Pak. J. Bot., 48(5): 1999-2004, 2016.

DIRECT REGENERATION AND EFFICIENT *IN VITRO* ROOT DEVELOPMENT STUDIES IN LENTIL (*LENS CULINARIS* MEDIK)

TASAWAR SULTANA[1], NADIA MAJEED[1], FARIHA KHAN[2], ATA UR REHMAN[3] AND S.M. SAQLAN NAQVI[1]*

[1] Department of Biochemistry, PMAS Arid Agriculture University Rawalpindi, Pakistan
[2]Department of Biosciences, COMSATS Institute of Information Technology, Islamabad, Pakistan
[3] Functional Grain Centre, Charles Sturt University, Wagga Wagga, NSW, Australia
[*]Corresponding author e-mail: saqlan@uaar.edu.pk

Abstract

Lentil is a self-pollinating annual crop with increasing demand all over the world due to its high protein content and easy digestibility. However, like many other crops lentil too needs improvement for which conventional as well as biotechnological tools are to be employed. This study was aimed at development of tissue culture protocol especially targeting improved root development to ensure their establishment in soil in order to use their potential towards genetic manipulation. Two Pakistani lentil cultivars, Masoor-2002 and Manshera-89 were used to obtain cotyledonary nodes, epicotyl and hypocotyl explants. The explants were cultured on shoot regeneration medium containing different concentration of kinetin, BAP and tyrosine with the addition of GA3, with or without charcoal for shoot development. Masoor-2002, showed the highest frequency of shoot development on MS medium containing 5.5 mg/L tyrosine, 0.25 mg/L kinetin, 1.0 mg/L BAP, 0.1 mg/L GA3, using cotyledonary node as explant. The addition of 2 g/L of charcoal in shoot medium resulted in healthier plants, but the number of shoots were reduced. Regarding the effect of age of explants on regeneration frequency, cotyledonary nodes of age 4-6 days had higher regeneration potential. Well-developed shoots were shifted to rooting medium containing different concentration of auxin with or without charcoal. Healthier and more roots were observed on medium containing 4 mg/L IAA with addition of 2 g/L charcoal. Plants were better established (70% survival) in a soil mix containing perlite, vermiculite and peat moss in 1:1:1 ratio.

Key words: Regeneration, Explant, Cytokinin, Auxin, Rooting, Charcoal, Lentil.

Introduction

Lentil (*Lens culinaris* Medik) is an excellent source of protein, soluble fiber, iron, folic acid, minerals, antioxidant compounds and vitamin B1. Lentil also plays important role in increasing soil fertility through nitrogen fixation (Duranti & Gius, 1997). The major lentil producing countries are Canada, India, Australia and Turkey. After chickpea lentil is the second major Rabi pulse crop in Pakistan with 9,883 metric tons production (Anon., 2013). Due to narrow genetic base and non availability of resistance sources in the lentil germplasm, its yield is affected by various pathogens among which fungal and viral diseases at various stages of their growth are of major concern (Taylor *et al.*, 2007). Introducing foreign beneficial genes, characterized to be stress responsive in different plant species (Naz *et al*., 2015), through genetic transformation approach can produce plants resistant to biotic and abiotic stresses. Efficient and reliable regeneration protocols for a particular plant are, therefore, necessary for stable plant transformation.

Regeneration of shoots in lentil has been reported from various lentil explants such as epicotyls (Williams and McHughen, 1986), stem nodes (Ahmed *et al.*, 1997), decapitated embryo, embryo axis and immature seeds (Polanco & Ruiz, 2001), cotyledonary node (Gulati *et al*., 2001, Sarker *et al.*, 2003, Omran *et al*., 2008). However, rooting of *in vitro* obtained lentil shoots, are a very slow and difficult process that usually generates weak roots with browning and drying of plant. The process of root development is also genotype and explants dependent. Moderate success in root formation was observed on MS medium containing 0.25 mg/L IBA (Khawar & Ozcan, 2002a).

