0.4, 0.6, 0.8, 1.0, or 1.2 mg/litre). The average number and length of shoot was collected.

### Species and inbreds

Shoot formation capabilities of the following 10 inbreds of four lentil species were tested using MS medium supplemented with 0.2 mg/litre BA—*L. culinaris*: ‘Titore’, ‘Olympic’; *L. nigricans*: W6 3210, W6 3218, W6 3221, and W6 3208; *L. ervoides*: W6 3173, W6 3176, and W6 3192; and *L. odemensis*: W6 3244.

### Elongation of induced shoots

Stumps (the remaining part after initially excising long shoots (>2.5 cm)) were transferred to fresh medium of the same composition of the multiple shoot induction medium, and a basal MS medium with 0.5 mg/litre GA<sub>3</sub> or 0.05 mg/litre NAA. Two weeks later, data were collected for the number of long shoots (>2.0 cm)/seed.

### Root induction and transfer plants to soil

The developing shoots (>2.0 cm) from seeds cultured on MS medium containing 0.2 mg/litre BA were excised, and used for root induction. Shoots of cultivar ‘Titore’ were cultured on basal MS medium supplemented with varying concentrations of NAA (0.2, 1.0, or 1.5 mg/litre) or indole-butyric acid (IBA) (1.5 or 2.0 mg/litre). MS basal medium containing 1.5 mg/litre NAA was used to root the shoots from other inbreds. After 4 weeks, data were collected for the number of rooted shoots.

After rooting, plantlets were removed from culture vessels, washed thoroughly with tap water to remove the remaining medium and planted to plastic pots containing sterilised soil mixture. Plantlets were then transferred to the glasshouse. For the first 2 weeks, plantlets were covered with glass

beakers to maintain high humidity. They were watered once a day using 20% strength MS salt solution.

All media were supplemented with 3% sucrose and solidified by 8% agar. They were adjusted to pH 5.7 before sterilisation by autoclaving (122 kPa, 20 min). Growth regulators were sterilised by filtration and then added to autoclaved medium. Plastic tissue culture vessel of 150 ml volume was used with c. 50 ml medium per vessel. All cultures were kept in a culture room with 60 μmol m<sup>-2</sup> s<sup>-1</sup> at 24°C with 16-h days.

### Experimental design and statistical analysis

A randomised complete block design with three replicates of five culture vessels per treatment (combination) was used for all experiments. For shoot induction, 10 seeds were used per container, but only five shoots were used per vessel for root initiation. The data were analysed based on vessel means using the general linear model (GLM) function of SAS<sup>®</sup> (1990). Percentage data were arcsine-transformed to satisfy the requirement of normality.

