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Original Article

Hormonal Combinations, Genotypes and Explants Influence Regeneration Efficacy of Lentil

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Abstract *- Lentil is considered a vital vegetable worldwide and impacts the national economy. It ranks among the most nutritious legumes, with its seeds containing over 18% protein. The research focused on callus induction and regeneration ability of five lentil cultivars using various explants. The explants were cultured on MS medium mixture with various concentrations of plant hormones. Callus induction was recorded as 78.89% from cotyledons. Among the cultivars, Binamasur-6 produced the maximum callus (74.45%), while Binamasur-3 gave the maximum number of shoots (23) from the shoot tip. Callus induction was achieved at 72.45% when MS medium contained 1.0 mg/l 2,4-D. MS added with 2.0 mg/l BAP, 2.0 mg/l Kn and 1.0 mg/l NAA produced maximum shoots in Binamasur-3. The regenerated shoots of Binamasur-3 produced roots within 20 days when cultured on MS supplemented with 20 mg/l IBA. The survival rate of seedlings (53.13%) after hardening was highest in Binamasur-6. This study developed a regeneration protocol of lentil via callus production, which will aid in the selection of desired somaclonal variants and support genetic transformation or gene editing endeavors.*

Keywords *- Lentil, Explant, Phytohormone, Callus induction, Somaclone.*

1. Introduction

From an economic standpoint, legumes are considered the second most important crop after Gramineae, accounting for approximately 27% of global crop production (Graham and Vance 2003). In many developing countries, the importance of legumes has increased due to a significant lack of animal protein production and the prevalent issue of protein malnutrition. In Bangladesh, the average daily consumption of pulses is about 12.0 g per capita, which is far below the FAO/WHO recommendation. In particular, those living in poverty often suffer from marasmus and kwashiorkor due to inadequate protein intake (Torun et al. 1981).

Lentil (*Lens culinaris* M.), referred to locally as "Masur," is one of the most essential crops in Bangladesh, covering roughly 1.54% of the total cultivated area. It plays a vital role in human and animal nutrition, and it aids in improving soil fertility (Sarker et al. 2011; Frederick et al. 2006). They can be enjoyed as a main dish, a side dish, or incorporated into salads. The flour made from lentils is used in the preparation of soups, stews, and purees, and it can be mixed with grains to create bread and cakes; it is also appropriate for baby food (Muehlbauer et al. 1985). Lentils are abundant in vital amino acids such as lysine, arginine, leucine, and sulfur-containing amino acids, in addition to carbohydrates, calories, fiber, vitamin A, calcium, starch, iron, phosphorus, copper, and manganese. Nevertheless, progress in the enhancement of lentils through traditional breeding techniques (hybridization and selection) is hindered by a lack of genetic diversity, mainly due to high rates of self-pollination and the unavailability of desirable resistance genes in the existing breeding germplasm.

The process of crossing lentils is difficult because of the small flower size, and hybridization poses challenges due to asynchronous flowering (Muehlbauer et al. 1980). Genetic variation aimed at improving any crop can be attained through recombination of allelic variation or by gene mutations. Nevertheless, all attempts to create genetic variability in lentils through introduction, selection, hybridization, and mutation have not yielded the desired results. In addition to sexual breeding methods, there are various strategies for generating variability, such as inducing somaclonal variation through tissue culture, somatic hybridization, and genetic engineering. The tissue culture technique can greatly enhance genetic variability by inducing

somaclonal variation or mutation (via radiation or chemical mutagens) at an unexpectedly high rate, potentially providing novel sources of genetic variability in different plant species (Saxena and King 1987).

Somaclonal variation is a quick and dependable method for enhancing target traits of a crop (Mroginski and Kartha 1984). Selection of somaclonal variants is a powerful tool for selecting target plants with improved traits (Rubiales et al. 2015). The *in vitro* regeneration of callus culture possesses the capability to induce both genetic and epigenetic changes in the regenerated plants. As a result, improving this crop through somaclonal variation is essential for achieving the desired performance. Establishing a dependable and reproducible regeneration protocol is one of the most critical criteria for the genetic modification or gene editing of any crop. A limited number of effective regeneration protocols have been reported for lentils (Polowick and Yan 2023). Therefore, it is crucial to enhance this crop through somaclonal variations and mutations to achieve the desired performance.