Activated charcoal is a strong adsorbent of toxic and inhibitory compounds from culture medium and improve cell development. It is frequently used in plant tissue culture medium to prevent death of plants due to browning. Barikissou & Baudoin (2011) found that addition of activated charcoal in root inducing media increased rooting rate up to 90% as compared to 53% in the absence of charcoal in common bean. However, use of activated charcoal has not been reported for regeneration studies in lentil. The objective of present study was to investigate the effect of different factors on the regeneration of viable shoots and roots in lentil for development of whole plant, such as explant source and age, genotype, concentration of cytokinin, tyrosine and activated charcoal in culture medium. Present study successfully established the regeneration protocol for lentil from an explant to root, which can be used for *in vitro* gene manipulation.

Materials and Methods

Lentil seeds of two varieties, one each of a macrosperma and a microsperma type i.e., Mansehra-89 and Masoor-2002 respectively, were obtained from NARC, Islamabad, Pakistan.

Seeds sterilization and germination: Seeds were rinsed with liquid detergent for few minutes followed by washing in excess tap water. Seeds were soaked for one minute in 70% ethanol followed by dipping in a 30% commercial bleach solution for 15 min. Subsequently they were washed four times with autoclaved distilled water for about five minutes each. Seed germinated on MS medium containing 3% sucrose, solidified with 0.6% agar and pH was adjusted

at 5.8. Explants including hypocotyl, epicotyl and cotyledonary nodes were excised from aseptically grown seedlings under sterilized conditions. The explants in culture tubes were incubated in a plant growth chamber at 25 ± 2°C in a 16/8 h light/dark photoperiod, in a fluorescent tube light of approximately 2000 lux.

Culture medium for regeneration of shoots: Explants obtained from 5-days old seedling were cultured on Murashige & Skoog (1962) basal media supplemented with different concentration and/or combinations of benzyl amino purine (BAP), kinetin, gibberellic acid (GA3) and tyrosine. Activated charcoal was added into medium independently. Data for shoots development was taken after 4 weeks.

Culture medium for formation of roots: Healthy shoots were transferred to rooting media without cutting or damaging after 3-4 weeks and allowed to develop roots. Rooting media were containing MS salts and different concentrations of auxins i.e., indole acetic acid (IAA) and indole butyric acid (IBA). Plants were shifted to fresh media after 2-3 weeks. In order to promote rooting, 2 g/L activated charcoal was added in rooting media containing various concentrations of IAA (1, 2, 4, 6 mg/L). Data was taken after 10 weeks.

Acclimatization and establishment in soil: Healthy rooted plants were removed from the media, washed under tap water and shifted to small pots with different types of soil mix like coconut bark, garden soil, sandy soil and soil mix containing perlite, vermiculite and peat moss in 1:1:1 ratio separately. Plantlets were covered with plastic wrap for 1-2 week to retain high humidity for acclimatization to the external environment.

Regeneration from nodal fragments: Nodal fragments were obtained from Masoor-2002, 21 days old seedlings and cultured on MS media having 0.1 mg/L GA3 and BAP (1.0, 2.0 mg/L). Plantlets were shifted to rooting media with 1.0 mg/L IAA after the formation of shoots. Data for roots development was taken after 10 weeks.

Statistical analysis: All the experiments were done in triplicates. 25 explants were cultured for each treatment. Analysis of variance (ANOVA) was performed using statistical software MSTAT-C (Michigan State University, USA).

Results

Effect of explant source, genotype and concentration of cytokinin on regeneration of shoots: Regeneration potential of cotyledonary node explant was more pronounced than epicotyl and hypocotyl on all media combinations tested. Epicotyl was second best while hypocotyls showed no regeneration on any type of media (Fig. 1). The regeneration response of explants differed significantly from each other. There was no significant difference in regeneration potential of two lentil varieties, however, Masoor-2002 developed more shoots per explants and the plants were healthier as compared to Manshera-89. There was no single media, which could support all types of explants and all varieties. SM4 apparently gave higher regeneration for cotyledonary node of Masoor-2002 i.e., 100% whereas SM1 was the best for epicotyl of Masoor-2002 with 67%. In Manshera-89 cotyledonary node and epicotyl gave higher regeneration on SM2. Statistical analysis showed no signnificant difference in regeneration response on different shoot media used. Cotyledonary node of Masoor-2002 developed multiple shoots i.e. at average 6.5 shoots per explant while Manshera-89 developed at average 5.6 shoots per explant on best supportive media i.e. SM4 (Table 1). Some of the cotyledonary explants of Masoor-2002 even developped more that 10 shoots. The average lengh of shoots was 3.1 cm for Masoor-2002 on SM4 medium whereas 2.8 cm for Manshera-89 on same medium. Generally 2-3 shoots developed on each epicotyl with average length of 3.4 cm for Masoor-2002 on SM1 medium whereas 2.0 cm for Manshera-89 on same medium.

