http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences, 1 (4): 335-338, 1998 Research Article

Influence of Microsperma Lentil Genotypes on Tissue Culture Responses

N. Altaf, J. Iqbal* and M. S. Ahmad
Nuclear Institute for Agriculture and Biology, P.O. Box No. 128, Jhang Road, Faisalabad
*Botany Department, Punjab University, Quaid-e-Azam Campus, Lahore

Abstract
In vitro growth responses of various lentil genotypes varied. The best callussing response was noticed in variety ILL-4605. The genotype AARI-L-89503 had a tendency for embryogenesis. The genotype AARI-L-86642 had the best rooting ability. This genotype also showed *in vitro* flowering response. Masoor-85 and Pant-L-406 had moderate morphogenic responses, Meristem multiplication, shoot development, shoot bud formation from callus and adventitious rooting from shoot cuttings was obtained under appropriate growth regulators combinations.

Key words: Microsperma lentil, genotypes, tissue culture

Introduction

Lentil *(Lens culinaris* Medik) is an important pulse crop. Several work has been reported on macrosperma lentils. 'Cultivar Eston' gave best regeneration from shoot apex (Williams and McHughen, 1986), while in Spanish cultivars (Verciina, Pardina and Castellana), the best morphogenetic responses were from nodes (Polanco *et al*., 1988). Saxena and King (1987) reported somatic embryogenesis in cultivar 'Laird'. This shows a strong influence of macrosperma genotypes on morphogenetic responses of explant cultures. Not much work is done in the area of lentil cell and tissue culture and especially in case of microsperma lentils grown in Pakistan. This paper reports the tissue culture responses of some of the lentil genotypes of microsperma type, that are grown in Pakistan.

Materials and methods

Nine genotypes (Masoor-85, ILL-4605, Pant -L 406, AARI-L-89503, AARI-L-88527, AARI-L-86520, AARI-L-86632, AARI-L-87515, AAR1-L-865791 from Ayub Agricultural Research Institute, Faisalabad were utilized for in *vitro* growth responses.

Culture media: In the present work, MS salt mixture with 3% sucrose was used. Asserted combinations of amino acids, vitamins, growth hormones and other additive were applied in the medium and are mentioned separately. Growth harmones i.e. IAA, IBA, GA when required were filter sterilized before adding to the medium after autoclaving. The pH of the medium was adjusted to 5.7 with 0.1 N KOH or HCI. Difco bacto-agar (1%) was used to solidify the medium. Some experiments were conducted in liquid medium. The medium (15, 40 mL, respectively) was either dispensed into the Pyrex test tubes (25 × 150 mm) or Erlenmeyer flasks (100 mL). The tubes and flasks were plugged with cotton and autoclaved at 20 psi for 10 min. The test tubes were sterilized separately and sterile medium was dispensed into the tubes. After autoclaving, the medium was left to cool at room temperature.

Preparation of plant material: All the operations were performed aseptically in a Laminar flow cabinet. Sterilized instruments (needles, forceps, scalpel blades) were used. Healthy seeds of lentil were washed with detergent and disinfected with 0.5% $HgC1_2$ for 20 min or with NaOCl (7%) for 30 min. The seeds removed and washed in sterile distilled water to remove the disinfectant. Seeds were allowed to germinate on MS basal medium with 3% sucrose at 25°C for 5 days.
Aseptically grown seedlings raised from disinfected seeds were a source of shoot tips, epicotyl, hypocotyl, cotyledons, intercotyledonary stem tissue as explants. The explants were gently removed and cultured immediately on agar medium. The explants were placed on the nutrient medium with basal cut end facing the medium horizontally.

Culture environment: Growth room had a light intensity of 3000-4000 lux, with light and dark cycle of 16/8 h. and temperature varying from 22-26°C. Some illuminated growth incubators had light intensity of 2000 3000 lux and temperature could be adjusted to 20-22°C.

