EFFECT OF CYTOKININ PRECONDITIONING ON *IN-VITRO* MULTIPLE SHOOT REGENERATION OF LENTIL CULTIVAR

Anil Kumar Chawla, P. Cheena Chawla and Seema Chaudhary*

Chimera Gentec Pvt. Ltd, 34, Knowledge Park 1, Greater Noida, Uttar Pradesh- 201310
Email: puja.smp@gmail.com

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Abstract: This study was aimed to establish a protocol for enhancing shoot proliferation, rooting percentage during the regeneration of lentil cultivar and also to demonstrate that pre-culturing of seedlings stimulates production of multiple shoots from cotyledonary nodes and shoot tips of Lentil cultivar. The highest direct shoot regeneration (79%) with an average of 15-16 shoots/explant were obtained when cotyledonary node explants were excised from seedlings germinated on Murashige and Skoog modified (MSM) media supplemented with benzyl adenine (BAP) 5 mg l^{-1}, and subsequently cultured on MS modified media with 0.5 mgl^{-1} benzyl adenine (BAP) . Pre-culturing of seedlings, at the time of seed germination with high BAP concentration results in fast and multiple shoot regeneration followed by culturing the explants on lower concentration of BAP. For rooting, different concentration of IBA, IAA and NAA were used and highest rooting was recorded on half strength MS medium supplemented with 0.3mg l^{-1} IBA. The rooted plantlets were hardened initially in culture room at 27±2°C and then transferred to *in-vivo* environment. The highly regenerative system developed in the present investigation for this important legume crop could be a useful tool for genetic transformation.

Keywords: Cotyledonary node, *In vitro*, Lentil L-4076, Multiple shoots, Roots regeneration

INTRODUCTION

Lentil is a good source of cholesterol-lowering fiber edible pulse and an essential source of inexpensive protein in many parts of the world, especially in West Asia and the Indian subcontinent, which have large vegetarian populations. Lentils also help in managing blood-sugar disorders since their high fiber content prevents blood sugar levels from rising rapidly after a meal. The low levels (5%) of Readily Digestible Starch (RDS) and high levels (30%) of Slowly Digested Starch (SDS) make lentils of great interest to people with diabetes. Lentil is often a preferred crop in the water deficient areas because of its drought tolerant nature.

Lentil (*Lens culinaris* Medik.) is the third important cold-season food legume, after pea and chickpea grown all over the world in 4.2 million hectare area with yield of 1083Kg/Ha (FAOSTAT 2012), for its high nutritional value (20-36 % protein). Pulse crops, such as lentils, have long been considered to be recalcitrant to cell and tissue culture and are among the most difficult legumes from which to regenerate whole plants due to problems of root induction. The frequency of root formation in lentil is dependent on cultivar and growing medium with supplements. On MS Modified medium, indirect regeneration of lentil was found (Bagheri *et al.*, 2012).

Direct regeneration and multiple shoot formation have been achieved from intact seedling cultures, shoot tips, the first node, and the first pair of leaves in media supplemented with BAP and NAA (Malik & Saxsena 1992; Bajaj & Dhanju 1979; Singh & Raghuvanshi 1989; Khanam 1994; Polanco *et al.*, 1988 and Sarker *et al.*, 2003). Prolific adventitious shoots after the initial callus stage from cotyledonary node using TDZ is reported in lentil (Khawar *et al.*, 2004). Pre-culturing of seedlings with high dose of cytokinins has been reported to improve subsequent regeneration efficiency in various plants, including grain legumes (Gurel *et al.,* 2011; Amutha *et al.*, 2006). Similar results were achieved by (Muhammed *et al.*, 2013) from plumular apices of chickpea using seeds preconditioned with 10mg/l BAP for 10 days on MS medium.

(Khentry *et al.*, 2014) also conducted *in vitro* propagation for six genotypes of lentil (*Lens culinaris* ssp.) Mature seeds were initially cultured on Murashige and Skoog (MS) medium supplemented with 4 mg/1 of benzyladenine (BA). The maximum number of shoots per seed was 4.13+0.33. (Fethi *et al.*, 2014) aimed to develop efficient and reliable protocol for in vitro plant regeneration. Shoot tip, stem, hypocotyl, cotyledon and root as used as explants. The MS medium containing 4 mg/l BAP induced maximum number (8.25) of shoots per shoot tip explant. However, IBA derived shoots were easy to root on MS medium containing 1.87 mg/l NAA but still the rooting percentage is quite low.

