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EFFECT OF PLANT GROWTH REGULATORS AND PHYSICAL FACTORS ON IN VITRO HIGH FREQUENCY REGENERATION OF GRASS PEA

S. Barpete[1*], S. F. Özcan[2]., K. M. Khawar[1] and S. Özcan[1]

[1]Department of Field Crops, Faculty of Agriculture, Ankara University, Ankara, Turkey;
[2]Central Research Institute for Field Crops, Ministry of Food, Agriculture and Livestock, Ankara, Turkey
[*]Corresponding author's email: surendrabarpete@gmail.com

ABSTRACT

Grass pea (*Lathyrus sativus* L.) is highly nutritious and protein rich legume crop; however, presence of ODAP (β-N-Oxalyl-L-α, β-diaminopropionic acid) has restricted its use for human and animal consumption. There is need for accelerated breeding of low or zero ODAP cultivars to ensure safer and wider use of grass pea through conventional and biotechnological approaches. The study investigated solidified MS medium containing a combination of thidiazuron (TDZ) and α-naphthalene acetic acid (NAA) concentrations for improved regeneration of grass pea using stem with two nodes (binodal) explant under photosynthetic photon flux density (PPFD) of 20 µmol m^{-2} s^{-1} light intensity. The results showed that combination of low light intensity, phytagel as gelling agent and TDZ - NAA was very effective for high frequency shoot regeneration after 3 weeks culture. Maximum number of 37.6 shoots per explants was noted on MS medium containing 0.75 mg/L TDZ - 0.25 mg/L NAA, with 96.66% shoot induction. Healthy, well growing shoots were rooted on ½ × MS medium (micro, macro elements and vitamins) containing 0.25 mg/L Indole-3-butyric acid (IBA) that resulted in 83.60% rhizogenesis. Peat moss potted plants induced hundred percent acclimatization and survival. Compared to other rooting substrates, peat moss acclimatized plants were more vigorous, flowered and set seeds under glass house conditions. High regeneration and acclimatization percentage signifies importance of this work for biotechnological based grass pea breeding.

Key words: Binodal stem; grass pea; *in vitro; Lathyrus sativus* L.; Light intensity; Phytagel.

INTRODUCTION

Legumes are major source of dietary proteins of people worldwide specially those living in the South East Asian; the Indian Ocean and the Mediterranean region countries. The genus Lathyrus includes 187 annual or perennial species and Turkey is an important center for *Lathyrus* with 73 taxons, 23 of which are endemic (Basaran and Acar, 2014). Among grain legumes, high protein grass pea (*Lathyrus sativus* L. 2n = 2x = 14) plants occupy a prominent position (Barpete *et al.*, 2012).

Grasspea is one of the species that widely cultivated as a food and forage crop in the genus Lathyrus, whereas other species *L. cicera* and *L. ochrus* are cultivated to a less significant amount (Barpete, 2015). Moreover, it has great agronomic potential as a forage and grain legume in the fragile agro-ecosystems, because of its ability to tolerant adverse climatic conditions like drought, heat, cold, flood and salinity. It is also highly resistant to insect pests (Kumar *et al.*, 2013; Piwowarczyk *et al.* 2016). The crop has not made much progress in terms of genetics and genomic developments. It is often included among underutilized and neglected crops primarily owing to presence of β-N-Oxalyl-L-α,β-diaminopropionic acid (ODAP) in seeds. ODAP cause a neurological disorder (Lathyrisum) in human and animal limbs (Khawar *et al.*, 2010; Kumar *et al.*, 2011; Onar *et al.*, 2014).

Biotechnological approaches for improvement of grass pea are limited due to recalcitrant nature of grass pea (Khawar *et al.*, 2010; Barpete *et al.*, 2014 a, b). Delgado-Montero and Moreno (1985) used induced callus using various tissues. Contrarily, Gharyal and Maheshwari (1980); Mukhopadhyay *et al.*, (1980); Sinha *et al.*, (1983); van Dorrestein *et al.*, (1998) used juvenile tissues for regeneration of buds and shoots from callus. Development of genetic and genomic resources is of paramount importance for grass pea improvement (Kumar *et al.*, 2013). It would be desirable to develop methodologies for improved regeneration of grass pea cultivars. It is understood that light and kind of gelling agent play an important role in plant development and regeneration of recalcitrant plants belonging to leguminosae. Previous report use light intensity of more than 35 µmol m^{-2} s^{-1} and agar as gelling agent that inhibits regeneration potential of the plant. This study reports effects of reduced light intensity and phytagel on regeneration from binodal stem explant of grass pea for the first time. Establishment of a protocol will be helpful in genetic transformation and biotechnological based grass pea breeding in future.

