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SEED VARIABILITY IN CALLUS REGENERATED PLANTS OF LENTIL (*LENS CULINARIS* MEDIK.)

Nafees Altaf

Nuclear Institute for Agriculture & Biology, P.O. Box. 128 Faisalabad, Pakistan
E.mail: nafeesdcs@yahoo.com Tel: 92 -41-2650316, Fax: 92-41-2654213

ABSTRACT

Microsperma lentil is an important grain legume of Pakistan. This detailed study tells the seed variability in size and color from callus regenerated plants of lentil cv. Masoor-85. Cotyledonary nodes with cotyledons from germinating seedlings were promising explants especially with scrapping of axillary portions of basal nodes that considerably enhanced callus and regenerations in MS medium. These developed callus and shoot primordia in the presence of BA (5 mg l^{-1}) and 3% fructose. Cotyledonary nodes from germinating seeds produced healthy calli in dark conditions in K (10 mg l^{-1}) + GA (1 mg l^{-1}). The maximum callus mass gave maximum regenerations in light in the same medium. The number of elongated shoots were very low as compared to the green dots that appear on the callus surface. Bud dormancy was a problem. Auxins had stimulatory effects on rooting. However, auxins were removed for root elongation. Four days exposure of auxins IBA 0.008% + NAA 0.002% in simple talc dusted to shoot bases seemed optimum for rooting. Rooting speeded up by high humidity. There was no or poor rooting in continuous dark. General health of shoots also influenced rooting. Removal of leaves had adverse effects and active shoot growth promoted rooting. Basal stem in regenerated shoot had maximum rooting potential. Polarity requirement were necessary. The survival rate of callus regenerated plants (R_0) in the field was 12.1%. Variability was observed in R_0, R_1, R_2, R_3 and R_4 as compared to control Masoor-85 in vegetative and yield characteristics. A plant was selected in R_2 for large seed size with lighter seed color as compared to control and the stability of seed characters was observed till R_4. It was the most important seed clone of callus regeneration. This study clearly indicated the importance of somaclonal variation for improvement of lentil cultivar.

Keywords: Lentil, Explant Culture, Callus, adventitious rooting, regenerated plants, somaclonal variation,.

INTRODUCTION

Lentil is a cold season annual legume. Winter hardiness appeared to be under polygenic control with additive loci (Kahraman *et al*., 2004). Lentil is the second major pulse crop after chickpea in Pakistan. Lentil is grown on 43.4 hectares with 26 tones production. The average yield is 597 kg hectare^{-1} (*MINFAL. 2004-2005). Lentils are subdivided into micro-sperma and macro-sperma on the basis of seed size. Micro-sperma is very well adapted in Pakistan but is not in demand on the world market as compared to macro-sperma which has a very low adaptability in our conditions. Lentil genotypes varied significantly for yield due to genotype X environment interactions (Sabaghina *et al*., 2006).

A number of factors are responsible for low yield. Among these are production cost, domestic lentil prices and annual rainfall (as lentil is grown mainly as a rain fed crop) are important factors. There is potential to increase yields by utilizing germplasm that is adapted to rainfed conditions especially maximum root length at different growth phases (Shrestha *et al*. 2004). Lentil has weak stems and the plant lodges at full maturity which results in grain and straw losses. Lentil crops is highly infested with different kinds of winter weeds with a reduction in grain yield as lentil is slow growing as compared to weeds. Losses due to pathogens and insects also cannot be ignored.

The broad objective of this study was the improvement of micro-sperma lentil which is a continuous effort within a limited gene pool of species. The improvement can be achieved in particular by variation recovered through the regeneration of plants from cell and tissue culture which have come to be seen as a possible path for the isolation of new variants. The regenerations will enhance and release somatic variability in plants. Very little is known about lentil cell and tissue culture especially micro-sperma lentils. This study will help to understand various aspects of lentil organogenesis, embryogenesis leading to plant formation and to study soma clones. The specific objective were to develop a system for *in vitro* culture of lentil tissues leading to the recovery of regenerated shoots with high frequency, to optimize the conditions influencing the establishment of plants in the soil and to compare the characters of regenerated plants with control seed grown populations and select somaclonal variants if any.