Establishing a plant regeneration protocol from explants is crucial for harnessing advancements in genetic transformation (Williams and Mchughen 1986). The regeneration process from callus also provides the opportunity to select somaclonal variants in lentil (Altaf et al. 1999, Sultana et al. 2012). Furthermore, direct regeneration techniques in lentil were pointed out by Sarker et al. (2003) and Sarker et al. (2012). However, all of the developed protocols are specific to certain genotypes. Despite numerous reports being available on lentil regeneration protocols, regeneration through callusing has yet to be established. Therefore, this experiment was taken to develop an easy and reproducible method for indirect *in vitro* shoot and root regeneration of lentils utilizing different explants and hormone concentrations, particularly with the advanced lentil cultivars released by the Bangladesh Institute on Nuclear Agriculture (BINA).

2. Materials and Methods

2.1. Plant Materials

Five advanced varieties, namely Binamasur-1, Binamasur-2, Binamasur-3, Binamasur-5 and Binamasur-6, were selected as a source of explants. Seeds weighing fifty grams from each variety were obtained from the BINA seed gene bank upon request.

2.2. Explant preparation

Cotyledons, hypocotyls, epicotyls, shoot tip and embryo were collected from the plants of the varieties that were grown in axenic culture. For axenic culture, 100 seeds of each were taken and thoroughly washed with distilled water in a 50 ml beaker. The seeds that were submerged were used for axenic culture. The selected seeds were then surface sterilized in two steps. At first, 70% ethanol and a drop of Tween 20 were added for surface sterilization of seeds. The seeds were agitated continuously for 5 min with a sterile glass rod. The seeds were dipped in 0.1% Hg_2Cl_2 for 5 min for sterilization, subsequently 3 times with sterile water. Surface-sterilized seeds were dried on a sterile filter for 10 min. Sterilization was conducted inside a laminar air flow cabinet. Twenty sterile seeds were placed for germination on half-strength MS (Murashige and Skoog, 1962) medium. The cultures were kept in incubation, maintained at 26 °C, 60% RH and 14h light conditions. After 10-12 days of incubation, sterile plants with 4-5 cm long were used as sources of explants for the experiment.

2.3. Callus Induction and Somatic Embryogenesis

Explants were dissected and cut into 2-3 mm pieces with an aseptic scalpel. The pieces of the plant parts were transplanted onto MS medium, which had the mixture of 0.5 mg/l Kn + 2 mg/l BAP, 0.5 mg/l Kn + 1 mg/l 2,4-D, 1.0 mg/l BAP + 1.0 mg/l NAA and 1.0 mg/l 2,4-D.

2.4. Plantlet and Root Regeneration

Proliferic calli were developed within 4-6 weeks and were divided and sub-cultured on the shooting medium for shoot induction. The culture was placed at 25 ± 2 °C under 16 h light conditions. The regenerated tiny plants were separated from the mass of regenerants and sub-cultured on a rooting medium. Then, the plantlets with abundant and healthy roots were transferred to soil.

3. Results and Discussion

3.1. Effect of Hormone, Explants and Genotypes on Callus Induction

A suitable protocol for callus induction of lentil was developed to determine the effectiveness of callus induction from different hormonal combinations, explants, and genotypes. Callus formation was noted within a culture duration of 20 - 40 days, leading to a significant mass of callus. The highest induction rate (91.67%) was observed in the cotyledon of the Binamasur-6 genotype, followed by the epicotyl and hypocotyl of the same genotype (see Table 1 and Plate 1a-c). Regarding hormonal combinations, the maximum callus (97.78%) was attained with 1.0 mg/l 2,4-D in the Binamasur-6 genotype.