MATERIALS AND METHODS

Seed sterilization, explant selection and shoot multiplication: The seeds of grass pea cv. Gurbuz were obtained from the Central Field Crop Research Institute, Ankara, Turkey. They were treated with 70% (v/v) ethanol for 1 min and 30 % (v/v) H_2O_2 (hydrogen peroxide) for 20 min to achieve surface sterilization. They were rinsed 3×5 min with sterile distilled water and blotted on tissue paper. The seeds were germinated in test tubes containing paper bridges in water culture. Binodal (stem with two nodes) explant were sectioned (Fig. 1a) from one week old growing seedlings under aseptic conditions and cultured on MS (Murashige and Skoog, 1962) regeneration medium supplemented with 3% (w/v) sucrose and 0.3% (w/v) Phytagel™. These were manipulated with 5 combinations of Thidiazuron (0.50, 0.75, 1.0 and 2.0 mg/L) with or without 0.25 mg/L α-Naphthalene acetic acid (NAA) contained in petri dishes (100 × 10 mm) or Magenta™ GA7 culture vessels containing 25 and 35 mL regeneration medium respectively.

Observations were recorded for callus induction, frequency of shoot induction, number of shoots per explant and shoot length after 3 weeks of culture. Total number of 24 explants was cultured on each treatment divided into three replicated groups. All culture media were autoclaved for 20 min at a temperature of 121 °C, 1.06 kg cm−2 after adjusting pH to 5.7 ± 0.1 with 1 M NaOH or 1 M HCl. All cultures were grown at 25 ± 2 °C with 16 h light and 8 h dark photoperiod. Light intensity was maintained at photosynthetic photon flux density (PPFD) of 20 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps.

Rooting: Vigorous and well growing shoots were used for rooting on ½ ×MS (micro, macro elements and vitamins) medium with or without 0.25, 0.50 and 1.0 mg/L Indole 3-butyric acid (IBA) to evaluate their effects on rooting. Total number of 24 explants was cultured on each rooting treatment divided into three replicated groups. Data on root induction (%), number of roots per explant and root length were recorded after three weeks of culture.

Acclimatization: The healthy plantlets of 6 to 8 cm length with well-developed roots and shoot were taken from the culture for acclimatization. They were washed in running tap water to remove phytagel sticking to roots before transfer to plastic pots (12 cm) containing 1 dm^3 each of peat moss and peat moss: clay loam soil.

Peat moss had and EC of 0.1 dS m^{-1}, porosity of about 68.5 % (v/w) and pH 6.0 and had low bulk density of 0.1 mg m^{-3} that allowed high water absorption Clay loam soil used in the study had 30% (w/w) sand and 40% (w/w) clay with 53% water saturation percentage. It had EC 1.24 dS m^{-1}, CEC of 30 cmol/kg and pH of 7.56. It contained 0.07% total nitrogen, 1.34% organic matter (w/w), and 0.78%, organic carbon 0.04% (w/w) total salts, , 5.18% (w/w) lime, 1746.7 kg ha^{-1}potassium and 137.3 kg ha^{-1} phosphorus. Each pot was covered with a transparent polyethylene bag to create a high relative humidity. The polythene bags were gradually opened after the plants began to grow. The transplanted plants were maintained under 12 h ambient daylight conditions in the greenhouse.

Statistical analysis: Data given in percentages were converted to square root (√X) transformation for statistical analysis (Snedecor and Cochran 1967). All data were subjected to one-way analysis of variance (ANOVA, SPSS for Windows v. 12.0, SPSS, USA), followed by Tukey's b test to compare means. The treatments were arranged in a completely randomized design.

