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HIGH FREQUENCY AXILLARY SHOOTS INDUCTION IN GRASS PEA (*LATHYRUS SATIVUS* L.)

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Abstract

Grass pea (*Lathyrus sativus* L.) is highly recalcitrant and difficult to regenerate under *in vitro* conditions. An efficient and reliable protocol was developed for induction of effective *in vitro* regeneration of grass pea using embryos with two cotyledons. The maximum shoot regeneration frequency of 89.80% and number of 15.83 shoots per explant was induced on MS medium containing 2 mg/l BAP and 1 mg/l NAA. The mean shoot length decreased proportionately with each increase in TDZ concentration irrespective of the IBA. The regenerated shoots produced roots on MS medium containing 0.5, 1.0 and 2.0 mg/l IBA. However, rooted plantlets flowered in the rooting media.

Introduction

Grass pea (*Lathyrus sativus* L.) is an important legume crop in India, China, Iran, Pakistan and Turkey (Kumar *et al.* 2011, Barpete 2015); where, it is mainly used as stock feed and human consumption. Grass pea has potential as an alternative legume in many cropping systems around the world (Kumar *et al.* 2013), due to its ability to survive under extreme climatic conditions like drought, cold, water stagnation and heat stress (Kumar *et al.* 2011). The knowledge that the excessive consumption of grass pea containing β-N-oxalyl-L-α,β diaminopropionic acid (β-ODAP) can cause neurological disorder in humans as well as animals has discouraged adaptive research on this underutilized and neglected crop. Therefore, conservation and sustainable use of genetic resources are of paramount importance for grass pea improvement (Wang *et al.* 2015, Barpete *et al.* 2014a, 2016). A review of previous reports for the last 50 years suggests limited research on improvement and development of breeding strategies for *Lathyrus* (Kumar *et al.* 2013, Hailu *et al.* 2015). Consequently, there is little progress in breeding of the crop to make it safer as a food or forage crop.

Direct regeneration of plants from explants is a faster and a time saving approach for obtaining whole plants without the callus interphase to decrease somaclonal variations (Dhingra *et al.* 2009, Ochatt *et al.* 2013). Direct shoot regeneration or organogenesis from a mature seed embryo explant has been reported in other legumes such as the chickpea (Polisetty *et al.* 1997), mungbean (Harisaranraj *et al.* 2008) and hairy vetch (Aasim *et al.* 2011). Apart from this, there have been a few reports on *in vitro* regeneration of shoots and roots for grass pea (Barik *et al.* 2005, Barpete *et al.* 2014b,c), but *in vitro* multiple shoot induction from mature seed embryo with two cotyledons has to be reported yet. Considering that establishment of regeneration protocols is a fundamental step for the utilization of these methodologies, the main goal of the present work was to develop highly potent *in vitro* regeneration system. The present protocol will be helpful in genetic transformation and biotechnological based grass pea breeding in future.

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Materials and Methods

The seeds of grass pea cv. Gurbuz were obtained from Department of Field Crops, Ankara University, Ankara (Turkey). They were washed in running tap water for a few minutes followed by surface sterilization using 25% H_2O_2 containing three drops of Tween-20 for 10 min. This was rinsed 3 × 5 min with sterile distilled water and dried on sterilized tissue paper. The sterilized mature seed embryos with two cotyledons were inoculated onto MS medium (Murashige and Skoog 1962) containing 0.1 - 2 mg/l BAP, 1 and 2 mg/l NAA or 0.10 - 2 mg/l TDZ with or without 0.10 mg/l IBA supplemented with 30 g/l sucrose and 3 g/l gelrite for shoot multiplication. Thereafter, elongated and multiplied shoots were induced on mature seed embryos with two cotyledon explants for the rooting experiment. The MS medium containing 0.50, 1.0 and 2.0 mg/l IBA was used to evaluate their effects on rooting. All cultures were kept at 25 ± 2ºC in 16/8 hrs light-dark cycle with light intensity of 25 µmol/m^2/s by cool-white fluorescent lamps in the growth chamber. Each replication contained 5 - 6 multiplied shoots and each treatment was replicated thrice.

Healthy plantlets with well developed roots and shoots were selected for acclimatization. They were removed from culture medium, washed in running tap water to remove agar sticking to the roots followed by transfer to plastic pots containing peat moss-perlite (2 : 1). Each vessel or pot was covered with a transparent polyethylene bag to create a high relative humidity; afterwards, the bags were gradually removed. Transplanted plants were maintained under ambient daylight conditions in the greenhouse.

