Jahangirnagar University J. Biol. Sci. **4**(2): 1-8, 2015 (December)

In vitro Regeneration of Grass Pea (*Lathyrus sativus* L.)

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Abstract

In vitro regeneration protocol for grass pea (*Lathyrus sativus* L.) was optimized using different concentrations and combinations of growth regulators. Direct shoot regeneration obtained through shoot organogenesis from different explants of grass pea cultured on MS medium supplemented with Gamborg B_5 vitamin containing 6-benzylaminopurine (BAP), Thidiazuron (TDZ) and α-naphthalene acetic acid (NAA). Highest percentage of shoots were obtained at 4.0 mg/l of BAP on nodal explants. Stunted multiple shoots were developed from nodal explants while 1.5 mg/l TDZ was used. About 56% of direct shoots were also obtained, while the combination of BAP (4.0 mg/l) and NAA (0.5 mg/l) were used. Regenerated plantlets were rooted most effectively (40%) in rooting medium containing half strength of MS basal medium containing 1.0 mg/l NAA. Well rooted plantlets were further successfully acclimatized to ambient humidity level and grown in controlled environment until hardening.

Key words: Acclimatization, direct shoot regeneration, grass pea, organogenesis, root initiation.

INTRODUCTION

Grass pea (*Lathyrus sativus* L.) is a leguminous crop plant cultivated extensively in various parts of the world especially the food-deficit countries due to its qualities of being a very cheap source of diet protein (Kenicer *et al.*, 2005). It is considered as fodder plant along with its excellent N_2-fixation properties and ever increasing global demand for food and feed (Tamburino *et al*., 2012; Makoi & Ndakidemi, 2011). It is a self-pollinated, annual and herbaceous legume rich in protein belonging to the Fabaceae family and Vicieae tribe (Biswas & Biswas, 1997). It is a native at Southern Europe and Western Asia. It is also found in North Africa, North America, Temperate South America and East Africa (Smartt, 1990). It is an efficient nitrogen fixer and improves soil fertility by adding around 67 kg/ha of nitrogen in a single season, thereby conferring yield and protein benefits for the subsequent non-legume crop (Wang *et al*., 2000).

It is one of the most important Rabi pulses in Bangladesh. Locally, it is known as Kheshari in Bangladesh (Banglapedia, 2014). It occupies the first position in terms of area and production among pulses in Bangladesh. Seed yield is about 385 kg/ha and it is cultivated in about 2,12,313 ha of land, and the total annual production is about 81,705 metric tons (BBS, 2009-2010).

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Although grass pea is endowed with many good features, the production of the species has not yet improved significantly due to its prominent susceptibility to biotic and abiotic stresses (Jaiwal & Gulati, 1995; Sahoo & Jaiwal, 2008). Physiological and molecular mechanism of its adaptation to different abiotic stresses are still not well studied (Piwowarczyk *et al*., 2015). Therefore, it shows significant yield losses every year. The potentiality of the *Lathyrus* species has not been fully utilized due to the presence of a neurotoxin, ß-N-oxalyl-L-a, ß-diaminopropionic acid (ODAP/BOAA) which can result in paralysis in human and lower limbs of animals by affecting central nervous system on prolonged consumption (Campbel *et al*., 1994).

Genetic transformation is an advanced tool for the production of new verities with desirable traits by incorporating suitable gene of interest. The availability of good regeneration protocols is a prerequisite for the transformation of any economically important crop plant (Ge *et al.*, 2006). On the other hand 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 regeneration in vitro and limited numbers of literatures are available on tissue culture and genetic transformation studies (Kendir *et al.*, 2009; Sahin-Demirbag *et al*., 2008; Khawar *et al*., 2004).

Therefore, the present investigation was attempted to standardize an efficient protocol for successful regeneration through organogenesis of leaflet, node and intermodal segments of grass pea (*Lathyrus sativus* L.).

MATERIALS AND METHODS

This study was carried out at the Plant Biotechnology and Genetic Engineering Laboratory of Biotechnology and Genetic Engineering Department, Jahangirnagar University, Savar, Dhaka.

Plant Materials: Seeds of grass pea *var*. BARI Kheshari II were obtained through the courtesy of Bangladesh Livestock Research Institute (BLRI). For this study explants e.g. leaflets, cotyledonary nodes, and internodal segments were used as explant's source from *in vitro* grown grass pea seedlings for direct shoot organogenesis.

Preparation of explants: The sterilized seeds were cultured on a solidified agar medium without any growth regulators (MS0) in sterile jars and incubated in the dark room for germination. Five to ten days after germination; leaflet, node and internode segment of seedlings were dissected under aseptic conditions and used as explant.