Polanco et al. (1988) similarly noted callus formation in all media containing 2,4-D in their research. Among the explants, the cotyledon generated the largest quantity of callus, followed by the epicotyl, while the excised embryo showed the least callus induction (refer to Table 1). The Binamasur-6 genotype produced the highest callus percentage (74.44%), followed by Binamasur-2, Binamasur-3, and Binamasur-5, with Binamasur-1 exhibiting the lowest results. These results clearly demonstrate that the efficiency of callusing is affected by the genotype, hormonal combinations, and the types of explants utilized.

De Jong et al. (1993) and Parrot et al. (1993) suggested that various explants could be utilized for the production of somatic embryos. Immature zygotic embryos are utilized as an important source for callusing rather than somatic embryo formation. Welander (1988), Fasolou et al. (1989), Yepes et al. (1994), and Pierik (1993) observed that younger plant parts displayed higher morphogenic potential. Callus induction was the highest from matured embryos cultured on MS medium added with 4 mg/l 2,4-D and 1 mg/l NAA (Turhan and Base 2004). Bagheri et al. (2012) examined the indirect regeneration potential of lentil plants using 13 dosages of phytohormones and 4 explants. One mg/l Naphthalene Acetic Acid (NAA) and 1 mg/l zeatin in MS medium showed optimal callus development.

3.2. Determination of Potentials of Hormonal Combinations, Explants and Genotypes in Shoot Development

The calli placed on MS medium began to develop somatic embryos within 15 - 20 days and started to produce shoots after 25 - 35 days. Shoot explants of Binamasur-3 produced the maximum number of shoots (23), followed by Binamasur-5 and Binamasur-6 from the same explant (Plate 1d-f). The excised mature embryo explant resulted in the lowest shoot initiation across all genotypes (Table 2 and Figure 1). Various hormonal combinations were tested for shoot induction, with the maximum number of shoots (104) being achieved from the MS medium supplemented with 2.0 mg/l BAP + 2.0 mg/l Kn + 1.0 mg/l NAA (Table 2). Among the various genotypes, Binamasur-3 demonstrated superior regeneration capabilities compared to the others. Polanco et al. (1988) documented the formation of shoots in lentil from shoot tips cultured on MS medium enriched with benzyl aminopurine (BAP) and NAA. Whereas Gulati et al. (2001) successfully induced shoots from different explants cultured on MS added with only BAP. Khanam (1994) accomplished shoot regeneration on MS medium containing 0.5 mg/l BAP, 100 mg/l Casein hydrolysate (CH), 0.5 mg/l Kn, and 0.2 mg/l NAA. The shoot tip explant might produce the maximum number of shoots as they are the meristematic tissue. Özcan et al. (1993) also achieved maximum plant regeneration on MS medium modified with BAP + Kinetin + a low concentration of NAA. Küplemez and Yıldırım (2020) evaluated the effects of cytokinins and auxins on the vascular tissues of the lentil and reported that lentil seedlings treated with BAP (1 mg/l) enhanced regeneration capacity.

3.3. Potentials of IBA Concentration, Explants and Genotypes in Root Induction in Lentil

Rooting from *in vitro* regenerated lentil shoots has proven to be a difficult task, marked by a low rooting frequency (Williams and McHughen 1986; Ye et al. 2002; Khawar et al. 2004; Sarker et al. 2003). Among the genotypes, Binamasur-3 produced the highest number of roots (65) when excised shoots were cultured on MS medium supplemented with 20 mg/l IBA, followed by Binamasur-6, Binamasur-5, and Binamasur-2 (Table 3, Plate 1g, h). In contrast, Binamasur-1 yielded the fewest roots and faced difficulties in developing complete plantlets. Root induction was decreased when using BAP, and/or BAP with NAA in the medium at any stage of plant regeneration. Küplemez and Yıldırım (2020) reported that BAP and NAA-treated shoots are hindered from rooting due to damage to root vascular tissue. The regenerated shoots achieved the highest root growth for developing strong and healthy plantlets. Conversely, the regenerated shoots from the cotyledon and hypocotyl showed limited root development, with some failing to produce any roots at all. A concentration of 20 mg/l IBA led to the development of numerous roots within 18 - 38 days post-subculture. Root growth was influenced by genotypes, explants, and the concentration of IBA. Binamasur-3 gave root formation within 18 days after subculture on MS medium with 20 mg/l IBA. Meanwhile, Binamasur-2 took 50 days to develop a root (Figure 2).