Table 1. Regeneration response of explants of lentil on MS media supplemented with growth regulators and tyrosine. Values are mean ± SE of three replicates. Means followed by the same letter indicate no significant difference ($p<0.05$).

Variety	Explant	Shoot media*	Mean shoot number per explant	Mean shoot length (cm)	Shooting frequency	Mean shooting frequency
Masoor-2002	Cotyledonary nodes	SM1	3.9 ± 1.1	2.5 ± 1.4	92 ± 6.9	94 ± 4.0[a]
		SM2	2.3 ± 2.1	2.0 ± 1.6	91 ± 0.9	
		SM3	4.1 ± 2.0	1.9 ± 0.9	94 ± 8.0	
		SM4	6.5 ± 1.8	3.1 ± 1.3	100 ± 0.0	
	Epicotyl	SM1	2.0 ± 0.8	3.4 ± 0.9	67 ± 10.8	59 ± 11.4[b]
		SM2	1.5 ± 1.0	1.8 ± 1.7	61 ± 11.5	
		SM3	1.0 ± 0.6	0.9 ± 0.5	65 ± 10.8	
		SM4	1.0 ± 0.9	1.3 ± 0.8	42 ± 8.9	
Manshera-89	Cotyledonary nodes	SM1	4.6 ± 1.9	2.7 ± 1.3	94 ± 3.7	87 ± 13.0[a]
		SM2	6.9 ± 3.2	2.9 ± 0.9	97 ± 3.7	
		SM3	6.3 ± 2.5	3.3 ± 1.5	68 ± 10.0	
		SM4	5.6 ± 2.7	2.8 ± 1.5	88 ± 10.6	
	Epicotyl	SM1	1.7 ± 0.7	2.0 ± 1.3	58 ± 6.1	48 ± 18.0[b]
		SM2	1.5 ± 0.5	1.9 ± 1.3	68 ± 8.6	
		SM3	1.3 ± 0.5	0.9 ± 0.3	28 ± 3.4	
		SM4	1.8 ± 0.8	1.0 ± 0.6	39 ± 4.2	

*SM1: 0.1 mg/L GA3, 5.5 mg/L Tyrosine, 0.5 mg/L Kinetin, 0.5 mg/L BAP, SM2: 0.1 mg/L GA3, 5.5 mg/L Tyrosine, 0.5 mg/L Kinetin, 1.0 mg/L BAP, SM3: 0.1 mg/L GA3, 5.5 mg/L Tyrosine, 0.5 mg/L Kinetin, 2.0 mg/L BAP, SM4: 0.1 mg/L GA3, 5.5 mg/L Tyrosine, 0.25 mg/L Kinetin, 1.0 mg/L BAP

Table 2A. Effect of tyrosine on shoot regeneration from cotyledonary node of Masoor-2002.

Media	Growth regulators (mg/L)	Tyrosine (mg/L)	Average shoot number per explant	Average shoot length per explants (cm)	Mean shooting frequency (%)
SM4	GA3 0.1+ BAP 1.0 + Kinetin 0.25	5.5	6.2 ± 1.0	3.8 ± 1.5	100 ± 0.0[a]
SM5	GA3 0.1+ BAP 1.0 + Kinetin 0.25	2.5	5.65 ± 0.5	3.85 ± 1.0	100 ± 0.0[a]
SM6	GA3 0.1+ BAP 1.0 + Kinetin 0.25	1.25	5.25 ± 1.0	3.95 ± 2.0	100 ± 0.0[a]
SM7	GA3 0.1+ BAP 1.0 + Kinetin 0.25	0	4.63 ± 0.2	3.65 ± 1.3	92 ± 2.9[a]

Table 2B. Effect of charcoal on shoot regeneration from cotyledonary node of Masoor-2002.

