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PLANT REGENERATION FROM PULSE-TREATED LONGITUDINALLY SLICED HALF COTYLEDON NODE EXPLANTS OF TURKISH OCHRUS CHICKLING [*LATHYRUS OCHRUS* (L.) D.C.]

S. SAGLAM

Department of Agricultural Biotechnology, Faculty of Agriculture, Ahi Evran University, 40100 Kirsehir, Turkey

Abstract - The forage legume ochrus chickling [*Lathyrus ochrus* (L.) D.C.] which is distributed in the Mediterranean region, is gaining importance in terms of economy and agriculture in Turkey. However, the full potential of the legume has yet to be realized due to the presence of neurotoxin, ß-N-oxalyl-L-a, ß-diaminopropionic acid (ODAP) causing lathyrism. This study aimed to develop an efficient micropropagation system using longitudinally sliced cotyledon node explants for use in *Agrobacterium*-mediated genetic transformation in the future. The results show that the maximum number of shoots per explant was achieved on MS medium solidified with 8 g/l isubgol gelled medium containing 0.30 mg/l BA-0.2 mg/l NAA. Well-developed shoots were rooted by pulse treatment with 50 mg/l IBA and culturing on an 8 g/l isubgol gel solidified MS medium. The results showed 60% rooting in the treated shoots. The rooted plantlets were transferred to pots containing sand and organic matter and acclimatized.

Key words: Pulse treatment, isubgol, forage plant, mass propagation, longitudinally sliced half cotyledon node, rooting

INTRODUCTION

The forage legume genus *Lathyrus* is receiving increased attention from scientists in response to an ever-increasing global demand for food and feed resources and the need to diversify modern cropping systems (Enneking, 1998). The genus *Lathyrus* L., family Fabaceae, consists of 189 species (Allkin et al. 1983), of which only a small number are cultivated. Besides other species, *Lathyrus ochrus* (L.) D.C. distributed in Mediterranean region at an altitude of 0-50 m above sea level is gaining importance in terms of economy and agriculture in Turkey.

The full potential of the *Lathyrus* species has not been realized due to the presence of a neurotoxin, ß-N-oxalyl-L-a, ß-diaminopropionic acid (ODAP) that causes lathyrism – a motor neuron disease responsible for paralysis of the lower limbs (Campbell et al., 1994). Conventional breeding and selection methods have failed to produce varieties free of this neurotoxin. *L. ochrus* is gaining interest as a grain legume crop in Mediterranean-type environments and production is increasing in Turkey as an alternative forage crop. For large-scale production as a commercial forage crop, the development of safer cultivars that express low or no levels of ODAP under the environmental conditions of Turkey is needed.

The availability of good regeneration protocols is a prerequisite for the transformation of any crop plant. Tissue culture and biotechnological research can help in the efforts to produce plants free of this neurotoxin. In general, grain legumes, including *Lathyrus* species, are very recalcitrant to shoot and root regeneration, and a very limited literature is

available about their tissue culture or genetic transformation (Kendir et al., 2009; Demirbag-Sahin et al., 2008; Khawar et al., 2004a,b). Only one report (Malik et al., 1992) suggests shoot regeneration from preconditioned epicotyl explants of *L. ochrus*. Limited progress in the development of plant regeneration systems has seriously impeded the application of gene transfer technology to this plant. Therefore, it is needed to supplement breeding activities through modern biotechnological means. This study aimed to develop a tissue culture protocol for the micropropagation of *L. ochrus* for using as a protocol for potential use in *Agrobacterium*-mediated genetic transformation in the future.

MATERIALS AND METHODS

Seeds of *L. ochrus* were obtained from the Osman Tosun Gene Bank, Department of Agronomy, Faculty of Agriculture, Ankara University, Ankara, Turkey. The seeds were surface sterilized with 100% commercial bleach (Ace-Turkey containing 5% NaOCl) for 15 min followed by 3 x 5 min rinsing with bi-distilled sterilized water and germinated on 35 ml of agar solidified MS basal medium (Murashige and Skoog, 1962) supplemented with 3.0% sucrose in 100 x 10 mm Petri dishes. Agar (0.65% - Duchefa Biochemie B.V., Haarlem, the Netherlands) was added to the culture medium after adjusting pH to 5.6-5.7 before autoclaving.