* Ministry of Food Agriculture & Live Stock

MATERIALS AND METHODS

Healthy seed of Masoor-85 were used to raise seedlings. Seeds were sterilized in 0.5% $HgCl_2$ for 20 minutes or NaOCl (7%) for 30 minutes. Disinfected germinated seedlings were a source of explants. The explants were gently removed and cultivated immediately on MS agar medium (Murashige and Skoog, 1962). The additives in MS medium tried for callus, shoots and rooting were: Kinetin (K), Benzyladenine (BA), Gibberrelic acid (GA), 2-4 Dichlorophenoxy acetic acid (2, 4-D), Zeatin (Z), Abscissic acid (Ab), Adenine sulfate (AS), were used in the study. For rooting mostly Indole butyric acid (IBA), Naphthalene acetic acid (NAA) and Indole acetic acid (IAA) were tried. In addition glutamine, proline, glutamic acid, mannitol, fructose, sucrose, maltose, lentil seed extract, yeast extract, malt extract were tried. Both light and dark conditions were tested for explants culturing at 18 – 25°C. A detailed study of lentil explants in MS medium with growth regulators and other additive was made and the best explants response in the specific culture medium is recorded in Table 1.

Table 1. Details of photographic presentation (Fig.1-15) of lentil callus, shoots, roots, field plants and selected clones.

Explants/ material	Growth medium	Morphogenic response	Figures
Cotyledonary Node (C.N.)	MS + Kinetin (10 mg l^{-1}) +GA (1 mg l^{-1}) in dark.	Callus	1
C.N. With cotyledons	MS+ BA (5 mg l^{-1}) +Glutamine (5 mg l^{-1}) +Fructose 3%	Callus + Shoot primordial	2
Callus	MS + Kinetin (10 mg l^{-1}) + GA (1 mg l^{-1})	Multiple shoots in callus	3
Callus	K (10 mg l^{-1}) + GA (1 mg l^{-1}) + Glutamic acid (2 mg l^{-1})	Green globular structures in callus	4
C.N. with 1/6th Cotyledons	K (10 mg l^{-1}) + GA(1 mg l^{-1}) + 10% seed extract	Shoots in callus	5
Shoot apices	MS + 2,4-D (0.5 mg l^{-1}) + BA (5 mg l^{-1})	Callus at the base + shoot growth	6
Shoots in C.N. Callus	MS +BA (5 mg l^{-1}) + Glutamic acid (10 mg l^{-1}) + seed extract 5%	Shoots + roots from callus	7
Shoots from callus	MS +IBA (1 mg l^{-1})	Rooting in shoots	8
Shoots	MS+IBA (1 mg l^{-1})	Rooting at the base of shoots	9
2 week old regenerated shoots	Bases cut and dusted with (IBA .008% + NAA .002% in simple talc) and shoots grown in peat	Rooting at the base	10
R_1 progeny	In field	Plants growing	11
Small seeded clone	In field	Selected in R4	12
Large seeded clone	In field	Selected in R4	13
Different large seeded strains	In field	Selected in R4	14
Different small seeded clones	In field	Selected in R4	15

RESULTS

Growth responses of explants from 3 – 4 day old germinating seedlings were good as compared to that of 1 – 4 week older seedlings. The most promising explant was scrapping of axillary portions of basal nodes for enhanced regeneration. Cotyledonary node with 1/6th cotyledon section attached gave better response towards callusing and regeneration. However stem segment with node gave better response and shoot apex of germinating seedling had vigorous callus growth. The basal nodes and the region adjacent to it had the maximum cell division capacity especially if the explants were from the germinating seedlings. These developed callus and shoot primordia in the presence of BA and fructose. These cultures continued shoot regenerations for 3 subcultures. Benzyl adenine and K both promoted multiple shoot formation. Kinetin and GA gave more shoots. Kinetin (10 mgs l^{-1}) + GA (1 mg l^{-1}) induced callus in explants of shoot apex, epicotyls stem and cotyledonary node from the germinating seedlings in

dark. Among the sugars tested, sucrose was the best for callusing and the callus started regenerations in light initially as green dots. Later these dots developed into shoot buds and elongated shoots. The texture of callus was important.