Interestingly, the development of roots in regenerated shoots exhibited a diminished potential at lower and higher concentrations of IBA compared to the 20 mg/l level. Sarker et al. (2003) reported that the acquisition of roots in lentil using a higher concentration of IBA, which agreed with the findings of the current study. Wiesman et al. (1988) reported that IBA was significantly more effective in promoting the formation of adventitious roots than IAA. Current findings are consistent with the observations made by De Klerk (1999), who recognized that IBA is the most potent auxin for rhizogenesis.

3.4. Transplantation, acclimatization and establishment of plantlets

The overall success of tissue culture depends on the adaptation potential of the transferred plants to soil. Plantlets with complete shoot and root systems were taken away from the culture vessels. Subsequently, plantlets were transferred into plastic pots within a growth chamber to facilitate proper hardening. Hardened plantlets were moved to earthen pots filled with a mixture of soil: sand: vermiculite (1:1:1, Plate 1i). Initially, a high-humidity environment was maintained by covering the seedlings with a plastic bag and periodically spraying water, gradually acclimatizing them to ambient conditions by increasing the number of perforations in the covered polythene bag. A similar hardening method was utilized for chickpea by Chaturvedi and Chand (2001). Survival rates of the plantlets were 0%, 39.29%, 39.13%, 37.04%, and 53.13% for Binamasur-1, Binamasur-2, Binamasur-3, Binamasur-5, and Binamasur-6, respectively. Sultana et al. (2016) reported a 70% survival rate of the plants maintained in the pot mixture of perlite, vermiculite, and peat moss in a 1:1:1 ratio. Plantlets were continued to grow until harvesting of the seeds for phenotypic characterization.

Table 1. Callus induction (%) potentiality of the genotypes and explants of the lentil cultured onto MS with different hormones

Genotypes	Explants	Hormonal combinations				Mean	Average
		0.5 mg/l Kn + 2 mg/l BAP	0.5 mg/l Kn + 1 mg/l 2,4-D	1.0 mg/l BAP + 1.0 mg/l NAA	1.0 mg/l 2,4-D		
Binamasur-1	Cotyledon	77.78	66.67	66.67	77.78	72.23	55
	Epicotyl	44.44	55.56	66.67	55.56	55.56	
	Hypocotyl	66.67	55.56	44.44	44.44	52.78	
	Shoot tip	44.44	44.44	44.44	66.67	49.99	
	Embryo	33.33	33.33	44.44	66.67	44.44	
Average		53.33	51.11	53.33	62.22		
Binamasur-2	Cotyledon	88.89	66.67	77.78	88.89	80.56	63.34
	Epicotyl	55.56	55.56	66.67	66.67	61.12	
	Hypocotyl	66.67	66.67	66.67	66.67	66.67	
	Shoot tip	55.56	66.67	55.56	55.56	58.34	
	Embryo	55.56	44.44	44.44	55.56	50.00	
Average		64.45	60.00	62.22	66.67		
Binamasur-3	Cotyledon	77.78	88.89	66.67	88.89	80.56	62.22
	Epicotyl	66.67	66.67	66.67	66.67	66.67	
	Hypocotyl	66.67	55.56	44.44	77.78	61.11	
	Shoot tip	44.44	66.67	44.44	66.67	55.56	
	Embryo	44.44	55.56	33.33	55.56	47.22	
Average		60.00	66.67	51.11	71.11		
Binamasur-5	Cotyledon	77.78	55.56	66.67	77.78	69.45	59.45
	Epicotyl	55.56	66.67	55.56	66.67	61.12	
	Hypocotyl	66.67	55.56	55.56	44.44	55.56	
	Shoot tip	44.44	55.56	66.67	77.78	61.12	
	Embryo	33.33	55.56	55.56	55.56	50.01	
Average		55.56	57.78	60.00	64.45		
Binamasur-6	Cotyledon	88.89	88.89	88.89	100	91.67	74.44
	Epicotyl	77.78	88.89	77.78	100	86.11	
	Hypocotyl	66.67	66.67	77.78	100	77.78	
	Shoot tip	55.56	55.56	55.56	100	66.67	
	Embryo	33.33	44.44	33.33	88.89	49.99	
Average		64.45	68.89	66.67	97.78		