The rooting of *in vitro* regenerated shoots present problems in achieving whole plant regeneration systems and there are contradictory reports for rooting in this plant. (Polanco *et al* 1988), used MS medium supplemented with NAA for rooting but the frequency of rooting was low. Similar results were reported by (Khawar and Ozcan 2004), on MS medium containing 0.25 mg l^{-1} IBA, showed root induction with frequency of about 25%. (Tavallaie *et al.*, 2011) evaluated Lentil regeneration by using explants including leaflets, stems, and cotyledons with and without embryo axis. Cotyledon with small part of the embryo axis was the superior explant.

*Corresponding Author

Over 40% of the elongated shoots produced roots in solid 1/4 BS media with 50 µM NAA for 3 days followed by 10 days in a mixture of liquid 1/4 BS, Vermiculite and sand. (Muhammad Aasim 2012) transferred regenerated shoots grown on MS medium containing 0.25 mg/l BAP on MS medium containing 0.25 to 1 mg/l IBA and IAA. The frequency of rooting was unsatisfactory. (Khentry *et al.,* 2014) reported that no root formation was observed on any of the six genotypes cultured on MS medium without the addition of NAA. Two varieties show little response to NAA, with roots formed from single nodes grown on a concentration of 1 mg/l. In rest of the plant unusual root and callus structures were reported. Thus, in this study, an efficient and reproducible protocol was developed for *in vitro* multiple shoot regeneration and rooting of explants in different concentration of cytokinins and Auxins.

MATERIAL AND METHOD

Lentil seeds (L-4076) obtained from IARI, Pulse Laboratory, PUSA, New Delhi. The seeds were surface-sterilized with 70% ethanol for 2 min followed by 0.2% (w/v) aqueous $HgCl_2$ solution for 5 min and finally rinsed 5-6 times with sterilized distilled water. The sterilized seeds were germinated by soaking in sterile distilled water for 16 hrs in the dark on a orbital shaker at 200 rpm, near about 95% seeds germinated. Germinated seedlings were pre-cultured on semisolid MS Modified medium containing BAP (5 mg l^{-1}) up to 2 days at 27±2°C under light conditions for fast germination. Cotyledonary node and shoot tips explants were excised from germinated seedlings and cultured on MS Modified medium supplemented with growth regulators such as BAP in different concentrations. All the cultures were incubated in a culture room at 27 ± 2ºC under a 16/8-hrs light/dark photoperiod. Observations on the induction process were scored after a regular interval.

Multiple shoots (1.5-2 cm) originating from in and around of preconditioned explants region were separated and sub cultured on to fresh media for shoot elongation. The remaining portion of the explant along with shoot buds (< 1 cm) was transferred again on to fresh MSM media supplemented with hormones and used repeatedly up to 2-3 cycles. The effect of basal medium was also assessed by culturing the cotyledonary node and shoot tip explants on MS Modified basal medium (without hormones) containing 3% (w/v) sucrose.

The regenerated shoots (3-4 cm) were rooted on half strength MS medium supplemented with different concentration (0.1, 0.2, 0.3, 0.4 and 0.5) mg/l of IBA, IAA and NAA in test-tubes respectively. All the test-tubes were incubated in a culture room at 27 ± 2ºC under a 16/8-hrs light/dark photoperiod. Each treatment was performed in replications for root regeneration. Observations were recorded and scored after a regular interval.

After 4 weeks, *in vitro* grown rooted plants were removed from the adhering gel, washed thoroughly with tap water to remove the remaining medium and planted to culture boxes containing mixture of soilrite (soil: sand: peat moss) and nursery soil, irrigated with 1/4 MS salt solution at regular interval and covered with the transparent plastic bags (punctured to enable aeration) to avoid desiccation of the plantlets. They were acclimatized in controlled environmental conditions of culture room. After 3-4 weeks, plantlets were transferred in mixture of soilrite and nursery soil in pots and established in *in-vivo* conditions.

RESULT AND DISCUSSION

Two day old explants, excised from pre-cultured seedlings on BAP, were used for multiple shoots formation with different concentrations of BAP (0.25, 0.5, 1.0 and 1.5 mg l^{-1}). Explants were transferred higher (pre-culturing) concentrations to lower concentrations of BAP increased the number of shoots. Explants isolated from normal seedlings were used for 2 to 3 times for the induction of multiple shoots and Maximum number (15-16) of total shoot formation in two to three subcultures was found on 0.5 mg l^{-1}BAP in cotyledonary node (figure.1) and shoot tip (6-7) per explants. About 79 % cotyledonary node and 66 % shoot tips explants developed shoots at this concentration (Table.1).