The cotyledon nodes were excised from 15-day-old *in vitro*-grown seedlings. They were pulse-treated with 10 mg/l BA for seven days. Then they were longitudinally sliced into two and cultured on 0.3 mg/l BA with and without 0.2 mg/l NAA using 6-8-10 g/l isubgol solidified MS medium. The pulse-treated explants were also cultured on MS medium solidified with 6, 8, 10 g/l isubgol (control).

The pH of all media was adjusted to 5.6 – 5.7 using 0.1 N KOH or 0.1 N HC1 before autoclaving under pressure of 119 kPa for 20 min and solidified by 0.65% agar. All cultures were incubated in growth chamber at 24 ± 2°C with 16 h light photoperiod.

Well-developed shoots were excised under aseptic conditions after six weeks of regeneration. They were pulse-treated with 50 mg/l IBA for 5 min and then rooted on 35 ml of MS in Magenta GA7 vessels for four weeks. The rooted shoots then were carefully removed from the isubgol-containing media very carefully under continuous flowing tap water. The tissue-cultured plants were transferred to pots containing vermiculite, organic matter and sand (1:2:1) in the greenhouse at room temperature where they were subjected to intermittent mist-water spray with a mist humidifier for 24 h. Relative humidity was maintained at 80% during the first seven days, which helped to maintain a film of water on the plant leaves to avoid wilting. The humidity was gradually reduced to 40% over 15 days. Thereafter, the plants were transferred to the greenhouse for growth development and seed set.

All treatments of the regeneration experiments had three replicates containing five explants each and all experiments were repeated twice (3 x 5 x 2 = 30 explants per treatment). The frequency (%) of shoot regeneration, mean number of shoots per explant, shoot length and frequency of rooting were recorded and analyzed using univariate analysis with statistical software SPSS 17.00 for Windows. The *post hoc* tests were performed using Duncans Multiple Range test. Data given in percentages were subjected to arcsine transformation (Snedecor and Cochran, 1967) before statistical analysis.

RESULTS

The explants began to swell and elongate, followed by axillary shoot regeneration at the axillary end on all the pulse-treated longitudinally sliced half cotyledon node explants. Well-developed shoots could be observed on the regenerating explants after 3 weeks of culture at any concentration of isubgol as gelling agent, and at any concentration of BA, with and without NAA (Fig. 1a). Analysis of variance after eight weeks of culture showed significant differences ($p<0.05$) among the frequencies (%) of shoot regeneration, frequency (%) of callus induction and