Media	Growth regulators (mg/L)	Charcoal g/L	Average shoot number per explant	Average shoot length per explants (cm)	Mean shooting frequency (%)
SM8	GA3 0.1+ BAP 1.0 + Kinetin 0.25+ Tyrosine 5.5	3.0	1.25 ± 0.5	6.5 ± 1.6	98 ± 5.0[a]
SM9	GA3 0.1+ BAP 1.0 + Kinetin 0.25+ Tyrosine 5.5	2.0	2.10 ± 0.5	5.85 ± 1.8	100 ± 0.0[a]
SM10	GA3 0.1+ BAP 1.0 + Kinetin 0.25+ Tyrosine 5.5	1.0	2.35 ± 0.9	5.95 ± 2.0	100 ± 0.0[a]
SM11	GA3 0.1+ BAP 1.0 + Kinetin 0.25+ Tyrosine 5.5	0.5	2.05 ± 0.2	5.02 ± 1.5	100 ± 0.0[a]

Table 3. Effect of auxin and charcoal in root formation of plantlet of Masoor-2002 on rooting media.

Rooting media	Auxin (mg/L)	Charcoal (g/L)	Mean rooting frequency (%)	Average root number per plantlet	Average root length per plantlet (cm)
RM1	0.5 IAA	0	13 ± 4.6[cd]	4.5 ± 2.5	4.25 ± 1.6
RM2	1.0 IAA	0	42 ± 3.0[ab]		
RM3	1.5 IAA	0	15 ± 3.0[cd]		
RM4	2.0 IAA	0	22 ± 2.9[cd]		
RM5	4.0 IAA	0	20 ± 2.5[cd]		
RM6	0.25 IBA	0	27 ± 1.7[bc]		
RM7	1.0 IAA	2.0	21 ± 1.0[cd]	5.8 ± 0.87	5.9 ± 2.1
RM8	2.0 IAA	2.0	27 ± 4.8[bc]		
RM9	4.0 IAA	2.0	47 ± 4.2[a]		
RM10	6.0 IAA	2.0	10 ± 4.1[d]		

Table 4. Effect of growth regulators on shoots and root formation from nodal fragments of Masoor-2002 on shooting and rooting media.

Shooting media growth regulators (mg/L)	Mean shoots regeneration frequency (%)	Rooting medium	Mean roots formation frequency (%)
0.1 GA3, 1.0 BAP	100 ± 0.0	1 mg/L IAA	25 ± 5.25
0.1 GA3, 2.0 BAP	92 ± 0.9	1 mg/L IAA	22 ± 4.5

Role of tyrosine in regeneration of shoots: Cotyledonary nodes of Masoor-2002 appeared better for shoot regeneration, therefore, these were used as explants for further experiments with an aim of improvement of tissue culture protocol. Tyrosine is considered to aid in multiple shoot formation (Sarker *et al.,* 2003). To evaluate its role, media containing tyrosine at different concentrations were used. Regeneration response remained same for all the concentrations tested, but explants did differ. Cotyledonary node developed more shoots per explant i.e. 6.2 shoots on 5.5 mg/L tyrosine as compared to 4.63 shoots for media without tyrosine while no difference was observed in shoot length (Table 2A). The results indicated that tyrosine is supportive in the development of healthy and multiple shoots.

Role of activated charcoal in regeneration of shoots: Cotyledonary nodes of Masoor-2002 were cultured on shoot medium having different concentrations of charcoal. With the addition of charcoal, stems looked healthier and stronger and without any sign of drying, but number of shoots was greatly affected, Only one to two long shoots emerged in contrast to multiple shoots observed in the charcoal-free medium (Fig. 1). No significant difference was observed for four media combination used with charcoal in terms of shoot regeneration frequency (Table 2B).

Effect of age of explant on regeneration of shoots: In order to evlaute the influence of age of explants, cotyledonary node explant of Masoor-2002 was obtained from seedling of age ranging from 4 to 14 days and cultured on SM4 medium. Significant difference was found in regeneration response of explants of different age (Fig. 2) as younger explants showed more regeneration potential than older ones. Explants 4, 6 and 8 days of age, had maximum regeneration frequency with no significant difference among each other. Hence explant of 4-8 days age are good choice for tissue culture experiments of lentil.