Table 1. Regeneration of multiple shoots from explants of Lentil (L-4076) on MS Modified medium with different concentrations of BAP and Kinetin.

Explants	MS Modified medium with Supplements BAP (mg l^{-1})	No. of explants inoculated	No. of responsive explants	No. of shoots/explants (Mean value)	% of responsive explants
Shoot Tip	0.00	50	16	1	32
	0.25	80	42	2-3	52.5
	0.5	80	53	6-7	66.25
	1.0	80	45	1-2	56.25
	1.5	80	38	1	47.5
	0.0	50	12	1	24
	0.25	80	51	3-4	63.75

Cotyledonary Node	0.5	80	63	15-16	78.75
	1.0	80	53	4-5	66.25
	1.5	80	46	2-3	57.5

Explants inoculated on basal MS modified medium (without hormone) formed about only 32% shoots and 24% shoots from shoot tips and cotyledonary node explants respectively. Without hormones multiple shoots were not formed, only one shoot developed per explants (Table.1).

The effect of cytokinin to achieve multiple shoot regeneration of lentil (L-40760) cultivar using cotyledonary node and shoot tips explants with different concentration of BAP were evaluated. BAP induces greater multiple shoot regeneration after pre-socking and pre-culturing of seedlings. The percentage of explants regenerating adventitious shoots and the number of shoots per explant were higher when explants were prepared from pre-cultured seedlings. It was observed that better response was obtained when the 16 h old germinated seedlings were pre-cultured on high concentration of BAP before the explants excision. BAP is among the most active cytokinins- like substances and it induces greater in vitro shoot proliferation than many other cytokinins in Lentil plant.

Figure:1. Effect of BAP concentration on cotyledonary node explant

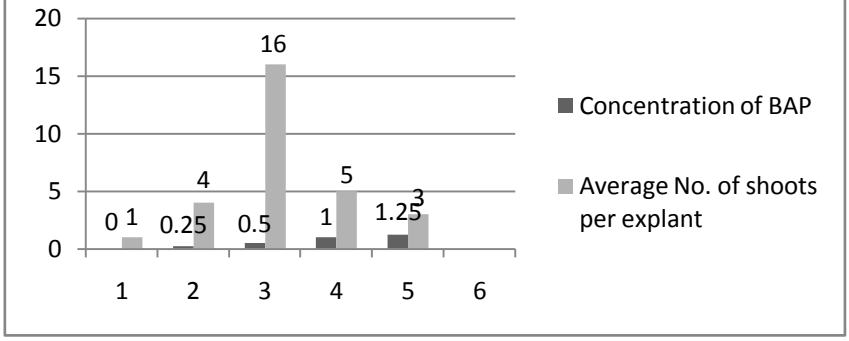
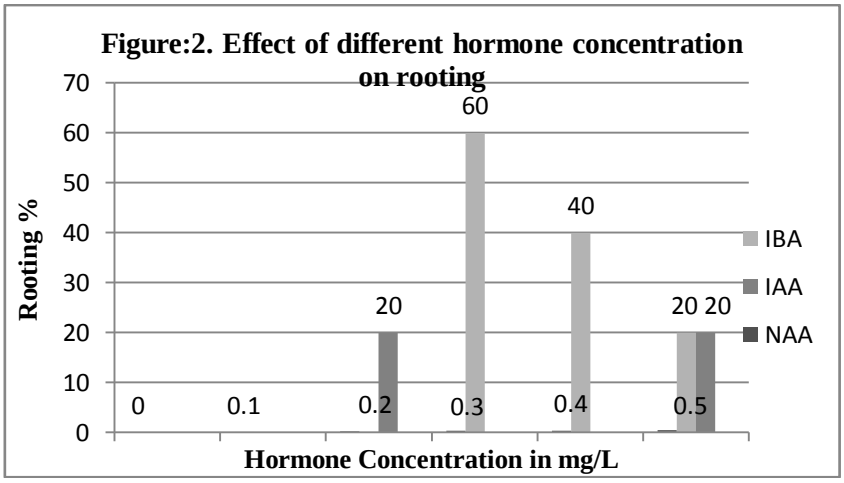


Figure:2. Effect of different hormone concentration on rooting



In vitro rooting is problematic in legumes since previous studies suggests difficulty in rooting of lentil microcuttings (Bajaj and Dhanju, 1979; Singh and Raghuvanshi, 1989; Polanco *et al.,* 1988; Mallick and Rashid, 1989; Malik and Saxena, 1992; Warkentin and McHughen, 1993; Fratini and Ruiz, 2002; Fratini *et al.,* 2003; Sarker *et al.,* 2003; Khawar *et al.,* 2004; Sevimay *et al.,* 2005). For rooting of regenerated shoots half strength MS medium supplemented with different concentration of auxins (IAA, IBA, and NAA) were used. The best rooting percentage, however, was observed on medium containing 0.3mg l^{-1} concentration of IBA, where rooting percentage was 60% followed by 0.4 mg l^{-1} concentration with 40% rooting percentage. Regenerated shoots rooted on medium containing IAA showed very low rooting percentage and there is no response on NAA supplemented medium (Table. 2).