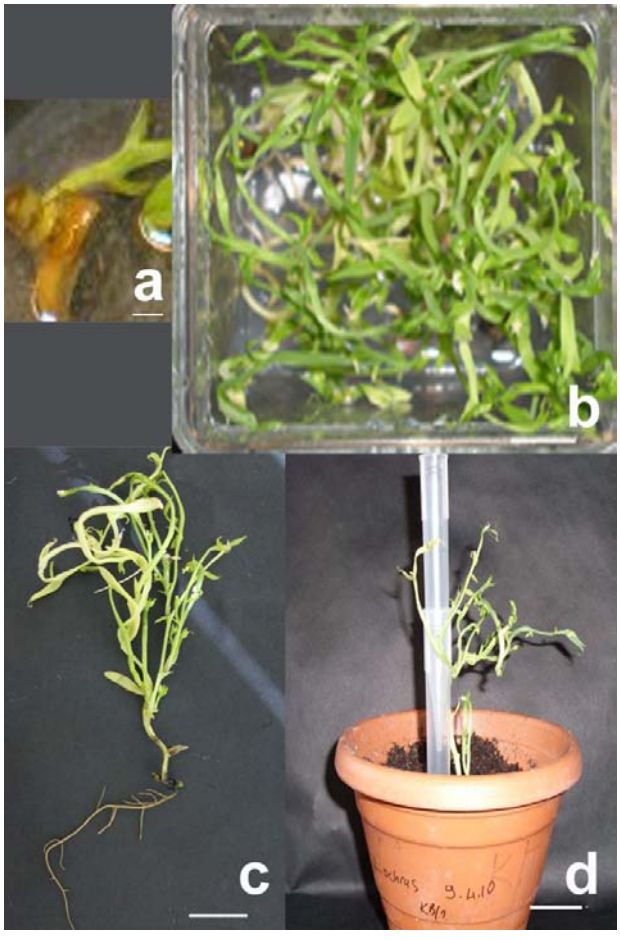

Fig 1. Shoot regeneration from half cotyledon node explant of *Lathyrus ochrus:* (a) axillary shoot regeneration after 3 weeks; (b) 8 weeks of culture 8 g/l isubgol containing 0.30 mg/l BA with 0.2 mg/l NAA; (c) rooted plant; (d) acclimatization in the greenhouse. Bar Fig 1a= 0.4 cm; Fig.1b=1 cm; Fig 1c=2.25 cm; Fig 1d= 2 cm.

number of shoots per explant. Moreover, no callusing was recorded on the MS medium containing 0.30 mg/l BA with any concentration of isubgol (Table 1). Similarly, no callusing was noted on MS medium containing 0.30 mg/l BA-0.2 mg/l NAA solidified with 6 g/l isubgol. However, 50.00 and 41.67% frequency of callus induction was noted on MS medium solidified with 8 and 10 g/l isubgol containing 0.30 mg/l BA-0.2 mg/l NAA. No shoot regeneration was recorded on any of the pulse-treated explants on MS medium solidified with 6, 8 and 10 mg/l isubgol (control).

The presence or absence of NAA in the culture medium exerted a significant effect on the mean number of shoots per explants. Comparing the effects of BA used singly or with 0.2 mg/l NAA, the pulse-treated explants cultured on 0.30 mg/l BA-0.2 mg/l NAA induced a greater number of shoots per explant. The maximum number of 5.92 shoots per explant was recorded on MS medium solidified with 8 g/l isubgol containing 0.30 mg/l BA with 0.2 mg/l NAA (Fig. 1b). It was followed closely by 4.00 shoots per explant on MS medium solidified with 10 g/l isubgol containing 0.30 mg/l BA with 0.20 mg/l NAA.

It was not difficult to root the well-developed shoots pulse-treated with 50 mg/l IBA for 5 min on MS rooting medium solidified with isubgol. Rooting started after 5-7 days of culture with a maximum rooting frequency of 60%. Rooted shoots (Fig. 1c) transferred to pots containing vermiculite, organic matter and sand (1:2:1) covered with polythene bags and were easy to acclimatize (Fig. 1d).

DISCUSSION

The development of new tissue culture protocols exploring new explants is a prerequisite for improvement in genetic transformation. Only one shoot regeneration protocol of *L. ochrus* has been reported previously, which indicates that extensive research is needed to develop new regeneration protocols for the plant that would facilitate breeding activities in this important forage legume. The results showed high regeneration potential of longitudinally sliced half cotyledon node explants on any concentration of isubgol-gelled media containing 0.30 mg/l BA with 0.2 mg/l NAA. These results support the findings of Malik et al. (1992), who used BA, kinetin, and TDZ for shoot regeneration from the cotyledon node explant of *L. cicera*, *L. ochrus*, *L. sativus* and *L. tingitanus* in MS medium. They successfully transferred the plants after rooting and acclimatization to external environmental conditions. Debnath et al. (2001) also induced multiple shoot regeneration from stem, rachis and leaf explants of *Lathyrus japonicus*. Ochatt et al. (2002) obtained regeneration from hypocotyl shoot nodes in *L. sativus.*

Table 1. Effects of BA with and without NAA in MS medium solidified with various concentrations of isubgol on shoot regeneration of *L. ochrus* using longitudinally sliced half cotyledon node explants.

Treatments			Frequency (%) of shoot regeneration	Frequency (%) of callus induction	Mean number of shoots per explant
Gelling agent isubgol (g/l)	BA (mg/l)	NAA (mg/l)			
6	0.30	0.00	25.00b	0.00b	0.33c
6	0.30	0.20	83.33a	0.00b	2.00bc
6	MS medium (control)		0.00c	0.00b	0.00d
8	0.30	0.00	66.67ab	0.00b	3.22bc
8	0.30	0.20	66.67ab	50. 00a	5.92a
8	MS medium (control)		0.00c	0.00b	0.00d
10	0.30	0.00	58.33ab	0.00b	2.56bc
10	0.30	0.20	50.00ab	41.67a	4.00ab
10	MS medium (control)		0.00c	0.00b	0.00d

Values within column followed by different small letters are significantly different at the 0.05 level by Duncan's test.