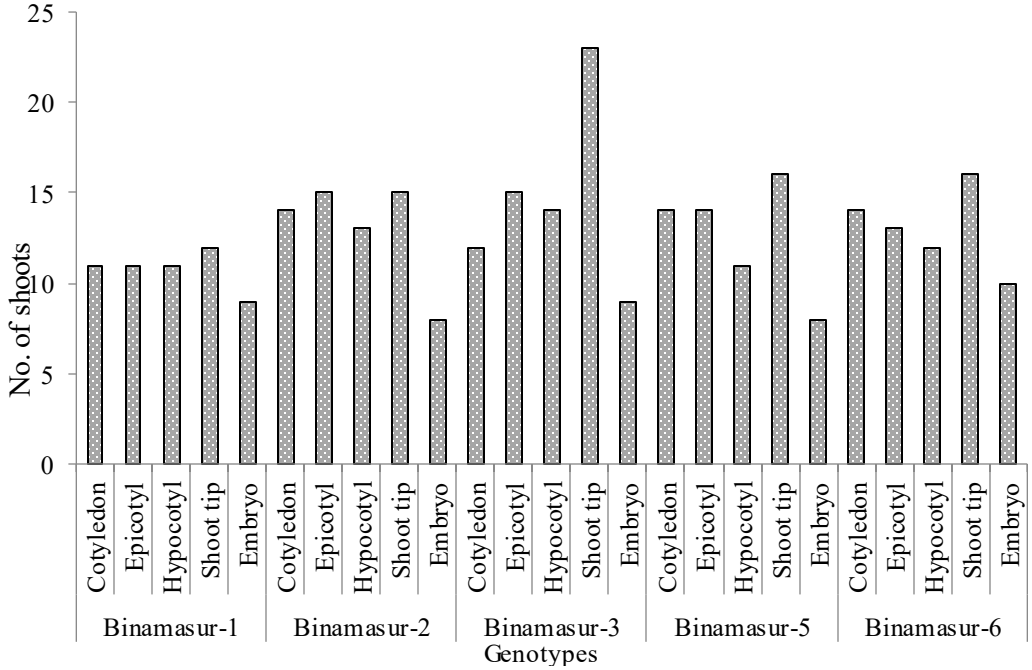

Fig. 1 Effect of genotypes on the efficacy of shooting of the explants.