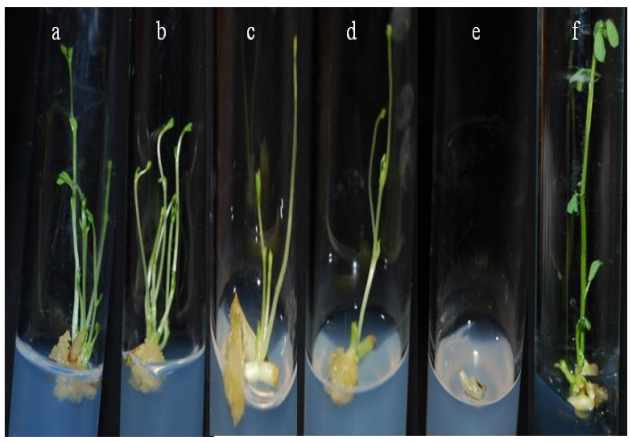

Fig. 1. Shoots regeneration response of explants on shooting media. a) Cotyledonary nodes of Masoor-2002, b) Cotyledonary nodes of Manshera-89, c) Epicotyl of Masoor-2002, d) Epicotyl of Manshera-89, e) Hypocotyl of Masoor-2002, a-e) Shoots regeneration from explants on shooting media SM4 without charcoal, f) Cotyledonary nodes of Masoor-2002 on charcoal containing shooting media (SM9).

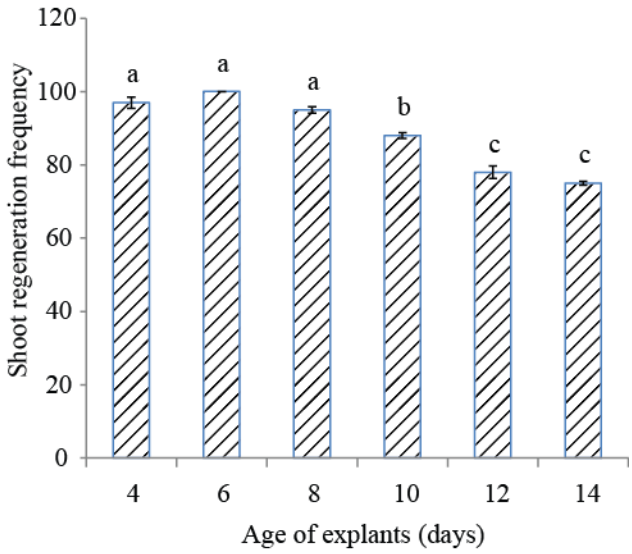

Fig. 2. Effect of age of cotyledonary node explant of Masoor-2002 on shoots regeneration frequency on SM4 shooting medium.