Influence of genotype and explant on callussing and regeneration: A comparative study to observe the influence of genotype and explant on microsperma lentil callussing and regeneration was also made All lentil genotypes were tested for callussing in K(10 mg L^{-1}) + GA(1 mg L^{-1}) and subcultured in the same medium containing seed extract (5-10%). These cultures were kept in continuous dark at 21 ± 1°C and cultures were regenerated after 2 subcultures in 16/8 h light/dark period. The comparative potential for callussing and regeneration (shoot, root, embryo) was noted down for these genotypes.

Determination of minimum period of growth regulators requirements for shoot and root regenerations: This experiment was with Masoor-85. Growth regulator requirements and specific time period for shoot and root regeneration response was studied. The time period of exposure of explants to specific growth regulators was noted for each regeneration response. For meristem multiplication and shoot development, the cotyledonary nodes with half cotyledons attached were cultured in BA 5 mg L^{-1} and then transferred to the basal medium, cotyledonary nodes with 1/6th cotyledons attached were cultured in two media; one containing K(10 mg L^{-1})+GAI 1 mg L^{-1}) arid the other containing, 2,4-D (1 mg L^{-1})+GA (1 mg L^{-1}) + BA (5 mg L^{-1}). The explants on these media were kept for 5,10,15,20,25 and 30 days. In another experiment, the bases of stem cuttings of two week old plants were treated with 0.2% NAA in simple talc and then washed after 1, 2, 3, 4, 5 or 6 day exposure and allowed to grow in auxin free peat plugs; arid the optimum time period of auxin exposure for rooting was observed on the

basis of further root development, health and growth of stem cuttings as well.

Genotype influence on rooting response: The influence of genotype on rooting response was studied using Masoor-85 and all accessions. One month old plants of these genotypes were taken from AARI fields and each plant was cut one inch above the cotyledons to remove its rooting system. The stem bases were dusted with rooting powder (simple talc +NAA 0.2% 4- IBA 0.8%) and twenty-five cuttings per genotype were grown in pots containing peat moss, sand and soil mixture (1:1:1). The pots were covered with polythene to retain humidity. The shoots were sprayed with water 10-12 times in the first ten day until the new leaves appeared. The data was recorded after one month of growth.

Results

Influence of genotype and explant on callussing and regeneration: The genotypes that were being tested for better field performance in Punjab were screened for callussing and regeneration responses alongwith the Masoor-85 (Table 1). These were screened for callussing in the dark in presence of K and GA. Genotype ILL-4605 gave the maximum callus among all genotypes tested. Genotype AARI-L-89503 and 88527 gave vigorous and healthy callus as compared to M-85, while Pant-L-406 and AARI-L-86520 were similar to M-85. Three lines AARI-L-86642, 87515 and 86579 showed poor proliferation for callussing as compared to the other genotypes. The explants from various genotypes in different media behaved differently. Genotype AARI-L-89503 produced callus with embryogenic structures. AARI-L-86642 had best rooting potential and a capability of *in vitro* flowering and podding. AARI-L-86579 had poor regenerative responses. Genotype AARI-L-88527 had a capability of giving shoot buds from the nodes alongwith the callussing. The cell mass continued to increase with the formation of green buds for at least two to three subculture stage. The elongated shoots were maximum in the initial culture in K 10 mg L^{-1} + GA 1 mg L^{-1}. The shoot buds remained dormant mostly in 2nd, 3rd subcultures. Bud dormancy is a problem in lentil cultures.

Determination of minimum period of growth regulators requirements for shoot and root regenerations: For meristem multiplication, shoot bud formation from callus, shoot development, adventitious rooting, there was need for exposing explant tissue to growth regulators for an optimum period as shown in Table 2. The explant cotyledonary nodes with 1/6th cotyledons attached were incubated in MS medium with BA, glutamine and fructose for 10-15 days, when transferred to MS medium without BA, showed meristem multiplication and shoot development. This showed that prolonged exposure of BA was not necessay for shoot elongation. The same explant in MS containing K + GA + seed extract in dark for 21-25 days produced shoot buds from callus. The shoot buds from callus were also produced in MS 1-2,4- D +BA +GA +Glutamine when the same explants were kept for 28 days. The shoots induced adventitious rooting after exposure to NAA for 4 days.