## RESULTS

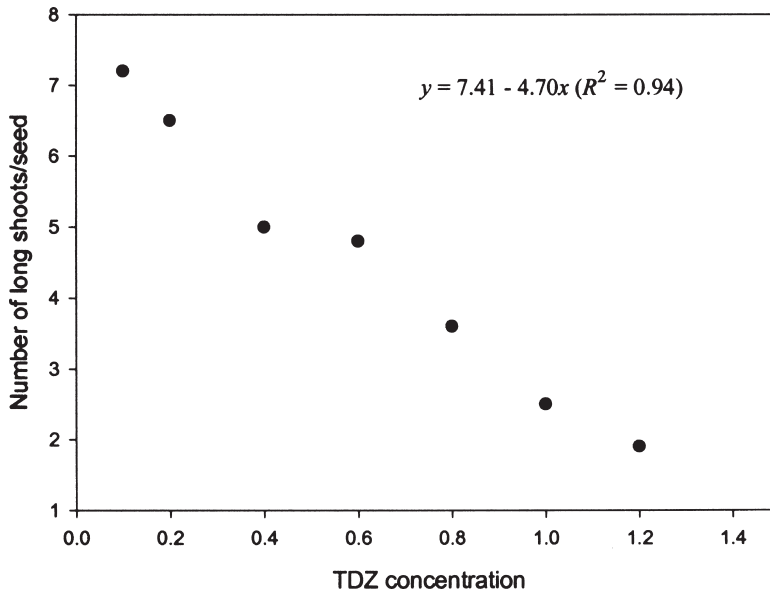
### Multiple shoot induction

Multiple shoots were induced on each of the four media and at every BA concentration (Table 1). The effects of medium, BA concentration, and their interaction on shoot induction were significant both for the number of shoots and the number of long shoots/seed (>2.0 cm). The contribution of the interaction variance to the overall variance was much less than those of medium and of BA concentration. For the number of shoots per seed the MS salts were superior to B5 salts, regardless of the concentration of BA tested. For the number of long shoot per seed, B5 medium is better than MS when BA concentration is 0.8 mg/litre. B5 with 750 mg/litre CaCl<sub>2</sub> produced significantly more (long) shoots than B5. Double the content of CaCl<sub>2</sub> of MS medium improved shoot induction only slightly. Generally, more long shoots were produced at lower BA concentrations, whereas more shoots were induced in higher BA concentrations (Table 1).

Shoot-tip necrosis (STN) of main shoots was observed (Table 2). A higher frequency of STN occurred in the B5 and MS medium. The most serious STN happened to B5 with 0.2 and 0.4 mg/litre BA. Compared to the results of B5 and B5 with

**Table 2** Effects of medium and benzyladenine (BA) concentration on shoot-tip necrosis frequency (data collected after 3 weeks of culture). (MS = Murashige & Skoog (1962); MSC = MS + 440 mg/litre CaCl<sub>2</sub>; B5 = Gamborge et al. (1968); B5C = B5 + 750 mg/litre CaCl<sub>2</sub>.)

	BA (mg/litre)			
	0.2	0.4	0.6	0.8
Medium				
MS	85.0	70.0	55.5	48.5
MSC	2.5	2.5	0.0	0.0
B5	90.5	87.3	75.0	72.5
B5C	18.3	15.5	4.8	5.2



**Fig. 1** Relationship of number of long shoots per seed and thidiazuron (TDZ) concentrations.

**Table 3** Effect of kinetin on the formation and development of multiple shoots (data collected after 3 weeks of culture). Basal medium was Murashige & Skoog (MS) salts and vitamin with 3% sucrose; means were from 120–150 seeds. Error degrees of freedom was 82.

Kinetin (mg/litre):	0.2	0.4	0.6	0.8	1.0	1.5	LSD <sup>2</sup>
No. of shoots	1.05	1.05	1.75	4.45	4.63	2.90	1.14
No. of long shoots (>2.0 cm)	1.00	1.05	1.50	2.75	2.45	1.90	0.86
Long shoot length (cm)	5.85	6.37	6.65	5.22	5.15	5.36	0.83

750 mg/litre of CaCl<sub>2</sub>, and those of MS and MS with doubled content of CaCl<sub>2</sub>, it was obvious that the addition of Ca largely overcame STN. However, on B5 medium with 750 mg/litre CaCl<sub>2</sub> and MS medium with doubled content of CaCl<sub>2</sub> the percentage of STN was very different, though the Ca content was the same.

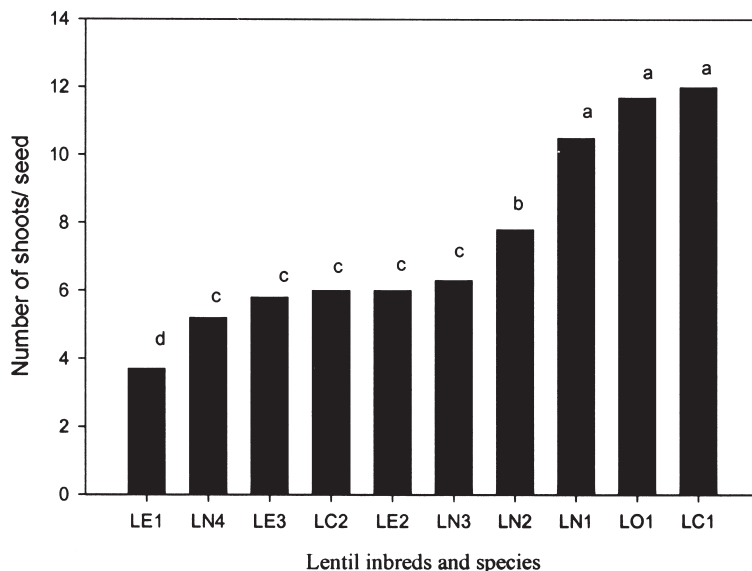
Kinetin induced multiple shoots from seed explants as well (Table 3). Statistically significant differences among the concentrations tested were found for the number of shoots and long shoots/seed. The concentration of 0.8 and 1.0 mg/litre were better for the total and the useable number of shoots per seed. Comparing the number of shoots induced using optimal concentration of kinetin (0.8 mg/litre) (Table 3) with that using BA (Table 1), it was clear that fewer shoots were produced by using kinetin. However, the shoots induced by using kinetin were longer, although no designed comparison was made.