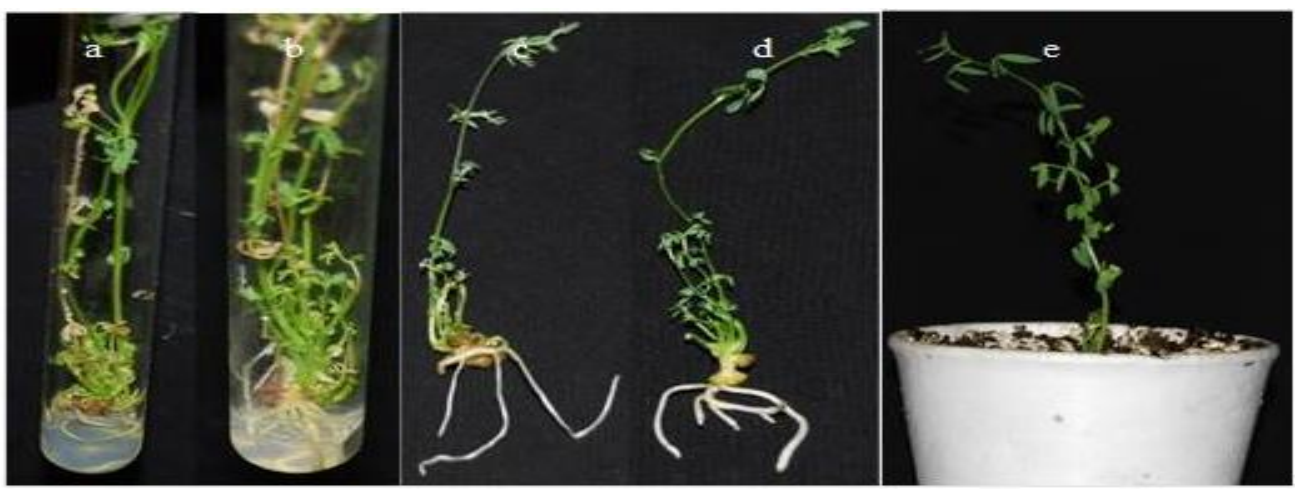

Fig. 3. Root formation by cotyledonary node regenerated plantlet of Masoor-2002.
a) Root formation on RM1, b) Root formation on RM2, c) Root formation on charcoal containing rooting medium RM8 d) Root formation on charcoal containing rooting medium RM9, e) Plants established in soil mix composed of perlite, vermiculite and peat moss in 1:1:1 ratio.

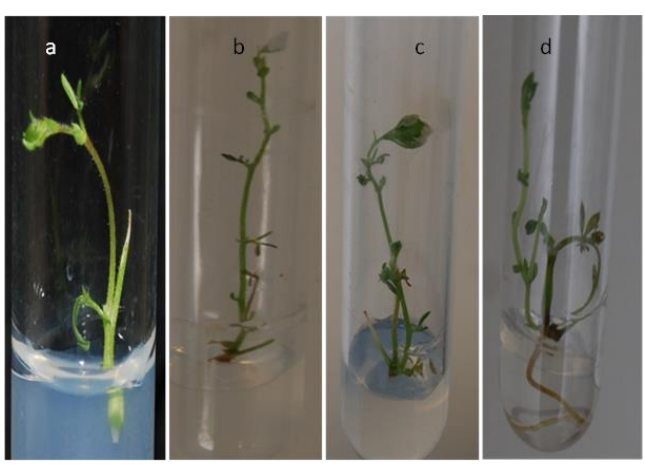

Fig. 4. Regeneration in nodal fragments of Masoor-2002.
a) Regeneration of shoots from nodal fragments, b) Flower formation on shoots, c) Pod formation, d) Rooting of regenerated shoots.

Rooting of regenerated shoots: Regeneration is not much valuable until a complete plant with roots can be obtained. Lentil is quite recalcitrant toward roots formation. Shoots of Masoor-2002 and Manshera-89 were shifted to rooting media with different concentrations of auxin. A significant difference was found in rooting frequency on different rooting media. Highest rooting response was obtained from media RM2 for Masoor-2002 shoots which was 42% (Table 3). No root development was observed in Manshera-89 plantlets. Data were taken after 8 to 10 weeks on rooting media. Healthy and apparently normal roots were developed with IAA while on IBA a mesh work of roots and in some cases abnormally thick roots were observed; therefore IAA was preferred for root formation (Fig. 3). Due to the long time period required for root formation subsequent drying and yellowing of plants were also observed on some media combinations.

Role of activated charcoal in rooting: For assessing the role of activated charcoal in root induction and protection from browning, regenerated shoots from the cotyledonary node of Masoor-2002 on SM4 shooting medium was shifted to four combinations of IAA with 2 g/L charcoal. Activated charcoal is reported to absorb growth regulators available in medium (Thomas, 2008) so slightly higher concentration of IAA was used. Activated charcoal promoted good root formation (47%) on medium having 4 mg/L IAA (RM9) (Table 3). Roots developed after 4 to 6 weeks on rooting media. Plants were healthier and greener due to shorter time period for root formation in charcoal and also had a better survival rate after shifting to soil (Fig. 3). The best survival (70±4.8%) was observed in 1:1:1 perlite, vermiculite and peat moss mix, as compared to 1:1 soil and sand mix (10±1.7%), and coconut bark (15± 2.9%). Amazingly, no plant could survive in routine garden soil.

Regeneration from nodal fragmentation for micro-propagation: In legumes, rooting and hardening is difficult and percentage of plant survival during this process is also very low. Through nodal fragmentation plantlets may be multiplied *in vitro*, which is liable to increase the chances of successful establishment of a particular plants in soil, especially for valuable transgenic lines. Nodal fragments of Masoor-2002 were regenerated successfully with 100% frequency on shooting media with 1.0 mg/L BAP and 0.1 mg/L GA3 (Fig. 4). These plantlets were able to produce roots on 1 mg/L IAA with 25% rooting frequency (Table 4).