Genotype influence on rooting responses: Different genotypes were tested for adventitious rooting ability (Fig. 1). It was observed that AARI-L-86642 was much better (52%) for rooting response as compared to Masoor-85 (28%). Two genotypes AARI-L-87515 (32%) and AARI-L-88527 (28%) were at par with Masoor-85 (28%) for adventitious rooting ability. However, two genotypes AARI-L-86579 (16%) and AARI-L- 86520 (8%) were some what inferior to rooting response as compared to Masoor-85 (28%). D ifferences in rooting of stem cuttings of the same genotype were also noticed. The overall differences among the genotypes were more clear.

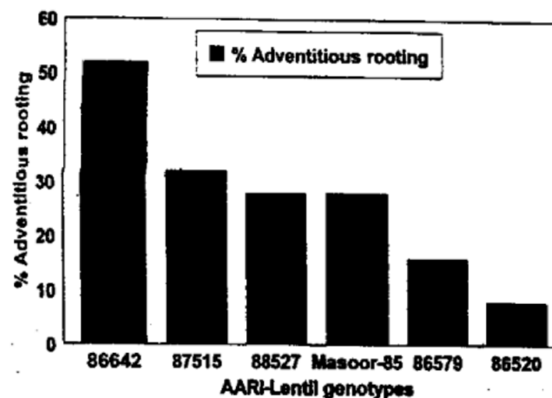

Fig. 1: Rooting in various lentil genotypes

Discussion

Microsperma lentil genotypes tested in this study revealed a strong genotype influence on callussing, callus regenerations, rooting and culture ability of various explants. Various genotypes also showed explant and culture medium interaction. Some microsperma genotypes had better culture response for callussing and shoot regenerations and some had less callussing response as compared to Masoor-85. The genotype responses were clearly observed for rooting, *in vitro* flowering, embryogenic callus and cell proliferations. Primary work in the microsperma lentil was to see the growth regulator interaction with meristem and it was observed that both K and BA are capable of inducing shoot buds. However, K with GA was better shoot inducer and 2,4-0 increased the cell proliferations and explant mass by callussing. The basal stem portion along with the basal nodes from the just germinating seedlings had maximum callussing and cell proliferation capacity. The same type of explants from older seedlings did not show the similar potential. Age of the explant along with the explant type was important. In this case, normal polarity of the explant was important during culturing in agar medium.

The size of the explant in microsperma lentil cultures was also important. Too small and too large explant size was not very suitable for the start of tissue cultures. It was observed from the experiments that almost all the seedling segments had a potential for callussing. This potential was maximum from 3-4 day seedlings. The cell proliferations were more with cytokinin and 2,4-D. In the initial cultures of microsperma lentil, the liquid medium worked well as compared to agar solidified medium. Similar results have also been obtained in meristem explants of bananas (Bhagyalakshmi and Singh, 1995). However, K and GA induced slow growing callus in lentil explants under dark conditions that regenerated into shoot buds and those elongated into shoots when this callus was shifted under light conditions. Seed extract addition to this medium enhanced shoot bud regenerations. Almost all microsperrna genotypes tested in this study responded to this condition,

Table 1: Influence of genotype and explant on microsperma lentil callussing and regeneration capacity