Thidiazuron induced the formation of multiple shoots at all concentrations. The number of long shoots/seed decreased with TDZ concentration, seven shoots at 0.1 mg/litre compared with two at 1.2 mg/litre (Fig. 1). Moreover, the proportion of abnormal shoots (stunted and hyperhydric) increased with increased concentration. Most of the shoots from media containing higher TDZ concentrations became chlorotic and died within 2 weeks after transfer to shoot elongation or rooting media (data not shown).

#### Comparisons between lentil inbreds

Multiple shoots were induced from all inbred/accessions tested, and a minimum of 3 shoots/seed were produced (Fig. 2). Shoot formation capability varied among and within species. The most responsive inbred ('Titore' of *L. culinaris*, W6 3210 of *L. nigricans*, and W6 3244 of *L. odemensis*)

**Fig. 2** Shoot formation from seeds of 10 inbreds of four lentil species. Differences among inbreds labelled by the same letter(s) were not significant at  $\alpha = 0.05$  level. LC1: ‘Titore’ of *L. culinaris*; LC2: ‘Olympic’ of *L. culinaris*; LN1–LN4: inbreds W6 3210, W6 3218, W6 3221, and W6 3208 of *L. nigricans*; LE1–LE3: inbreds W6 3173, W6 3176, and W6 3192 of *L. ervoides*; and LO1: inbred W6 3244 of *L. odemensis*.



**Table 4** Elongation of shoots from different shoot induction media on different elongation media. Differences among treatments within a column (row) labelled by the same right (left) superscript letter(s) were not significant at  $\alpha = 0.05$  level. (GA<sub>3</sub> = gibberellic acid; TDZ = thidiazuron; NAA =  $\alpha$ -naphthaleneacetic acid; BA=benzyladenine.)

Elongation medium	Shoot induction medium			
	0.4 TDZ	0.1 TDZ	0.8 BA	0.2 BA
Fresh medium	a3.1 a	a,b11.3 a	a5.4a	a,b10.5 a
MS + 0.5 GA <sub>3</sub>	a7.6 a,b	a,b11.5 a	a,b10.3a,b	a,b10.5 a
MS + 0.05 NAA	a7.6 a,b	a,b12.1 a	a,b11.7a,b	a,b12.6 a

produced 3 times more shoots/seed than the least responsive inbred (W6 3173 of *L. ervoides*).