Discussion

The target of the present study was to standardize an efficient and reliable tissue culture system for lentil from initial explant to establishment in rooting medium. The process of direct organogenesis through different explants of seedling was selected as success had been reported for shoot organogenesis from various explants of lentil cultivars (Williams and McHughen, 1986, Ahmed *et al*., 1997, Polanco & Ruiz, 2001, Sarker *et al*., 2003). It was observed that among three explants used, cotyledonary node had a higher regeneration frequency than epicotyl and hypocotyl. Cotyledonary node of Masoor-2002 had more of healthy regenerated shoots than Manshera-89 demonstrating the genotypic effect on regeneration of shoots for lentil. Genotype effect on shoot regeneration was also reported by Khawar & Ozcan, (2002b) when they observed Akm 362 outperforming another 20 genotypes. Shoot regeneration is known to be highly affected by a dose of cytokinin, so different concentrations of BAP and Kinetin were used. Lower concentration of BAP and kinetin were used to avoid the subsequent inhibitory effect of higher concentration of BAP on root development (Omran *et al.*, 2008). Among shoot media used, SM4 better supported regeneration than other combinations. The study also indicated that addition of tyrosine with growth regulators GA3, kinetin and BAP is supportive in shoots regeneration. An addition of supplementary amino acid in the medium has beneficial effects on growth, development and enhances morphogenesis, such as induce direct adventitious shoot formation (George *et al.*, 2008). Tyrosine is involved in regulating receptor kinase mediated signaling in plants which contribute to plant growth and immunity (Macho *et al.*, 2015). Addition of GA3 in shooting media promoted shoot formation as Gibberellins reported to involved in a range of developmental processes of plants such as stem elongation, germination and flower formation (Sajid *et al*., 2016).

Activated charcoal is involved in the absorption of inhibitory compounds like toxic metabolites and phenolic exudation present in tissue culture media. Nayanakantha *et al.* (2010) observed that the addition of charcoal in MS media reduces browning of explants, promote adventitious buds formation and root development. It was found in the present study that the addition of charcoal in shooting media SM4 severely decreased number of shoots emerging from a given explant, however the shoots developed on charcoal containing medium were healthier. It was suggested by Ebert *et al.* (1993) that activated charcoal adsorbs and then slowly releases the growth regulators. This may explain the less but vigorous shoots on charcoal containing media.

Rooting is a major problem in regenerated plantlets of lentil. Auxins are reported to induce root initiation. The localized accumulation of auxin in epidermal cells of the root initiates the formation of lateral or secondary roots. (Daphne *et al.*, 2005). In present study 1 mg/L IAA in rooting media supported root formation better than other concentrations of IAA and IBA but root formation takes 8 to 10 weeks as well as browning of regenerated shoots was also observed. Charcoal was added in rooting medium which had a positive effect on root formation, as 47% root formation was observed on RM9 media while plants looked healthy and time period for root formation was also reduced. The growth promoting influence of activated charcoal is not only attributed to adsorption of inhibitory phenolic compounds released from wounded tissue in tissue culture media, but also to decrease in accumulation of brown exudate, adjustment of pH of medium optimal for morphogenesis (Thomas, 2008). Plants after root formation were transferred in different potting mixtures. A six to seven time better survival rate (70±4.8%) was observed in 1:1:1 perlite, vermiculite and peat moss mix followed by to 1:1 soil and sand mix and coconut bark. Amazingly, no plant could survive in routine garden soil.

Conclusion

The lentils regeneration response was influenced by explant type, genotype and plant growth regulator concentration. During regeneration experiments, cotyledonary node explant of Masoor-2002 were found better in regeneration response. Charcoal addition in rooting medium exerted a positive effect on root formation by increased rooting frequency with more green and healthier plants. Rooted plants were able to survive in soil mix composed of perlite, vermiculite and peat moss in 1:1:1 ratio.

Acknowledgement

This research work is funded by Higher Education Commission under Indigenous 5000 Fellowship Program (No. 17-5-4(Ps4-279) HEC/Sch/2007). We are thankful to Dr. Asghar Ali (NARC) for providing lentil seeds.

References

Ahmad, M., A.G. Fautrer, D.L. McNeil, G. Hill and D.J. Burritt. 1997. *In vitro* propagation of *Lens* species and their F1 interspecific hybrids. *Plant Cell Tiss. Org*., 47(2): 169-176

Anonymous. 2013. Food and Agricultural Organization of the United Nations. Statistic division, on line database.