It would appear that 8 g/l isubgol provided a better diffusion of media components to the plant tissues, resulting in higher shoot regeneration compared to 6 and 10 g/l isubgol. This could be also be ascribed to a better contact between the explants and the culture medium due to the concentration of isubgol, which increased the availability of plant growth regulators and other nutrients in the respective media and contributed to enhanced induction of shoot regeneration.

Ozel et al. (2008) who used the Samsun tobacco variety in their experiments to compare adventitious shoot regeneration on different blends of agar-isubgol, gelrite-isubgol, phytagel-isubgol or isubgol alone, found that the maximum number of shoot per explant was recorded on MSD4X2 medium gelled with 7 g/l isubgol. The longest shoots were recorded on MSD4X2 medium gelled with 9 g/l isubgol. Similarly, Saglam and Ciftci (2010) also used isubgol in *in vitro* regeneration of woad (*Isatis tinctoria* L.) from leaf and hypocotyl explants. They obtained maximum shoot regeneration of 17.80 shoot per leaf explant on isubgol-gelled MS medium containing 0.50 mg/L BA. Maximum shoot regeneration of 20.55 shoots per hypocotyl explant on isubgol-gelled medium was recorded on MS medium containing 1.00 mg/l BA.

The results are also in agreement with Aasim et al. (2009a, b), who reported the positive effect of 10 mg/l BA pulse-treated plumule explants of cowpea on callus and shoot induction in the presence of NAA in the culture medium. Contrarily, Aasim et al. (2008) reported the negative effects of NAA in the regeneration medium on the shoot regeneration potential of explants. The results are also in agreement with the previous findings of Demirbag-Sahin et al. (2008), who induced *in vitro* shoot regeneration of Turkish dwarf chickling (*Lathyrus cicera* L.) using immature zygotic embryo explants. Similarly, Kendir et al. (2009) in *Vicia narbonensis* also used BA-NAA with and without ascorbic acid for shoot regeneration from immature zygotic embryos.

The application of auxins is used to induce rooting. The recalcitrant regeneration behavior of legumes towards rooting has made it very difficult to apply plant biotechnology to the improvement of legume crops (Khawar and Ozcan, 2002; Khawar et al., 2004a; Sevimay et al., 2005).

The results on rooting showed the clear effect of isubgol concentration on the hardening and rooting of shoots. These results were supported by the previous findings of Ozel et al. (2008) recorded the highest number of roots on MSO medium gelled with 7

g/l isubgol. Similarly, Saglam and Ciftci (2010) found that isubgol-gelled medium could be effectively used for *in vitro* rooting of woad. They obtained rooting on full strength MS medium supplemented with 0.75 mg/L IBA. They further recommended the use of isubgol as a cheaper gelling agent for use in tissue culture.

The development of a successful regeneration protocol from pulse-treated longitudinally sliced half cotyledon node explants, followed by rooting and plant establishment, indicates that this protocol could be applied to this forage plant in breeding and improvement programs.

REFERENCES

Aasım, M., Khawar, K.M. and *S. Ozcan* (2008). *In vitro* micropropagation from shoot meristems of Turkish cowpea (*Vigna unguiculata* L.) cultivar Akkiz. *Bangladesh J. Bot*, **37**(2), 149-154.

Aasım, M., Khawar, K.M. and *S. Ozcan* (2009a). *In vitro* micropropagation from plumular apices of Turkish cowpea (*Vigna unguiculata* L.) cultivar Akkiz. *Scientia Horticulturae*. **122,** 468–471.

Aasım, M., Khawar, K.M. and *S. Ozcan* (2009b). Comparison of shoot regeneration on different concentrations of Tdz from shoot tip explant of cowpea on gelrite and agar containing medium. *Not. Bot. Hort. Agrobot. Cluj*. **37** (1), 89–93.