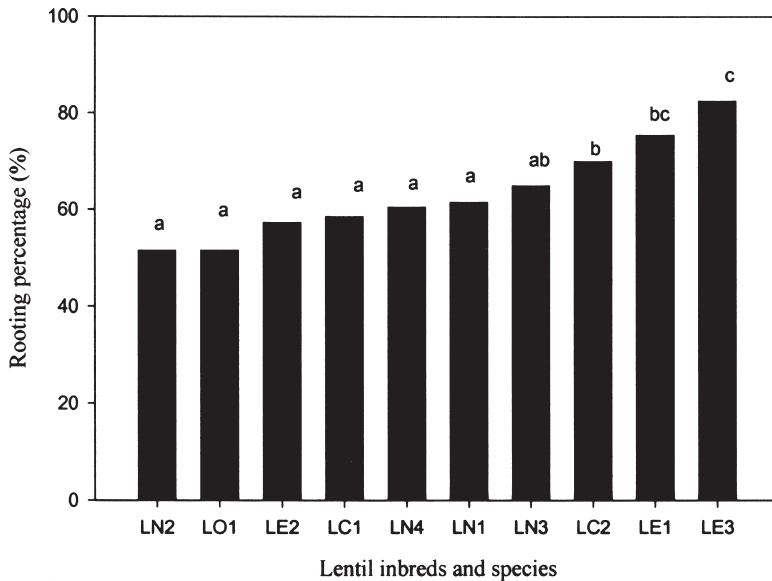
### Elongation of induced shoots

Both of the original shoot induction medium, and the medium used for elongation affected the number of long shoots per stumps produced (Table 4). After 2 weeks on fresh medium of the same compositions, on average stumps from medium containing 0.2 mg/litre BA and medium containing 0.1 mg/litre TDZ produced 10.5 and 11.3 long shoots, respectively. Stumps from medium containing 0.8 mg/litre BA and medium containing 0.4 mg/litre TDZ produced five and three long shoots, respectively. Using MS basal medium supplemented with 0.5 mg/litre GA<sub>3</sub> or 0.05 mg/litre NAA greatly increased the number

of long shoots on stumps from medium containing 0.8 mg/litre BA (10.3 and 11.7, respectively), or 0.4 mg/litre TDZ (7.6). However, these two elongation media did not significantly improve the shoot elongation of stumps from medium containing 0.2 mg/litre BA or 0.1 mg/litre TDZ.

In addition to the elongation of small shoots, many new shoots/shoot meristem were induced around the connecting site of the cotyledons and the hypocotyl, irrespective of whether fresh medium of the same composition, or MS basal medium supplemented with GA<sub>3</sub> or NAA was used.

After one round of subculture, the stumps became too large to be easily transferred. It was required to subdivide stumps into three or four small clusters. Continual shoot production from these



**Fig. 3** Rooting percentages of induced shoots from 10 inbreds of four lentil species. Differences among inbreds labelled by the same letter(s) were not significant at  $\alpha = 0.05$  level. LC1: 'Titore' of *L. culinaris*; LC2: 'Olympic' of *L. culinaris*; LN1–LN4: inbreds W6 3210, W6 3218, W6 3221, and W6 3208 of *L. nigricans*; LE1–LE3: inbreds W6 3173, W6 3176, and W6 3192 of *L. ervoides*; and LO1: inbred W6 3244 of *L. odemensis*.

**Table 5** Effect of plant growth regulator and shoot source on rooting percentage (data collected after 4 weeks of culture). Differences among treatments within a column (row) labelled by the same right (left) superscript letter(s) were not significant at  $\alpha = 0.05$  level. (NAA =  $\alpha$ -naphthaleneacetic acid; IBA = indole-3-butyric acid.)

	NAA (mg/litre)			IBA (mg/litre)	
	0.2	1.0	1.5	1.5	0.2
Adventitious	<sup>a</sup> 13.3 <sup>a</sup>	<sup>b</sup> 43.5 <sup>a</sup>	<sup>c</sup> 58.5 <sup>a</sup>	<sup>a</sup> 10.0 <sup>a</sup>	<sup>a</sup> 6.7 <sup>a</sup>
Primary	<sup>a</sup> 32.5 <sup>b</sup>	<sup>c</sup> 66.7 <sup>b</sup>	<sup>c</sup> 75.0 <sup>b</sup>	<sup>b</sup> 60.0 <sup>b</sup>	<sup>a</sup> 25.0 <sup>b</sup>

clusters were obtained using a MS basal medium supplemented with 0.5 mg/litre GA<sub>3</sub> or 0.05 mg/litre NAA. Up to 20 long shoots/stamp could be harvested in 3 weeks.

### Rooting and transfer to soil

The proportion of rooted shoots varied widely with different NAA and IBA supplements of the MS medium (Table 5). From 14% (on basal MS medium without growth regulator) to 75% (on MS supplemented with 1.5 mg/litre NAA) of the shoots rooted within 4 weeks. NAA was more effective than IBA. Usually only one well developed root with several short secondary roots were produced/shoot within 4 weeks, but three or four roots with secondary roots were obtained after a further 2 weeks of culture in a fresh medium with the same composition.