Genotypes	Callussing and regeneration apices in MI	Explant source	Medium/Regeneration Response
ILL 4605	+ + + +	Apex, cotyledonary nodes	M1/Vigorous callus in dark node Shoot buds in light Multiple shoots
AARI L 89503	+ + +	Germinating seed	M2/Cream callus in light with green globular to torpedo shaped embryos
AARI-L-88527	+ + +	Germinating seed Seedling, half Cotyledons root apex removed	M3/Direct shoot buds regenerating callus in light
Masoor-85	+ +	Apex, cotyledonary nodes	M1/Callus in dark. Shoot organogenesis in light
Pant-L-406	+ +	Apex, cotyledonary nodes	M1/Callus in dark. Shoot organogenesis in light
AARI-L 86520	+ +	Apex, cotyledonary nodes	MI/Callus in dark. Shoot organogenesis in light
AARI-L-86642	+	Cotyledonary nodes	M4//n *vitro* flowering and podding after 5 weeks
AARI-L -87515	+	Cotyledonary nodes	M5/Adventitious rooting + + +
AARI L 86579	+	Apex, cotyledonary nodes	M1 M5 Poor regeneration

Response: + + + + very good. + + + good, + + moderate, +poor; various media addition (mg L^{-1}) to MS:
MI:K(1 0) +GAM +seed extract (10%1; M2 ;BA(5) + 2.4-D(2),GA(1) + glutamine(100);
M3:BA(5)+2,4-0(1) + IAA(1)-1-glutamine(50); M4:BA(2.5)+'1BA(1); M5:IBA10.51
Callus of each genotype was grown separately in each of the five media. The responsive medium is indicated only

Table 2: Regeneration response upon tissue transfer from growth regulator media to basal medium

Culture medium	Days of exposure	Regeneration response
MS BA(5) glutamine (100) fructose 3%	10-15	Meristem multiplication shoot development
MS K(1 0) + GA(1) seed extract in dark	21-25	Shoot buds from callus
MS + 2,4-D(1) BA (5) GA(1) Glutamine(100)	28	Callus
0.2% NAA in simple talc quantities in ( ) are mg L^{-1}	4	Adventitious rooting

* Explant tissue is cotyledonary nodes with 1/6th cotyledons attached

although callussing and regeneration potential varied among the genotypes.

The seed had a very good potential of callussing. However, callussing and shoot primordia formation could be increased with the addition of seed extract in all callus inducing media including K and GA. The later medium had regeneration potential for more than one year as long as the callus continued growing in it. Almost all the explants from the germinating seedlings formed callus in K and GA and also regenerated buds. The number of buds regeneration depended on callus surface. For the larger callus surface, there were more regenerations and the surface increased with the increase in mass by subculturing passages.

In lentil, most of the work in the world is on macrosperma genotypes. In case of cultivar 'Eston', regeneration of shoots occurred only in calli derived from the shoot meristem and the epicotyl, cotyledonary calli did not regenerate in K and GA (Williams and McHughen, 1986). Regeneration from cotyledonary node explants has been reported in cultivar 'Laird' using BAP. In this case, epidermal and subepidermal cells had potential for shoot organogenesis (Warkentin and McHughen, 1993).

Regeneration of plants from cultured shoot tips has been reported in microsperma lentil (Bajaj and Dhanju, 1979). Plants by embryo rescue has been reported by Cohen *et al.*, 1984. Polanco *et al.* (1988) used three spanish cultivars, Verdina, Pardina and Castellana and observed that from shoot tip, leaf and node explants tested, the best morphogenetic responses were obtained from nodes and the poorest from the leaves.

Induction of multiple shoots from cotyledonary nodes of microsperma lentil proved possible on medium with BAP but other parts of the seedlings i.e. root, leaf, hypocotyl tailed to regenerate. On isolation, these parts underwent callussing on medium rich in cytokinin or auxin and the tissue formed was able to regenerate only roots (Mallick and Rashid, 1989).

Nodal segments and shoot tip explants produced a single shoot from the pre-existing meristem and then root in 4 weeks on hormone free MS medium in microsperma lentil. However, only shoots regenerated on media containing K and multiple shoots formed without intervention by callus or root formation. Best callus formation was on media containing 1 mg L^{-1} K and 10 mg L^{-1} 2,4-D and shoot buds formed on transfer to media containing only K. These shoot buds were transferred to MS basal medium for plantlet formation (Singh and Raghuvanshi, 1989). Cultivar 'Laird' from macrosperma was cultured on medium containing 25-500 fM, PAA but habituation of cultures was common. PAA was not toxic to lentil tissues at concentration as high as 500 fM (Leuba and LeTourneau, 1990). In lentil, excised root tips showed elongation in sucrose medium, pH 5.2. The maximum response was 25-30 percent in 1 muM abscissic acid containing medium (Gaither *et at.*, 1975).