RESULTS

Multiple shoot induction: Grass pea binodal stem explant behaved variably on MS medium containing ten different combinations of TDZ (0.25 - 2.0 mg/L) with or without NAA (0.25 mg/L). The analysis of variance results testified that plant growth regulators significantly ($p \leq 0.01$) affected frequency (%) of callus induction ($F= 13.55$; $df = 9, 20$), number of shoots per explant ($F= 3.55$) and shoot length ($F= 7.20$). No callus but shoot induction was noted on any concentration of TDZ containing medium in the absence of NAA (Table 1). Apart from this, callus mediated shoot regeneration was registered on all regeneration media containing TDZ - NAA; where frequency of callus induction ranged 27.7 to 47.5 %. Maximum callus induction was noted on MS medium containing 1.00 mg/L TDZ - 0.25 mg/L NAA. TDZ plus NAA promoted shoot regeneration compared to TDZ used singly. The shoot induction ranged 56.6 - 94.4 % with maximum shoot induction on MS medium containing 0.75 mg/L TDZ when TDZ was used singly. The shoots per explant changed from 7.3 to 24.3 with maximum numbers of shoots on MS medium containing 1 mg/L TDZ. Whereas, when variants of TDZ with 0.25 mg/L NAA were used the shoot induction ranged 81.1 – 96.6% with maximum shoot induction on MS medium containing 0.75 mg/L TDZ – 0.25 mg/L NAA. The shoots per explant ranged 14.6 to 37.6 and maximum numbers of shoots (Fig. 1b) on MS medium containing 0.75 mg/L TDZ - 0.25 mg/L NAA.

Low light was considerably effective to promote abundant shoot regeneration on MS medium containing TDZ with 0.25 mg/L NAA. Shoot organogenesis was less promoted when auxin was omitted. Presence of 0.25 mg/L NAA significantly promoted shoot regeneration and induction of shoots on all explants.

Shoot length ranged 2.2 to 3.1 cm and had inconsistency on MS medium containing variants of TDZ with maximum shoot length of 3.1 cm. Each increasing dose of TDZ with 0.25 mg/L NAA was inhibiting and resulted in reduced shoot length. Whereas, shoot length on MS medium containing TDZ plus NAA ranged 2.9 to 5.4 cm. Maximum shoot length (5.4) was noted on MS medium containing 0.25 mg/L TDZ- 0.25 mg/L NAA. The explants cultured on 1.0 & 2.0 mg/L TDZ and 0.75, 1.0 & 2.0 mg/L TDZ with 0.25 mg/L NAA beyond maximum required culture period (3 weeks) tended to induce partial hyperhydricity with gradually developing chlorosis on shoots (Fig. 1c) hyperhydric in range of 4.4 to 22.8 %. The affected explants were transferred to MS medium containing 1.5 % sucrose to recover the plants.

Rooting and *ex-vitro* acclimatization: The analysis of variance results indicated significant differences ($p \leq 0.01$) among treatment means for root induction percentage. The rooting started on 1/2 × MS medium containing 0.25, 0.50 & 1.0 mg/L IBA after one week of culture (Table 2). No root induction was noted on ½ × MS medium in the absence of IBA (control). Increasing concentrations of IBA in ½ × MS medium had significant inhibiting effects on root induction. The average root induction percent ranged 24.32 to 83.63% with maximum induction on 0.25 mg/L IBA containing rooting medium (Fig. 1d). Moreover, IBA - a rooting plant growth regulator also induced variable number of shoots on lower node of cultured shoots in the rooting media. Mean number of roots per explant were significantly affected by increasing concentrations of IBA in half strength MS medium. The average number of roots per explant varied 2.46 to 6.33 per rooted shoot. The longest roots were recorded on ½ × MS medium containing 0.25 mg/L IBA. Each increasing concentration of IBA in the rooting media had sharply negative effect on root length. The root length increased from 1.56 to 5.03 cm with the longest roots (5.03 cm) on ½ × MS medium containing 0.25 mg/L IBA.