The data were subjected to ANOVA and the post hoc Tukey's b test using SPSS for Windows v. 18, SPSS, USA. Data given in percentages were subjected to arcsine (√X) transformation (Snedecor and Cochran 1967) before statistical analysis.

Results and Discussion

Plant regeneration by seeds is the major *in vitro* tissue culture technique in legume especially *Lathyrus*, because it can provide the possibility to rapid multiplication, gene transfer or somaclonal variations using biotechnology tools (Ochatt *et al.* 2013, Hailu *et al.* 2015). Direct shoot regeneration from a mature seed explant has been reported in other legumes such as the hairy vetch (Aasim *et al.* 2011), narbon vetch (Kendir *et al.* 2009), mungbean (Harisaranraj *et al.* 2008) and chickpea (Polisetty *et al.* 1997).

In this background, cytokinins combined with auxins were tested for multiple shoot induction from mature seed embryos with two cotyledons. The explants cultured on MS basal medium containing BAP (0.10 - 2 mg/l) - NAA (1 and 2 mg/l) or TDZ (0.1 - 2 mg/l) with/without IBA (0.10 mg/l) induced prominent swellings after one week of culture followed by multiple shoot regeneration (Fig. 1a).

The explants showed 100% germination in all MS media followed by single shoot regeneration (Table 1). The results are in agreement with Aasim *et al.* (2011), who noticed swelling of explants in MS medium supplemented with TDZ irrespective of the absence or presence of IBA in the medium, followed by single shoot regeneration after 10 - 12 days. Callus induction appeared late and was recorded only on the radical part of explant. Visual observation of the results showed callus induction on 5 out of 10 MS medium supplemented with BAP and NAA. The present findings are in agreement with Harisaranraj *et al.* (2008), who reported callus induction from half seed explants on media containing cytokinins and auxins in mungbean.

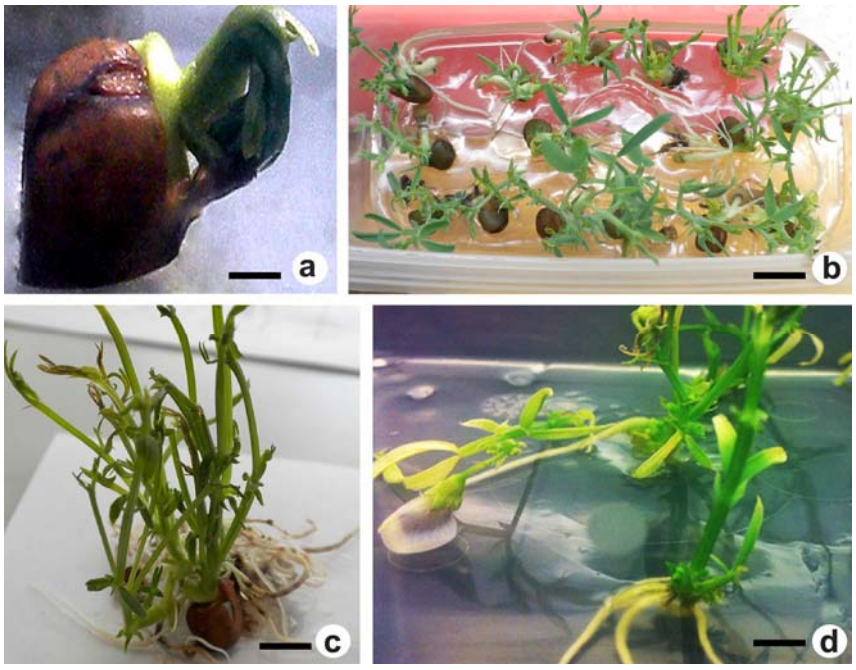

Fig. 1. *In vitro* regeneration from mature embryo with two cotyledons of grass pea: (a) Induced prominent swellings after one week of culture, (b) the maximum shoot regeneration was noted on MS medium containing 2 mg/l BAP and 1 mg/l NAA, (c) maximum number of shoot per explant were induced on MS medium containing 2 mg/l BAP - 1 mg/l NAA after two subcultures and (d) maximum number of roots per explant was noted on rooting medium containing 2 mg/l IBA with scanty flowering. (Bar Fig. 1a = 0.2 cm, Fig. 1b = 1.3 cm, Fig. 1c = 0.5 cm, Fig. 1d = 0.6 cm).