Barikissou, E. and J.P. Baudoin. 2011. Refinement of an *In vitro* culture technique for the rescue of globular eembryos using microcutting for *Phaseolus vulgaris* L. and *Phaseolus coccineus* L. *Tropicultura*, 29(4): 218-224.

Daphne, J.O. and M.T. McManus. 2005. Hormones, signals and target cells in plant development. Cambridge University Press. Cambridge, New York, pp.158.

Duranti, M. and C. Gius. 1997. Legume seeds: protein content and nutritional value. *Field Crops Res*., 53(1): 31-45.

Ebert, A., F. Taylor and J. Blake. 1993. Changes of 6-benzylaminopurine and 2, 4-dichiorophenoxyacetic acid concentrations in plant tissue culture media in the presence of activated charcoal. *Plant Cell Tiss. Org*., 33: 157-63.

George, E.F., A.H. Michael and G.J. De Klerk. 2008. The components of plant tissue culture media I: macro-and micro-nutrients. *Springer, The Netherlands*, pp. 65-113.

Gulati, A., P. Schryer and A. McHughen. 2001. Regeneration and micro-grafting of lentil shoots. *In Vitro Cell Dev. Biol. Plant*, 37(6): 798-802.

Khawar, K.M. and S. Ozcan. 2002a. Effect of indole-3-butyric acid on *In vitro* root development in lentil (*Lens culinaris* Medik). *Turk. J. Bot.*, 26: 109-111.

Khawar, K.M. and S. Ozcan. 2002b. High Frequency shoot regeneration from cotyledonary node explant of different lentil (*Lens culinaris* Medik) genotypes and *In vitro* micrografting. *Biotechnol Biotec. Eq.*, 16(1): 12-17.

Macho, A.P., R. Lozano-Durán and C. Zipfel. 2015. Importance of tyrosine phosphorylation in receptor kinase complexes. *Trends Plant Sci.*, 20(5): 269-272.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol. Plant.*, 15(3): 473-497.

Nayanakantha, N.M.C., B.R. Singh and A. Kumar. 2010. Improved culture medium for micropropagation of *Aloe vera* L. *Trop. Agric. Res. Exten.*, 13(4): 87-93.

Naz, S., H. Kausar, F. Saleem and A. Zafarullah. 2015. Characterization of abiotic stress genes from different species of Eucalyptus. *Pak. J. Bot.*, 47(4): 1217-1223.

Omran, V.G., Bagheri A. and N. Moshtaghi. 2008. Direct *In vitro* regeneration of lentil *(Lens culinaris* Medik.). *Pak. J. Biol. Sci*., 11: 2237-2242.

Polanco, M.C. and M.L. Ruiz. 2001. Factors that affect plant regeneration from *In vitro* culture of immature seeds in four lentil (*Lens culinaris* Medik.) cultivars. *Plant Cell Tiss. Org*., 66(2): 133-139.

Sajid, M., N. Amin, H. Ahmed and K. Khan. 2016. Effect of gibberellic acid on enhancing flowering time in *Chrysanthemum morifolium. Pak. J. Bot.*, 48(2): 477-483..

Sarker, R.H., B.M. Mustafa, A. Biswas, S. Mahbub, M. Nahar, R. Hashem and M.I. Hoque. 2003. *In vitro* regeneration in lentil (*Lens culinaris* Medik.). *Plant Tiss. Cult.*, 13(2): 155-163.

Taylor, P., K. Lindbeck, W. Chen and R. Ford. 2007. Lentil diseases. In: (Eds.): Yadav, S.S., D.L. McNeil and P.C. Stevenson, (Ed.) *Lentil: An Ancient Crop for Modern Times*. *Spinger, The Netherland*, pp. 275-289.

Thomas, T.D. 2008. The role of activated charcoal in plant tissue culture. *Biotech. Adv*., 26(6): 618-631.

Williams, D.J. and A. McHughen. 1986. Plant regeneration of the legume *Lens culinaris* Medik (lentil) *in vitro*. *Plant Cell Tiss. Org*., 7(2): 149-153.

(Received for publication 2 September 2016)