Healthy plantlets with well-developed roots and shoots were transferred for acclimation. Comparing acclimated plants on peat moss and peat moss: clay loam soil, the plant health, rate of growth and survival percentage on peat moss acclimation substrate was better compared to peat moss: clay loam soil mixture substrate grown plants in the glass house. The optimum survival percentage on peat moss remained 100% (Fig. 1e) and that on peat moss: clay loam soil, it remained 52%.

The plant acclimated on peat moss had strong root system with approximately 0.1 to 0.2 cm thick roots that tapered to hair like structures at growing ends by developing filamentous roots with lateral spread. All mature regenerated plants induced flowers, pod formation with induction of seeds (Fig. 1f) that were morphologically similar to the seeds obtained on non tissue cultured plants.

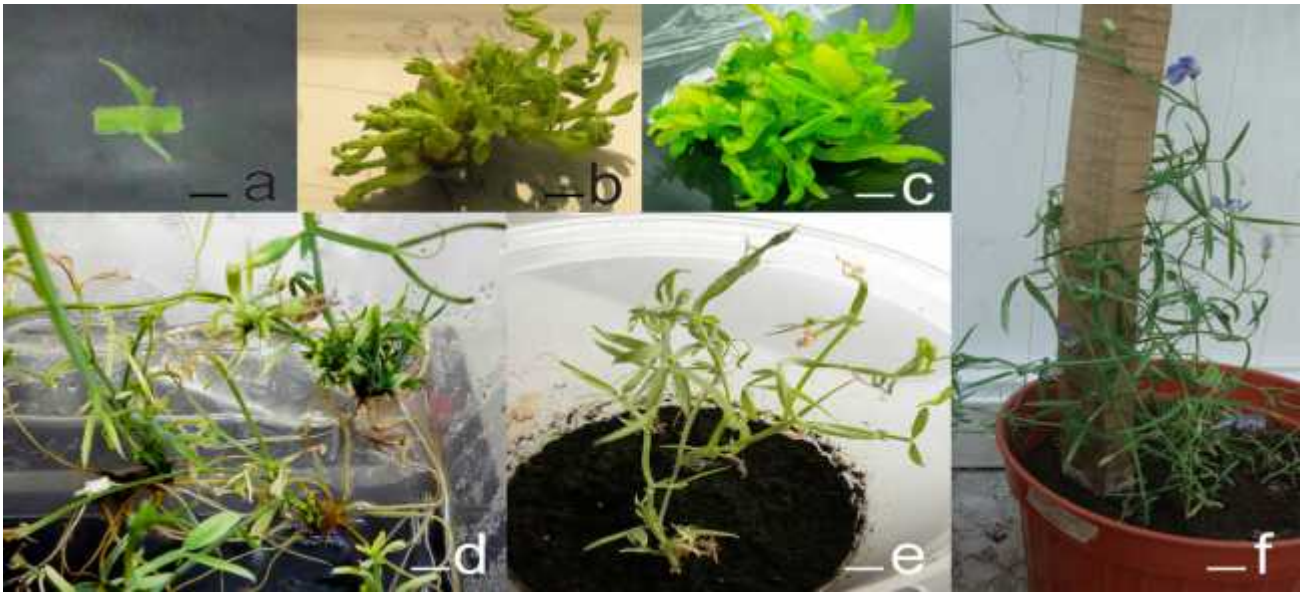

Fig. 1. *In vitro* plant regeneration in grass pea cv. Gurbuz. (a) Binodal stem explant, (b) Maximum numbers of 37.6 shoots noted on MS medium containing 0.75 mg/L - 0.25 mg/L NAA, (c) induction of multiple clumped, hyperhydric and chlorotic shoots on explants after 42 d of culture, (d) root induction on half strength MS medium containing 0.25 mg/L IBA, (e) acclimatized grass pea plant flourishing on peat moss substrate contained in pots, (e) The mature plant bearing flower and pods containing seeds after acclimatization under greenhouse conditions. Bar of Fig. 1a, b, c, d = 0.5 cm, Fig. 1 e = 1 cm, f = 1.7 cm

Table 1. Effect of MS medium containing a combination of TDZ (0.25 – 2.00 mg/L) and NAA (0.25 mg/L) on multiple shoot induction from binodal stem explant of *L. sativus.*