Table 1. Effect of MS medium supplemented with BAP (0.10 - 2.0 mg/l) and NAA (1 - 2 mg/l) on multiple shoot induction from embryo cotyledons of *L. sativus*.

MS + growth regulators		Shoot regeneration (%)	Number of shoots/explant	Shoot length	Callus morphology
BAP (mg/l)	NAA (mg/l)				
0.10	1.0	33.86 d	2.76 c	10.12 a	-
0.10	2.0	33.30 d	3.94 c	7.96 a,b	-
0.25	1.0	54.98 c, d	3.86 c	7.73 a,b	-
0.25	2.0	66.60 a,b,c	4.76 b,c	5.72 b,c	+
0.50	1.0	53.83 c,d	6.02 b,c	4.99 b,c	+
0.50	2.0	61.53 b,c	4.55 b,c	7.33 a,b	+
1.0	1.0	75.16 a,b,c	5.42 b,c	6.41 b,c	-
1.0	2.0	79.76 a,b	7.74 b	3.56 c	+
2.0	1.0	89.80 a	15.83 a	3.70 c	-
2.0	2.0	75.50 a,b,c	5.73 b,c	3.73 c	++

Values with in a column followed by different letters are significantly different ($p < 0.01$) using Tukey's b test; Callus morphology: - = Nil; + = Low; + + = High.

On the other hand, MS medium containing TDZ singly inhibited callus phase, while 2 out of 5 culture media containing TDZ supplemented with IBA induced low or poor callus. Similar results have also been reported by Aasim *et al.* (2011). They also noted very low callusing in hairy vetch using seed explants cultured on TDZ-IBA. A review of previous literature shows that BAP and TDZ are generally used for *in vitro* shoot regeneration and multiplication singly or in combination with auxins in legumes especially in *Lathyrus* (Barik *et al.* 2005, Barpete *et al.* 2014b).

The present study showed that variable multiple shoot induction frequency (%) on MS medium containing BAP - NAA or TDZ - IBA. Multiple shoot regeneration started from embryonic axis of seed explants. Shoot regeneration was not observed on the cotyledon portions. Comparing the shoot induction frequency, it varied on both tested media with range of 33.30 - 89.80% and 19.73 - 68.86% on MS medium containing BAP-NAA and TDZ - IBA, respectively. It was observed that multiple shoot induction was strongly influenced by higher concentration of the BAP with NAA. The maximum shoot regeneration frequency was noted on MS medium supplemented with 2 mg/l BAP and 1 mg/l NAA (Fig. 1b). All regenerated shoots were induced on embryos with two cotyledons explants are in agreement with Polisetty *et al.* (1997). They also achieved shoot/shoot bud differentiation on mature embryos with two cotyledon explants of chickpea. Multiple shoot regeneration on embryonic axes with no regeneration from cotyledons is in line with Kendir *et al.* (2009).

An analysis of results showed that mean number of shoots per explant showed inconsistent behavior and ranged 2.76 to 15.83 on MS medium containing BAP - NAA. Maximum number of shoots (15.83) per explant with 3.70 cm long shoots was induced on MS medium containing 2.00 mg/l BAP - 1 mg/l NAA after two subcultures with maximum number of rooting (Fig. 1c). The mean number of shoot regeneration was significantly ($p < 0.01$) higher on MS medium containing 2 mg/l TDZ - 0.1 mg/l IBA compared to 2 mg/l TDZ used singly.

Table 2. Effect of MS medium supplemented with TDZ (0.10 - 2.0 mg/l) - IBA (0.10 mg/l) on multiple shoot induction from mature seed embryo with two cotyledons of *L. sativus*.