Allkin, R., Macfarlane, T.D., White, R.J., Bisby, T.A. and *M.E Adey* (1983). Names and synonyms of species and subspecies in the vicieae. Issue 2. Vicieae Database Project, Publication No. 2, Southampton.

Campbell, C.G., Mehra, R.B., Agrawal, S.K., Chen, Y.Z., Abd El Moneim, A., Khawaja, H.I.T., Yadov, C.R., Tay, J.U. and *W.A Araya* (1994). Current status and future research strategy in breeding grasspea (*Lathyrus sativus*). *Euphytica*, **73**, 167–175.

Debnath S.C., Mckenzıe D. B. and *K.B., Mcrae* (2001). Callus induction and shoot regeneration from stem, rachis and leaf explants in beach pea (*Lathyrus japonicus* Willd). *J. Pl. Biochemist. Biotech*. **10**(1), 57–60.

Demırbag-Sahın N., Kendir H., Khawar K.M. and *C.Y. Cıftcı* (2008). *In vitro* regeneration of Turkish dwarf chickling (*Lathyrus cicera* L) using immature zygotic embryo explant . *African J Biotechnol*. **7** (12), 2030–2033.

Ennekıng, D. (1998). A bibliographic database for the genus Lathyrus. Co-operative Research Centre for Legumes in Mediterranean Agriculture. Occasional publication No **18.** ISSN 1-320-366 ISBN 0-86422-829-5.

Kendır, H., Sahın-Demırbag, N., Khawar, K.M. and *S. Ozcan* (2009). *In vitro* plant regeneration from Turkish grasspea (*Lathyrus sativus* L.) using immature zygotic embryo explant. *Biotechnol. & Biotechnol. Equip*. **23**(2), 177–1180.

Khawar, K.M. and *S. Ozcan* (2002). High frequency shoot regeneration from cotyledonary node explants of different lentil (*Lens culinaris* Medik.) genotypes and *in vitro* micrografting. *Biotechnol. & Biotechnol. Equip*. **16**(1), 12–17.

Khawar, K.M., Sancak, C., Uranbey, S. and *S. Ozcan* (2004a). Effect of thidiazuron on shoot regeneration from different explants of lentil *(Lens culinaris* Medik.) via Organogenesis. *Turk J. Bot*. **28**, 421-426.

Khawar, K.M., Gulbıtti-Onarıcı, S., Cocu, S., Erısen, S., Sancak, C. and *S. Ozcan* (2004b). *In vitro* crown galls induced by *Agrobacterium tumefaciens* strain A281 (pTiBo542) in *Trigonella foenumgraecum. Biologica Plantarum*. **48** (3), 441–444.

Malık, K.A., Alıkhan, S.T. and *P.K. Saxena* (1992). Direct organogenesis and plant regeneration in preconditioned tissue cultures of *Lathyrus cicera* L. *L. ochrus* (L.) DC and *L. sativus* L. *Ann. Bot*. **70**, 301–304.

Murashige T., and *F. Skoog* (1962). Revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*.**15:** 473- 497.

Ochatt, S.J., Muneaux, E., Machado, C., Jacas, L. and *C. Pontécaille* (2002). The hyperhydricity of *in vitro* regenerants is linked with an abnormal DNA content in grass pea (*Lathyrus sativus* L.). *J. Plant Physiol*. **159**, 1021–1028.

Ozel, C.A., Khawar, K.M. and *O. Arslan* (2008). A comparison of the gelling of isubgol, agar and gelrite on *in vitro* shoot regeneration and rooting of variety Samsun of tobacco (*Nicotiana tabacum* L.) *Scientia Horticulturae*. **117** (2), 174–181.

Saglam, S. and *C.Y. Ciftci* (2010). Effects of agar and ısubgol on adventitous shoot regeneration of woad (*Isatis tinctoria*). *Int. J. Agric. B*iol. **12** (2), 281–285.

Sevimay, C.S., Khawar, K.M. and *E. Yuzbasioglu* (2005). Adventitious shoot regeneration from different explants of wild lentil **(***Lens culinaris* subsp. orientalis). *Biotechnol. & Biotechnol. Equip.* **19**, 2 Suppl. 2, 46-49.

Snedecor, G.W. and *W.G. Cochran* (1967). Statistical Methods. The Iowa State University Press, Iowa, USA.