Treatments		Callus induction frequency (%)	Shoots induction Frequency (%)	Number of shoots/ explant	Shoot length (cm)	Hyperhydricity (%)
TDZ mg/L	NAA mg/L					
0.25	-	0.0 [b]	56.6	7.3 [b]	2.6 [bc]	0.0
0.50	-	0.0 [b]	58.8	14.0 [b]	2.2 [c]	0.0
0.75	-	0.0 [b]	94.4	19.3 [ab]	2.2 [c]	0.0
1.00	-	0.0 [b]	88.8	24.3 [ab]	2.6 [bc]	4.4
2.00	-	0.0 [b]	88.0	20.3 [ab]	3.1 [bc]	6.6
0.25	0.25	44.4 [a]	81.1	14.6 [b]	5.4 [a]	0.0
0.50	0.25	27.7 [a]	85.7	22.0 [ab]	5.1 [a]	0.0
0.75	0.25	40.0 [a]	96.6	37.6 [a]	4.6 [ab]	20.0
1.00	0.25	47.5 [a]	89.6	27.0 [ab]	3.5 [abc]	18.9
2.00	0.25	27.7 [a]	77.7	23.0 [ab]	2.9 [bc]	22.8

Each value is the mean of three replicates each with 8 explants
Values with in a column followed by different letters are significantly different ($p<0.01$) using Tukey's b test.

Table 2. The effects of half strength MS medium containing different concentrations of IBA on rooting of the shoots obtained from binodal stem explants

IBA Treatments (mg/L)	Root induction (%)	Number of roots per explant	Root length (cm)
Half MS medium*(Control)	0.00	0.00	0.00
0.25	83.63 [a]	6.33 [a]	5.03 [a]
0.50	63.50 [b]	3.66 [b]	2.36 [b]
1.00	24.32 [c]	2.46 [c]	1.56 [c]

Each value is the mean of three replicates each with 8 explants
b Values with in a column followed by different letters are significantly different ($p < 0.01$) using LSD test
* Half concentrations of macro, micro elements and vitamins of MS medium

DISCUSSION

Plant tissue culture techniques provide possibility to introduce new approaches for direct regeneration depending on strong competence of the genotype and *in vitro* culture conditions (Ochatt *et al.,* 2013).

The present study suggested that paper bridge water culture was better for grass pea seeds germination with no contamination in agreement with Barpete *et al.* (2014a). Lathyrus genotypes are recalcitrant to regeneration *in vitro* (Vaz Patto *et al.,* 20; Ochatt *et al.,* 2013; Piwowarczyk *et al.* 2016). MS medium supplemented with 3.42 µM TDZ plus 1.34 µM NAA provided optimum and high frequency shoot regeneration on binodal stem explant in the present study. Aforementioned, medium along with plant growth regulator combinations also encouraged induction of maximum number of shoots per explant in agreement with Kendir *et al.,* (2009). The researcher emphasized that MS medium containing TDZ was more potent to induce shoot regeneration on immature zygotic embryos. Similarly, Sahin-Demirbag *et al.,* (2008) also noted shoot regeneration from immature zygotic embryos of *L. cicera* using TDZ and 6-Benzylaminopurine (BAP)-NAA. In contradiction, Barik *et al.,* (2004) emphasized that BAP was more favorable for shoot regeneration compared to TDZ on agar gelled regeneration medium under 35 µmol m^{-2} s^{-1} light intensity provided by cool white fluorescent lamps. The present study reports 37.6 shoot per explant without any subculture in three weeks time on culture media gelled with phytagel under light intensity of 20 µmol m^{-2} s^{-1}. Whereas, Barik *et al.,* (2004) noted average number of ten to eleven shoots per explants after 3 - 4 subcultures of nine weeks to regenerate shoots. If we compare this study (using phytagel with previous study by Barik *et al.,* (2004) who used agar - , the possibility of increasing grass pea shoot regeneration competence to approximately four folds is very visible.