MS + growth regulators		Shoot regeneration (%)	Number of shoots/explant	Shoot length	Callus morphology
TDZ (mg/l)	IBA (mg/l)				
0.10	-	28.94 e,f	1.50 d,e	9.70 a,b	-
0.25	-	42.46 c,d,e	1.83 d,e	5.46 b,c	-
0.50	-	65.80 a,b	2.93 b,c,d	4.96 c	-
1.0	-	54.83 a,b,c,d	3.86 b	4.66 c	-
2.0	-	44.70 b,c,d,e	2.26 c,d,e	3.83 c	-
0.10	0.10	19.73 f	1.43 e	10.00 a	-
0.25	0.10	49.43 a,b,c,d,e	2.53 b,c,d,e	7.50 a,b,c	-
0.50	0.10	33.93 d,e,f	1.96 d,e	6.76 a,b,c	+
1.0	0.10	68.86 a	5.46 a	4.66 c	+
2.0	0.10	59.03 a,b,c	3.63 b,c	4.93 c	-

Values with in a column followed by different letters are significantly different ($p < 0.01$) using Tukey's b test; Callus morphology: - = Nil; + = Low; ++ = Moderate.

Contrarily, Sahin-Demirbag *et al.* (2008) also reported maximum shoot regeneration on MS medium containing 0.45 mg/l TDZ and their results suggested that a further increase in TDZ concentration inhibited shoot regeneration in the Hungarian vetch. The mean shoot length ranged

3.56 - 10.12 cm and 3.83 - 10 cm on MS medium with BAP - NAA and TDZ - IBA, respectively (Table 2).

The longest shoots (10.12 cm) were recorded on MS medium containing 0.1 mg/l BAP with 1 mg/l NAA. These results are in agreement with the findings of Aasim *et al.* (2009), who reported that shoot length was dependant on cytokinin type and concentration and presence or absence of auxin in the culture medium. Shoot length on MS culture medium containing BAP-NAA gradually increased with increase in BAP concentration. Brar *et al.* (1997) also reported the promotery effect of auxins in the culture media on shoot length in cowpea. On the other hand, the mean shoot lengths decreased with the each increased concentration of TDZ with/without IBA in the culture medium (Table 2). As reported by Aasim *et al.* (2009) each increase of TDZ concentration decreased the shoot length in Fenugreek. Sahin-Demirbag *et al.* (2008) also reported a maximum shoot length at 0.05 mg/l TDZ in Hungarian vetch. Kendir *et al.* (2009) also reported a decreased shoot length of the seed explant (zygotic embryo with two cotyledons) of Narbon vetch with increasing cytokinin concentrations.

The results suggested that MS medium (control) and 0.5 mg/l IBA were not appropriate for rooting; whereas, 51.85 and 70.37% rooting was noted on MS medium containing 1 and 2 mg/l IBA, respectively (data not shown). Maximum number of 3.9 roots per explant with root length of 2.3 cm was noted on rooting medium containing 2 mg/l IBA. The results are in partial agreement with Barpete *et al.* (2010); who reported maximum root induction (80%) on grass pea axillary shoots using 0.75 mg/l IBA medium supplemented with 100 mg/l activated charcoal. Barpete *et al.* (2014 a, b) emphasize that auxins must be used for root initiation in grass pea. However, Sahin-Demirbag *et al.* (2008) and Kendir *et al.* (2009) suggest pulse treatment of IBA for successful rooting in legumes. Subculture of regenerated plantlets when transferred on MS medium containing 2 mg/l IBA showed morphogenetic differentiation after two weeks of culture and induce scanty flowering (Fig. 1d) that continued flowers in the rooting medium and greenhouse. The rooted plantlets were acclimatized in the substrate containing peat moss-perlite in a 2 : 1 ratio. This may be due to the good water-retaining capacity of these substrates (Barpete *et al.* 2014a, b). The protocol will be useful for direct organogenesis by using mature seed embryos with two cotyledons as explants. The protocol can be used in future for breeding activities and genetic transformation studies in important but neglected plant of grass pea.

References

Aasim M, Khawar KM, Sancak C and Özcan S 2009. *In vitro* shoot regeneration of Fenugreek (*Trigonella foenumgraceum* L.). Am-Eu. Sustain. Agric. **3**: 135-138.

Aasim M, Sahin-demirbag N, Khawar KM, Kendir H and Ozcan S 2011. Direct axillary shoot regeneration from the mature seed explant of the hairy vetch (*Vicia villosa* Roth). Arch. Biol. Sci, Belgrade **63**: 757-762.

Barik, DP, Mohapatra U and Chand PK 2005. High frequency *in vitro* regeneration of *Lathyrus sativus* L. Biol. Plant. **49**: 637-639.