The effect of auxins to achieve our aim to increase rooting percentage of lentil (L-4076) cultivar using regenerated shoots with different concentration of IBA, IAA and NAA were evaluated. IBA at 0.3 mg l^{-1}, 0.4 mg l^{-1} and 0.5 mg l^{-1} concentration induced

rooting in half strength MS medium. The best rooting response was observed on 0.3 mg l^{-1} concentration of IBA with 60% rooting percentage. Similarly, half strength MS medium supplemented with IAA at 0.2 mg l^{-1} and 0.5 mg l^{-1} concentration induced rooting but the rooting percentage was very low as compared to IBA. Thus, IBA is among the most responsive auxins which induce *in vitro* rooting in Lentil plant.

Table 2. Effect of various concentrations of Auxins on root regeneration of Lentil (L-4076) in half strength MS medium.

Treatments	1/2 MS with different concentration of hormone (mg/l)	Number of shoots cultured in medium	Number of roots regenerated in medium	Frequency of rooting (%)
IBA	0.0	20	0	0
	0.1	20	0	0
	0.2	20	0	0
	0.3	20	12	60
	0.4	20	8	40
	0.5	20	4	20
IAA	0.0	20	0	0
	0.1	20	0	0
	0.2	20	4	20
	0.3	20	0	0
	0.4	20	0	0
	0.5	20	4	20
NAA	0.0	20	0	0
	0.1	20	0	0
	0.2	20	0	0
	0.3	20	0	0
	0.4	20	0	0
	0.5	20	0	0

All *in vitro* regenerated plantlets were successfully acclimatized in culture bottles containing mixture of soilrite and nursery soil, irrigated with 1/4 MS salt solution. Plantlets grown in media containing IBA hormone were successfully established under greenhouse condition where they flowered and set seeds but the frequency of whole plant establishment was relatively better (50%) in this study. Earlier (Polanco and Ruiz 1997), studied the inhibitory effect of BAP on *in vitro* and *in vivo* root formation of lentil, concluded that success depends on the kind of cytokinin, its concentration and the time elapsed during shoot formation on these media prior transferring to rooting media. However, the method presented here may be more feasible than others described earlier.

CONCLUSION

In this investigation, we found that cotyledonary node explants were more responsive that shoot tips. Regeneration of many shoots via new meristem organogenesis may provide an opportunity for potentially increasing the number of individuals produced per explant. In the present study, the effect of adding BAP at 5 mg l^{-1} during seedling germination (pre-culturing of the explant) proved to be beneficial for early multiple shoot induction from cotyledonary node. Although there is a report on multiple shoot induction from cotyledonary node explants using TDZ (Amutha *et al.*, 2006; Gurel *et al.*, 2011) and by using BAP hormone (Khentry *et al.*, 2014). The present study highlights the significance of BAP pre-culturing of seedlings which resulted to increase in number of shoots per explants. In rooting of regenerated shoots different combination and concentration of IBA and IAA were used by (Tavallaie *et al.*, 2011; Muhammad Aasim 2012). (Khentry *et al.*, 2014) also reported rooting in six genotypes of lentil with low frequency at 1 mg/l NAA. In our study, the root formation was observed significantly on half strength MS medium containing 0.3mg l^{-1} concentration of IBA followed by 0.4 mg l^{-1} concentration. Increasing hormones concentration decreases the rooting percentage due to inhibitory effect. 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 simple and efficient regeneration system can be adopted for mass propagation and for future genetic transformation studies in this economically important plant.