Possible explanation to this distinct variability in two results could be different types of positive or negative stresses due to genotypes, type of explant, gelling agent and the light intensity used in two studies that resulted array of changes on the explants in a complex way. It is well known that high doses of visible light induce oxidative stress in plant cells (Watanabe *et al.,* 2006) with increased release of phenolic compounds that hinder growth and development on micropropagated

tissues (Kirdmanee *et al.,* 1992, Li *et al.,* 2002). Stem segments on both sides of nodes inhibited development of phenolic compounds that provided amiable atmosphere for profuse regeneration on binodes. Furthermore, phytagel as gelling agent also provided an atmosphere aided by physiochemical characteristics for better nutrient diffusion rate in agreement with Nairn *et al.,* (1995) and Ozel *et al.,* (2008).

This frequency and induced number of shoots per explant is noticeable and exceptionally higher compared to that reported for grass pea by Sinha *et al.,* (1983); Barik *et al.,* (2005) and Kendir *et al.,* (2009). The present study further witness that shoots regeneration competence of TDZ increased considerably, when the regeneration medium also contained NAA. The results are in agreement with the findings of Yucesan *et al.,* (2007), who reported complimentary effects of TDZ with IAA on shoot regeneration from witloof chicory (*Cichorium intybus*).

The results clearly demonstrate importance of IBA in rooting of grass pea. The present study showed 83.33% rooting on ½ × MS medium containing 0.25 mg/L IBA that was optimum concentration for rooting. No rooting was noted on shoots cultured on half strength MS medium without IBA (control) in the present study. Increased concentration of IBA (1.0 mg/L) was inhibitory to rooting. The results are in agreement with Aasim *et al.,* (2009, 2013) who suggest that IBA was the most effective PGR in inducing rooting in cowpea and chickpea. Similar results were also reported by Jayanand *et al.,* (2003) in chickpea. Barpete *et al.,* (2010) reported 80 % root induction using 0.75 mg/L IBA in combination with 100 mg/L activated charcoal using axillary shoot explants of grass pea. In contrast, Barik *et al.,* (2005) reported rhizogenesis in 78 % of regenerated shoot of *L. sativus* cv. IC120487 on MS medium containing 2.85 µM indole-3-aceticacid.

The hyperhydric explants were transferred to MS medium containing 1.5 % sucrose to recover the plants. The results are in agreement with Barpete *et al.* (2014a). They observed that relatively higher or lower osmotic pressure caused sucrose concentrations. It is well established that osmotic pressure results in positive or negative water stress that causes increased or decreased photosynthesis in plants. Present findings have edge over previous studies in terms of rooting frequency, number of roots per rooted shoot and the mean root length of growing roots. These are also in agreement with Gurel and Wren (1995); Khawar *et al.,* (2004) and Aasim, (2012), who emphasize that auxin is necessary for the acquisition of the meristematic competence of the responsive cells. Once this competence has been established, excessive auxin concentrations are often inhibitory.

One of the major problems is that most *in vitro* derived plants experience a desiccation shock just after transplantation (Kozai, 1991; Barpete *et al.,* 2014b) that could be reduced to considerable extent by submerging *in vitro* raised plants in water for 1 to 4 h that helps in easy procuring of plants capable of surviving outside culture vessels. Strong root system of plants acclimated on peat moss helped in faster acclimation and better growth of plants due to highly efficient penetrating filamentous roots that absorbed more nutrients at the time of watering (Barpete *et al.,* 2014b). As both water and mineral cations adsorbed to negatively charged peat moss particles are released and show high availability. Other factors like pH, CEC, EC and porosity of peat moss also acted positively. Combination of all these factors lead to good growth of roots and consequently leaf area development; which had bearing on pod formation and acclimatization of plants as well. The results of the study are in agreement with Arvidsson (1999). Zamanidis *et al.,* (2013) who suggested that good growth depend on composition and quality of the substrate. Stronger root system also affected varied growth of crown development with development of greater number of leafy branches on above ground parts in positive root shoot interrelationship. This interrelationship was more bearing on peat moss with early flowering, pod and seed set.

Extension of this study in meaningful way will help to accelerate breeding low or zero ODAP cultivars in future. The present investigation is a preliminary study that put forwards possibilities of rapid and increased proliferation to *in vitro* conditions by offering a novel ways to improve grass pea regeneration and genetic transformation.

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