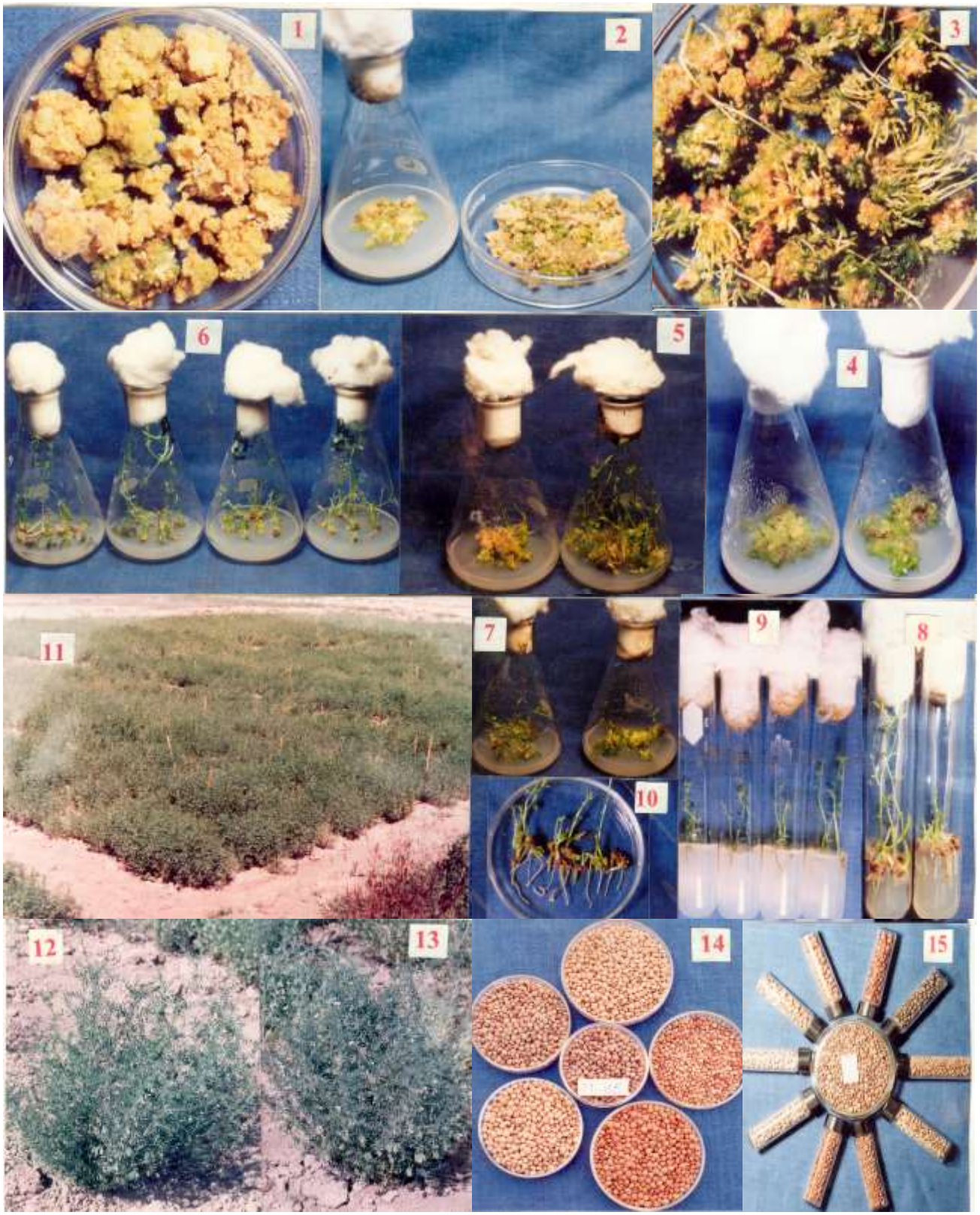

Fig. 1-15. Photgraphic presentation of Lentil callus, shoots, roots, field plants and selected clones.

The maximum proliferative callus mass gave maximum regenerations irrespective of the sub culturing passage. The maximum callus surface area regenerated in about one month time. Once callus formed, it was maintained up to one year by sub culturing. The addition of seed extract to K and GA medium considerably enhanced regenerations. Bud dormancy was a problem. The number of elongated shoots was very low as compared to the green dots that appeared on the callus surface. Elongation of the shoot did not depend on the original explants type. If the basal nodes were cut along with $1/6^{th}$ cotyledonary portion and incubated in K + GA medium, they produced more callusing as compared to the callus obtained from the explants of germinating seedling. Since the callus mass was more, the regenerations were more. This regeneration procedure was efficient. There were globular to torpedo shaped embryos in calli.

Meristem multiplication, shoot development, shoot buds from callus and adventitious rooting, all processes required growth regulator exposures for at least some minimum period. Auxins had stimulatory effect on rooting. However, auxins were removed for root elongation. IBA and NAA were important for rooting process. Four days exposure of auxins IBA 0.008% + NAA 0.002% to shoot bases seemed optimum for rooting. Rooting speeded up by high humidity. Maintenance of humidity was essential. There was no rooting or poor rooting in continuous dark. General health of shoots also influenced rooting. Removal of leaves had adverse effects and active shoot growth promoted rooting. Two to three week old shoot growth was optimum for rooting. Cuttings from plants especially ready to flower or from adult plants were extremely difficult for rooting. The basal stem portion had the maximum rooting potential and this distance from basal portion decreased rooting capability. Polarity requirements were necessary. Filter Paper Bridge was a better system for root induction as compared to the peat and soil types. Boron (20 – 30 mg l^{-1}) and MS Salts ($1/100^{th}$ strength) had positive effect on rooting. In field, low night temperatures (15-20^0C) favored rooting. The survival rate of regenerated plants (R_o) in the field was low (12.2%). Progenies of R_0, R_1, R_2, and R_4 along with Masoor-85 were studied in the field for vegetative and yield characteristics. Soma clones were selected for erect plant type, best physiological maturity at harvest time. Selections were made for larger seed size and lighter color of seeds as compared to Masoor-85 seeds size and color. The stability of large seed size and lighter seed color was confirmed and this was the most important somaclonal variant. However, some of the mature pods shatter before full physiological maturity. Seed and seedling characteristics were associated with seed mass of somaclones. The seedling weight and number of leaflets were higher from large seed as compared to the control.