The explant type has a definite influence on callus regenerations in seed legumes and other species. In microsperma lentil in this study, it was observed that cotyledonary nodes and the area adjacent to it had maximum regeneration potential. In *Phaseolus vulgaris* L. cotyledon and epicotyl and occasionally at the terminal end of the epicotyl, shoot buds were developed which were actually formed from the superepidermal parenchyma cells of the node, thus confirming the adventitious nature of these organ structures (McClean and Grafton, 1989). Regeneration from cotyledonary node explants has been reported in soybean (Wright *et at.*, 1986) and peas (Jackson and Hobbs, 1990). In all these cases, multiple buds and shoots arose in the axils of the cotyledonary petioles. The

initial shoots probably originated from the axillary bud rnerrstems. Histological studies revealed that further shoot development was from epidermal or subepidermal cells which had the potential for shoot organogenesis via induction by BA. Primary leaves including the intact petiole regenerated shoots via callussing in cowpea (Muthukumar *et al.,* 1995). Addition of seed extract improved callussing and regeneration in microsperma lentil, as it had been reported to improve regeneration in case of *Phaseolus vulgaris* L., where plants were regenerated on a medium containing bean seed extract of an undefined composition (Crocomo *et at*., 1976).

Cotyledonary node explain works well with the mungbean regeneration and the number of attached cotyledons and removal of a portion of one or both cotyledons affected shoot formation (Gulati and Jaiwal, 1994). Cotyledonary region has a very good regeneration potential not only in seed legumes but in other species as well. Cotyledonary protoplasts from seedlings of muskmelon was established and a high frequency division of the protoplasts derived cells was observed.

In this study, growth regulators were required for some period for meristem multiplication, shoot development, for callus induction in explants and shoot bud formation from callus and adventitious rooting in microsperma lentil regeneration experiments. Without growth regulators there was no organogenic response from the explants. The balance of auxins and cytokinins in a growth medium is generally thought to determine the type of organ produced. A high level of cytokinin to auxin ratio favours shoot formation, while the reverse favours root formation and this has been found for many plant species (Torrey, 1958). Temperature, light and gaseous atmosphere are the three main factors affecting organogenesis. The optimum temperature for organogenesis can vary. Lentil cultures have been grown by various workers at temperature ranging from 18-26°C (Williams and McHughen, 1986; Rozwadowski *et al*., 1990) and light varying from diffuse light to day light. In general. light was necessary for the formation of shoots.

Rooting of *in vitro* shoots is very important (Schiefelbein and Benfey, 1991). Profound perturbation of normal metabolism is required in order to regenerate the root system, to restore the thermodynamics of the whole plant and to sustain life (Haissig, 1985). Microsperma lentil needed auxins for root induction in the regenerated shoots. The basal stem sections had tendency to form roots but rooting was enhanced by auxins. Keeping for a longer time in cultures made the survival of shoots difficult during the root induction period. High humidity was very essential during weaning period. Under field conditions, January temperatures favoured rooting of shoots. The rooting response varied with the genotypes in this study. Not much work has been done on adventitious rooting of lentil. Adventitious rooting is a problem in lentil (Polanco *et al*., 1988) and rooting response is low (less than 10%) in macrosperma lentil cv. "Eston". May be adaventitious rooting responses were more influenced by genotypes (Williams and McHughen, 1986).

Rooting is a complex phenomenon in which several factors play a role and genotype is among the important factors.

References

Bajaj, Y.P.S. and M.S. Dhanju, 1979. Regeneration of plants from apical meristem tips of some legumes. Curr. Sci., 48: 906-907.