Callus formation was observed on the cut surface of shoots when NAA was applied. The higher the NAA concentration, the greater the callus development. However, roots were not initiated from callus.

Using MS basal medium supplemented with 1.5 mg/litre NAA, more than 50% of shoots from 10 inbreds rooted, although there were differences among inbreds (Fig. 3). The highest and lowest rooting were W63192 of *L. ervoides* (82.5%), and W63241 of *L. odemensis*, and W63218 of *L. nigricans* (51.5%), respectively.

The percentage survival of the plantlets after transfer to soil mixture was 90% for *L. culinaris* 'Titore'. For other inbred, survival percentage varies from 80 to 100%. However, no designed comparison among inbred was made because of the limited number of plantlets.

## DISCUSSION

Cytokinin BA, TDZ, and kinetin all induced multiple shoot formation. The effectiveness of BA on shoot induction in lentil tissue culture has been well documented (Saxena & King 1987; Polanco et al. 1988; Malick & Rashid 1989; Warkentin & McHughen 1993; Ahmad et al. 1997; Hallbach et al. 1998). Malik & Saxena (1992) reported that TDZ induced multiple shoots directly from intact seedlings of *L. culinaris*, *Pisum sativum*, *Cicer arietinum*, and *Phaseolus vulgaris*. Singh & Raghuvansi (1989) reported that multiple shoots directly formed from nodal segments and shoot tip explant using MS medium supplemented with kinetin. Kinetin has also been successfully used to induce shoot formation from lentil hypocotyls, epicotyls, and shoot meristem via callus phase (Williams & McHughen 1986).

Shoot-tip necrosis was observed in many media used for shoot induction. The addition of calcium (Ca) overcomes shoot-tip necrosis. Parh et al. (1998) first reported shoot-tip necrosis of *in vitro* lentil seedlings. They alleviated this problem by doubling the Ca content of the MS basal medium. Studies in other plant species have indicated that Ca deficiency, which can be easily promoted by *in vitro* conditions such as low transpiration rate and high humidity, may be the main reason for STN (Sha et al. 1985).

It was shown that BA-based seed culture could be used for inducing multiple shoots from a range of lentil germplasm, although intra- and inter-specific variations were obvious. However, the small number of cultivars/accessions of each species used did not allow us to accurately estimate the inter- and intra-specific variability of shoot regeneration capability. These results implied that a universal protocol for lentil *in vitro* culture could be developed based on seed culture using a BA-containing medium, and that cultivars screening for good multiplication performance will greatly improve the success rate of *in vitro* culture.

A common phenomenon when inducing shoots from seeds was inconsistent shoot development. Furthermore, short shoots (<2.0 cm) did not root well (Ye et al. 2000). Therefore, it was necessary to promote the development of the short shoots. Removal of longer shoots and the subculture of the stumps onto a suitable medium gave very good result. For stumps from medium with low BA (<0.8 mg/litre) or TDZ (<0.5 mg/litre), fresh medium of the same compositions could be used. Otherwise, MS medium supplemented with 0.5 mg/litre GA<sub>3</sub> or 0.05 mg/litre NAA was appropriate.

The rooting percentage in this study was higher than in all the previous studies except that of Ahmad et al. (1997). One reason may be the lower concentration of external cytokinins used. However, it is very hard to compare our results with previous studies because of the different types of explants used. Nevertheless, the high rooting ability of shoots produced from seeds gives seed culture another important advantage.

During the process of root induction, several shoots were produced simultaneously at the basal part of some shoots. When detached, these shoots were much easier to root than others. This observation can be exploited to increase the efficiency of multiplication in a future study.

## CONCLUSION

Cytokinin BA, TDZ, and kinetin all induced multiple shoot formation in lentil seed culture. For shoot formation, MS salts with doubled calcium chloride content and 0.2–0.4 mg/litre BA was recommended. Thidiazuron at 0.1 mg/litre was also efficient, but further experimentation is required to justify the use of this growth regulator.

The higher number of shoots/seed and higher rooting percentage make the system developed the most efficient one for *in vitro* culture of lentil. This suggests that this protocol is very universal. Therefore an efficient and universal protocol for *in vitro* culture of lentil is likely to be developed based on seed culture.

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