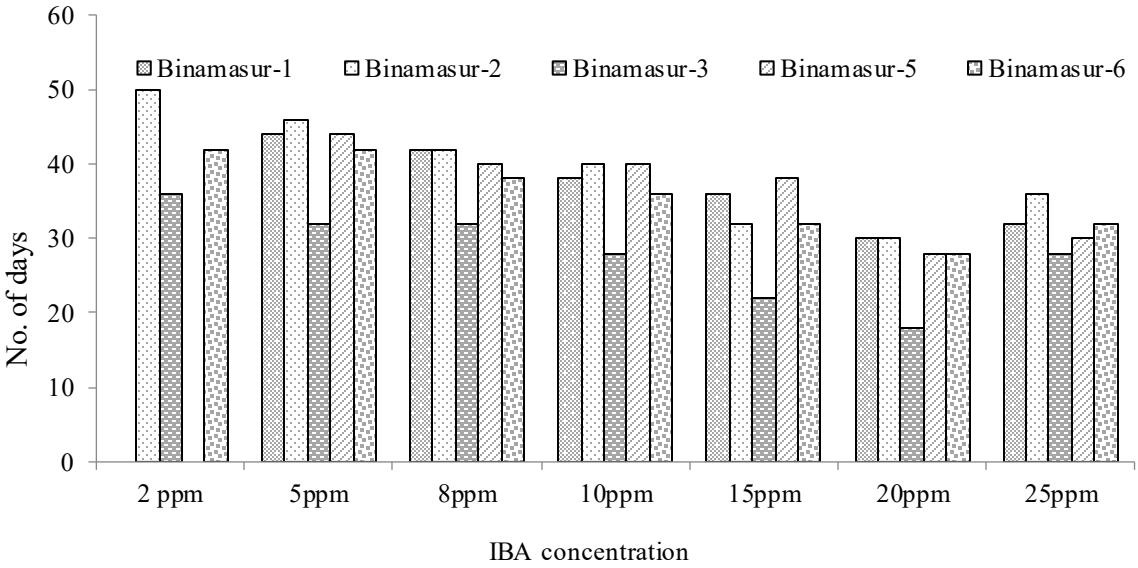

Fig. 2 Effect of hormonal combination and varieties on days required for root development in regenerated shoots

4. Conclusion

Lentils are widely recognized for their challenges in regeneration and rooting via tissue culture. A protocol was established to generate multiple shoots via callus induction utilizing five distinct explants derived from five lentil genotypes, thereby enabling the potential for somaclonal variation. The results of this investigation reveal that the most significant callus production was observed from cotyledon, epicotyl, hypocotyl, and shoot tip cultured on MS medium containing 1.0 mg/l 2,4-D. The ideal conditions for shoot regeneration were realized by transferring the calli to a medium that had 2.0 mg/l BAP, 2.0 mg/l Kn, and 1.0 mg/l NAA. In addition, 20 mg/l IBA facilitates the rooting of the regenerated shoots. This protocol is applicable for further research endeavors, including studies on genetic transformation and somaclonal variation, and is expected to support the breeding and genetic transformation of additional lentil varieties in the future.

Table 2. Effect of hormonal compositions (mg/l) on shoot regeneration from explants of lentil genotypes

Genotypes	Explants	2.0 BAP + 2.0 Kn + 1.0 NAA	2.0 BAP + 3.0 Kn	2.0 BAP + 1.0 NAA	2.0 BAP	Total
Binamasur -1	Cotyledon	3	2	3	3	11
	Epicotyl	4	3	2	2	11
	Hypocotyl	3	3	2	3	11
	Shoot tip	4	2	3	3	12
	Embryo	2	2	2	3	9
Average		3.2	2.4	2.4	2.8	
Binamasur -2	Cotyledon	5	4	2	3	14
	Epicotyl	5	3	3	4	15
	Hypocotyl	4	3	3	3	13
	Shoot tip	5	4	2	4	15
	Embryo	2	2	2	2	8
Average		4.2	3.2	2.4	3.2	
Binamasur -3	Cotyledon	4	4	2	2	12
	Epicotyl	4	5	3	3	15
	Hypocotyl	6	4	2	2	14
	Shoot tip	9	5	5	4	23
	Embryo	3	2	2	2	9
Average		5.2	4.0	2.8	2.6	
	Cotyledon	5	4	3	2	14
	Epicotyl	4	3	4	3	14

Binamasur -5	**Hypocotyl**	3	3	3	2	11
	Shoot tip	5	4	3	4	16
	Embryo	3	1	2	2	8
Average		4.0	3.0	3.0	2.6	
Binamasur -6	**Cotyledon**	5	4	2	3	14
	Epicotyl	4	4	3	2	14
	Hypocotyl	4	3	2	3	12
	Shoot tip	5	4	3	4	16
	Embryo	3	2	2	3	10
Average		4.2	3.4	2.4	3.0	

Table 3. Effect of IBA concentration on root induction of the shoots derived from explants of the genotypes