DISCUSSION

Microsperma lentil has full capability of explant callusing, shoot regenerations, adventitious rooting of regenerated shoots and producing seed variability from callus regenerated populations. Large seed size and higher seed mass provided healthy seedlings. In legumes, young meristematic tissues such as immature embryos and developing leaves have been used for obtaining somatic embryos (Imin *et al*., 2005). Mostly shoot buds are induced (Shahzad *et al*., 2007). The origin of shoot buds in our study as green dots in callus has been also reported in Glycine max (Tripathi, & Tiwari, 2005). One to three days after pollination, pods of *Phaseolus* species were cultured with maximum plant regeneration as 30% (Schryer *et al*., 2005). Growth regulators have a positive response on plant growth in green gram (Aurovinda and Rajendra, 2004). Cotyledonary nodes were best explants for multiple shoot formation in four lentil varieties of Bangladesh (Sarker *et al*., 2003).They also found BA, K, GA and IBA workable hormones in lentil regenerations. Thiadiazuron was most active cytokinin for *in vitro* shoot proliferation in lentil cultures. They also found that root formation and elongation are controlled by interactions between multiple factors and suspected that in lentil higher levels of IBA resulted in dormancy (Khawar *et al*., 2004). Thiadiazuron induced prolific shoot regeneration in mungbean cotyledonary node and hypocotyl explants (Amutha *et al*., 2006). The success of micrografting in several cultivars of lentil was independent of nature and concentrations of growth regulators used in shoot initiation medium and the time period for induction of shoots (Anju *et al*., 2001). *In vitro* propagation of *Lens* species and their F1 interspecific hybrids results showed that species differences, gibberellic acid, benzyl adenine levels have largest effects with only minor interaction effects (Ahmad *et al*., 1997). For successful rooting half MS salts and NAA 5.37 µM was best, IBA 0.25 mg l^{-1} showed 25% rooting response in shoots of lentil CV. Ali Day. The cytokinin had inhibitory effects on rooting in legumes. The frequency of whole plant establishment was relatively low in this study (Khawar & Ozcan, 2002). In lentil regenerated shoots in BA (0.225 mg l^{-1}) formed 4.6 – 39.9% roots in IAA 2 mg l^{-1}. Rooting success depended upon the time of exposure, concentration of BA during shoot formation prior to transplanting regenerated shoots to rooting medium (Polanco and Ruiz, 1997). Strong inhibitory effects of BA on root growth of lentil seedlings reflected in a drastic reduction of the mitotic index of root meristem. The efficiency of rooting medium varied upon the shoot regeneration medium and the cultivar tested for immature seed culture of lentil (Polanco and Ruiz, 2001). Nodal segments of lentil with an axillary bud cultured in an inverted orientation showed higher rooting frequencies

than explants cultured in normal orientation. Rooting was in MS with 3% sucrose and 5 µM IAA and 1µM kinetin. Medium aeration at the proximal end of the micro cuttings of CV. Digger is more important than shoot orientation for *in vitro* rooting of lentil micro cuttings (Newell *et al*., 2006). In lentils early flowering, pubescent peduncle and seed coat pattern are linked together (Sarker *et al*., 1999).

Variability is a universal feature of plant tissue culture. Soma clonal variation is a major obstacle in regenerations. Mutations happen all the time (Cassells and Curry, 2001). Soma clonal variation is manifested as cytological abnormalities, mutations, sequence change and gene activation and silencing. Epigenetic changes occur through the culture process. Soma clonal variation offers as opportunity to uncover natural variability in plants and to use its genetic variability of for new product development. However, soma clonal variation is undesirable if objective is clone propagation (Sahijram *et al*., 2003). This study concluded that plants can be obtained from callus regenerations of microsperma lentil and desirable selections can be made from variability in seed size and color of somaclones having stability of characters in seed generations.

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