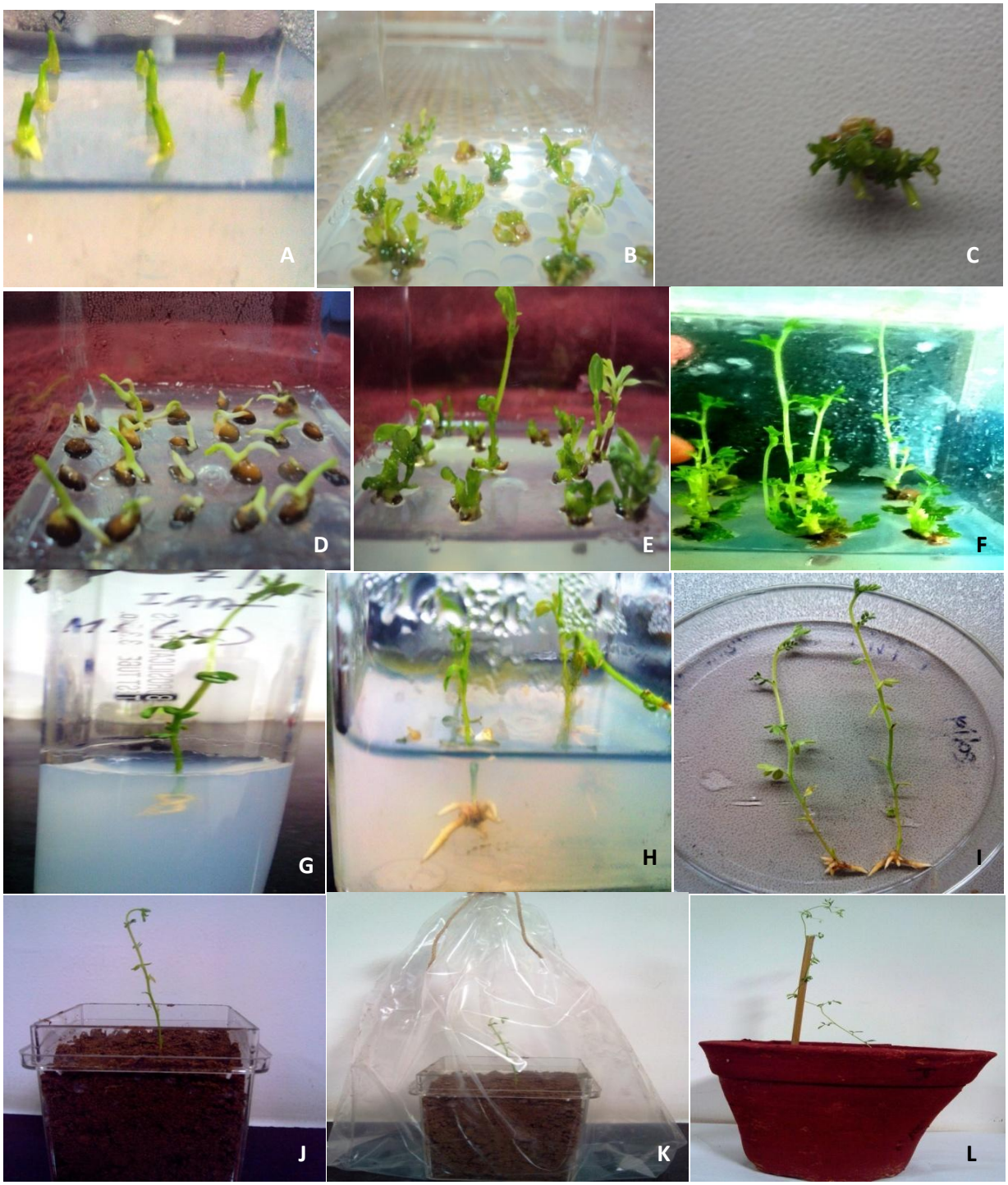


Figure: 3. ***In vitro*** **multiple shoots and roots formation from cotyledonary node of L-4076 on MS medium supplemented with hormones. (A) 2-d old Pre-cultured seedlings on BAP 5 mg l^{-1} (B) Inoculation of explants from and transfer on MSB media with 0.5 mg l^{-1} BAP. (C) Multiple shoots formation from cotyledonary node with 0.5 mg l^{-1} BAP (D) Explant with multiple buds (E-F) Sub-cultured shoot on fresh MSM media supplemented with hormones for elongation (G) Rooting of regenerated shoots in ½ strength MS medium supplemented with 0.5 mg l^{-1} IAA hormone (H) Rooting of regenerated shoots in ½ strength MS medium supplemented with 0.3 mg l^{-1} IBA hormone (I) Regenerated plantlets for establishment in soil (J-K) Establishment of regenerated plantlets in soilrite and nursery soil in** ***in vitro*** **conditions (L) Acclimatization of regenerated plantlets in** ***in vivo*** **conditions.**

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