Genotypes	**Explants**	**IBA**						
		2 mg/l	**5 mg/l**	**8 mg/l**	**10 mg/l**	**15 mg/l**	**20 mg/l**	**25 mg/l**
Binamasur-1	Cotyledon	0	0	1	2	3	6	3
	Epicotyl	0	0	1	1	3	9	8
	Hypocotyl	0	0	2	2	4	5	3
	Shoot tip	0	0	2	7	16	26	8
	Embryo	0	0	0	0	2	2	3
Average		0.0	0.0	1.2	2.4	5.6	9.6	5.0
Binamasur-2	Cotyledon	1	2	4	4	8	8	4
	Epicotyl	0	0	3	6	11	15	3
	Hypocotyl	0	0	0	0	9	11	2
	Shoot tip	0	1	4	11	25	46	13
	Embryo	0	0	0	0	1	4	0
Average		0.2	0.6	2.2	3.0	10.8	17.2	4.4
Binamasur-3	Cotyledon	2	2	4	5	9	18	3
	Epicotyl	0	0	6	4	4	10	6
	Hypocotyl	0	0	3	3	4	9	3
	Shoot tip	2	5	16	16	32	65	22
	Embryo	0	0	2	2	5	6	5
Average		0.8	1.4	6.2	6.0	10.8	21.6	7.8
Binamasur-5	Cotyledon	0	2	4	4	10	14	2
	Epicotyl	0	0	5	6	14	18	0
	Hypocotyl	0	1	3	2	9	13	0
	Shoot tip	0	2	6	12	20	47	11
	Embryo	0	0	3	2	3	8	0
Average		0.0	1.0	4.2	5.2	11.2	20.0	2.6
Binamasur-6	Cotyledon	1	3	3	5	12	22	3
	Epicotyl	0	6	5	6	15	19	7
	Hypocotyl	0	4	8	6	11	11	6
	Shoot tip	1	4	4	8	20	52	10
	Embryo	0	0	2	2	3	7	1
Average		0.4	3.4	4.4	5.4	12.2	22.2	5.4

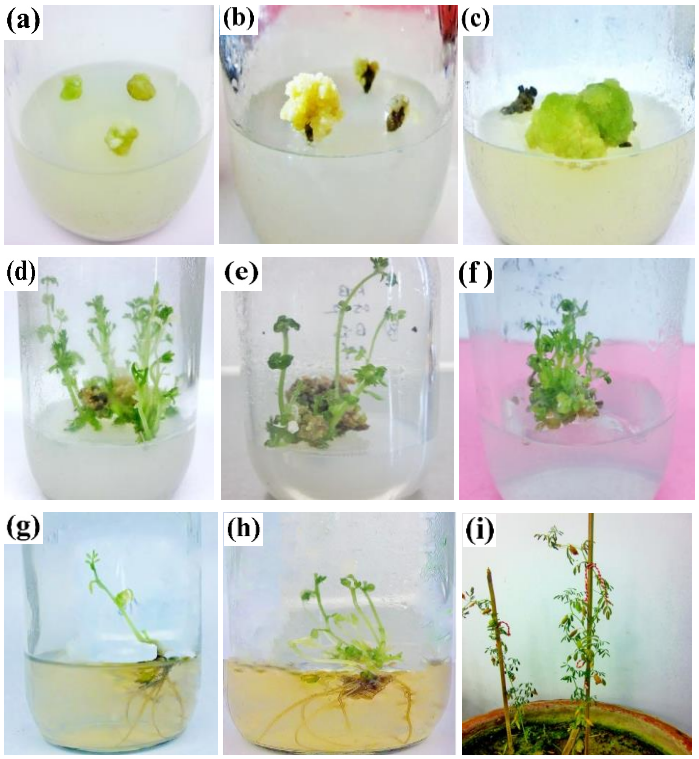

Plate 1. Callus induction to established plants of *in vitro* cultured lentil genotypes, a) callus induction in cotyledon explant; b) callus induction in shoot tip explant; c) callus induction in hypocotyl; d) initiation of shoots from callus of shoot tip; e) initiation of shoot from callus of epicotyl; f) initiation of shoot from callus of cotyledon; g) poor root induction from the regenerated shoots; h) profuse roots from regenerated shoot; i) established plants

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