Bhagyalakshmi and N.S. Singh, 1995. Role of liquid versus agar-gelled media in mass propagation and *ex vitro* survival in bananas. Plant Cell Tissue Org. Cult., 41: 71-73.

Cohen, D., G. Ladizinsky, M. Ziv and F.J. Muehlbauer, 1984. Rescue of interspecific *Lens* hybrids by means of embryo culture. Plant Cell Tissue Org. Cult., 3: 343-347.

Crocomo, O.J., W.R. Sharp and J.E. Peters, 1976. Plantlet morphogenesis and the control of callus growth and root induction of *Phaseolus vulgaris* with the addition of a bean seed extract. Zeitschrift Pflanzenphysiologie, 78: 456-460.

Gaither, D.H., D.H. Lutz and L.E. Forrence, 1975. Abscisic acid stimulates elongation of excised pea root tips. Plant Physiol., 55: 948-949.

Gulati, A. and P.K. Jaiwal, 1994. Plant regeneration from cotyledonary node explants of mungbean (*Vigna radiata* (L.) Wilczek). Plant Cell Rep., 13: 523-527.

Haissig, B.E., 1985. Metabolic Processes in Adventitious Rooting of Cuttings. In: New Root Formation in Plants and Cuttings, Jackson, M.B. (Ed.). Springer, New York, USA., ISBN: 9789400943582, pp: 141-190.

Jackson, J.A. and S.L.A. Hobbs, 1990. Rapid multiple shoot production from cotyledonary node explants of pea (*Pisum sativum* L.). *In vitro* Cell. Dev. Biol., 26: 825-835.

Leuba, V. and D. LeTourneau, 1990. Auxin activity of phenylacetic acid in tissue culture. J. Plant Growth Regul., 9: 71-76.

Mallick, M.A. and A. Rashid, 1989. Induction of multiple-shoots from cotyledonary node of grain legumes, pea and lentil. Biologia Plantarum, 31: 230-232.

McClean, P. and K.F. Grafton, 1989. Regeneration of dry bean (*Phaseolus vulgaris* L.) via organogenesis. Plant Sci., 60: 117-122.

Muthukumar, B., M. Mariamma and A. Gnanam, 1995. Regeneration of plants from primary leaves of cowpea. Plant Cell Tissue Org. Cult., 42: 153-155.

Polanco, M.C., M.I. Pelaez and M.L. Ruiz, 1988. Factors affecting callus and shoot formation from *in vitro* cultures of *Lens culinaris* Medik. Plant Cell Tissue Org. Cult., 15: 175-182.

Rozwadowski, K.L., P.K. Saxena and J. King, 1990. Isolation and culture of *Lens culinaris* Medik. cv. Eston epicotyl protoplasts to calli. Plant Cell Tissue Org. Cult., 20: 75-79.

Saxena, P.K. and J. King, 1987. Morphogenesis in lentil: Plant regeneration from callus cultures of *Lens culinaris* medik. via somatic embryogenesis. Plant Sci., 52: 223-227.

Schiefelbein, J.W. and P.N. Benfey, 1991. The development of plant roots: New approaches to underground problems. Plant Cell, 3: 1147-1154.

Singh, R.K. and S.S. Raghuvanshi, 1989. Plantlet regeneration from nodal segment and shoot tip derived explants of lentil. LENS Newslett., 16: 33-35.

Torrey, J.G., 1958. Endogenous bud and root formation by isolated roots of convolvulus grown *in vitro*. Plant Physiol., 33: 258-263.

Warkentin, T.D. and A. McHughen, 1993. Regeneration from lentil cotyledonary nodes and potential of this explant for transformation by *Agrobacterium tumefaciens*. LENS Newslett., 20: 26-28.

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

Wright, M.S., S.M. Koehler, M.A. Hinchee and M.G. Carnes, 1986. Plant regeneration by organogenesis in *Glycine max*. Plant Cell Rep., 5: 150-154.