Barpete S 2015. Genetic associations, variability and diversity in biochemical and morphological seed characters in Indian grass pea (*Lathyrus sativus* L.) accessions. Fresenius Environm. Bullet. **24**: 1-6.

Barpete S, Aasim M, Khawar KM, Özcan SF and Özcan S 2014a. Preconditioning effect of cytokinins on *in vitro* multiplication of embryonic node of grass pea (*Lathyrus sativus* L.). Turk. J Biol. **38**: 485-492.

Barpete S, Khawar KM and Özcan S 2014b. Differential competence for *in vitro* adventitious rooting of grass pea (*Lathyrus sativus* L.). Plant Cell Tiss. Org. Cult. **119**: 39-50.

Barpete S, Khawar KM, Özcan SF, Sancak C and Özcan S 2016. Evaluation of toxicity levels of micronutrient and macronutrient strengths on regeneration of grasspea under *in vitro* condition. International Conference on Pulses, Marrakesh, Morocco, 18-20 April, 2016. pp. 146-147.

Barpete S, Parmar D and Sharma NC 2010. Callus culture and plantlet formation using axillary shoot explant of grass pea (*Lathyrus sativus* L.). *In*: Pandey, B.N, S.P. Singh and R. Singh. (eds) Sustainable Management and Conservation of Biodiversity, Narendra Publishing House, New Delhi. pp. 55-68.

Barpete S, Sharma NC and Kumar S 2014c. Assessment of somaclonal variation and stability in vitro regenerated grass pea plants using SDS-PAGE. Legume Res. **37**: 345-352.

Brar MS, Al-Khayri JM, Shamblin CE, McNew RW, Morelock TE and Anderson EJ 1997. *In vitro* shoot tip multiplication of cowpea *Vigna unguiculata* (L.) Walp. In vitro Cell. Dev. Biol. **33**: 111-118.

Dhingra M, Barpete S, Parmar D and Sharma NC 2010. High frequency plant regeneration in *Carthamus tinctorius* L. Search Res. **2**: 112-116.

Hailu D, Abera S and Teka TA 2015. Effects of Processing on Nutritional Composition and Anti-Nutritional Factors of Grass pea (*Lathyrus sativus* L): A Review**.** *Food Sci. Qual. Manag.* **36**: 61-70.

Harisaranraj R, Babu SS and Suresh K 2008. Callus induction and plant regeneration of *Vigna mungo* (L.) Hepper via half seed explant. Ethnobot. Leaflets **12**: 577-585.

Kendir H, Sahin-Demirbag N, Aasim M and Khawar KM 2008. *In vitro* plant regeneration from Turkish Narbon Vetch (*Vicia narbonensis* L.). Afr. Biotech. **8**: 614-618.

Kumar S, Bejiga G, Ahmed S, Nakkoul H and Sarker A 2011. Genetic improvement of grass pea for low neurotoxin (ODAP) content. Food Chem. Toxicol. **49**: 589-600.

Kumar S, Gupta P, Barpete S, Sarker A, Amri A, Mathur PN, and Baum M 2013. Grass pea. *In*: Singh M, Upadhyaya HD and Bisht IS (eds) Genetic and Genomic Resources for Grass pea Improvement. pp. 269-293.

Murashige T and Skoog F 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. **15**: 473-497.

Ochatt SJ, Conreux C, and Jacas L 2013. Flow cytometry distinction between species and between landraces within *Lathyrus* species and assessment of true-to-typeness of *in vitro* regenerants. Plant Syst. Evol. **299**: 75-85.

Polisetty R, Paul V, Deveshwar JJ, Khetarpal S, Suresh K and Chandra R 1997. Multiple shoot induction by benzyladenine and complete plant regeneration from seed explants of chickpea (*Cicer arietinum* L.). Plant Cell Rep. **16**: 565-571.

Sahin-Demirbag N, Kendir H, Khawar KM and Aasim M 2008. *In vitro* plant regeneration from Hungarian vetch (*Vicia pannonica* Crantz) using cotyledonary node explants. Biotechnol. Biotechnol. Eq. **22**: 929-932.

Snedecor GW and Cochran WG 1967. Statistical methods. The Iowa State University Press, Iowa, USA. pp. 327-329.

Wang F, Yang T, Burlyaeva M, Li L, Jiang J, Fang L, *et al.* 2015. Genetic diversity of grass pea and its relative species revealed by SSR markers. PLoS One **10**: 1-14.

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