Nutrient medium and growth regulators: Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) supplemented with Gamborg B_5 vitamin (Gamborg *et al*., 1968) were used for plant regeneration. Different concentrations (0.0, 0.5, 1.0 1.5 and 2.0 mg/l) of cytokinin type growth regulators (hormone) e.g. 6-benzyl aminopurine (BAP) and Thidiazuron (TDZ) separately and different combinations of BAP and auxin type

hormone e.g. α-Naphthalene Acetic Acid (NAA) (0.0+0.0, 4.0+0.5, 4.0+1.0, 4.0+2.0) added to the MS medium for shoot primordia initiation and its elongation. Different concentrations (0.5, 1.0 and 2.0 mg/l) of auxin type hormones e.g. indole butyric acid (IBA) and NAA added to half-strength MS medium separately for root initiation and elongation of both shoots and roots.

Media sterilization and culture conditions: All the culture media were sterilized by autoclave machine at 121ºC for 15 minutes at 15psi. The explants were cultured in each media combination under aseptic conditions and incubated at 24±1ºC under dark conditions for germination and 16/8h light/dark regime for shoot initiation and plant regeneration.

RESULTS AND DISCUSSION

Different explants like leaflets, node and internodal segments were cultured on MS media with B_5 vitamin (MS-B_5) medium supplemented with benzylaminopurine (BAP) or thidiazuron (TDZ) alone and in combinations with BAP and auxins, α–naphthalene acetic acid (NAA) in varying concentrations and combinations. Direct shoots were obtained at different hormonal concentrations from different explants of grass pea. The efficiency of *in vitro* multiple shoot primordia formation and its regeneration was found in our study to be dependent on various parameters, *viz*. explant size, age of explant donor seedling, explant type, and growth regulators and its concentration (Gulati & Jaiwal, 1994).

Initiation of multiple shoots from all three explants in most of the treatments ranged between 7-11 days of inoculation. Rapid and early shoots initiation was observed in lower concentration of BAP i.e., 0.5 mg/l (Fig. 1A), however higher concentration of BAP showed delayed initiation response. Although the higher concentrations of growth regulator launched shoot initiation response delay but the better shoot proliferation (Fig. 1B) was observed in MS-B_5 medium containing higher concentration of BAP (4.0mg/l) (Table 1). The regeneration frequency increased with increases in concentration of cytokinins and 4.0 mg/l BAP was found to be optimal for maximum frequency of shoot regeneration (Fig. 1C-D). Nodal explants showed better response in comparison to other explants e.g. leaflet and internode (Table 1).

BAP is the most widely used and most effective cytokinin in legumes, including *Lathyrus* species according to Gulati & Jaiwal (1994); Sahoo *et al.* (2002). Franklin *et al.* (1998) obtained maximum of 49 shoots on medium containing 3.0 mg/l BAP from seedling explant cotyledonary node with shoot tip and only five shoots from cotyledonary node. Kumar *et al*. (1983) reported 20% of shoot bud regeneration from cotyledonary callus on Blaydes medium with 2.25 mg/l BAP and 14% from callus leaf tissue. Similar results were obtained by Polisettyet & Paul (1997) in chick pea. Nodal explant cultured on medium containing BAP showed better shoot regeneration compare to other explants indicates that regeneration frequency depends on explant type. Future experiments in relation to gene transformation study in grass pea would be carried out with nodal explants.

Different concentrations of TDZ had variable effect on shoot regeneration with a range of 49-74%. Maximum number of shoots per explant was recorded on MS-B_5 medium containing 1.5 mg/l of TDZ. Higher percentage of shoot regeneration (74%) was observed at the same concentration of TDZ (1.5 mg/l) (Fig. 1E-F). A sharp decline in the number of shoots and shoot length was recorded at other concentrations of TDZ (Table 1). In this experiment it indicates that lower concentrations of TDZ showed delayed shoot primordia initiation and regeneration (13 to 14 days), however, higher concentrations showed early regeneration (10 to 12 days). Even lower concentration e.g. 0.5 mg/l of TDZ initiated lower percentage (%) of shoot regeneration (49%). In presence of TDZ node explant showed better regeneration performance in comparison to other explants of grass pea (Table 1).

Table 1. Effect of different concentrations and combinations of plant growth regulators for the direct shoot regeneration from different explants of grass pea

Plant growth regulators (mg/l)	Days required for shoot initiation	Explants			% shoot regeneration
		Leaflet	Node	Internode	
BAP					
0.0	No response	No response	No response	No response	No shoot
0.5	7	+	++	+	35%
1.0	9	+	+++	+	43%
2.0	10	++	++	+	58%
4.0	**11**	**+**	**+++**	**+**	**63%**
TDZ					
0.0	No response	No response	No response	No response	No shoot
0.5	14	+	++	-	49%
1.0	13	+	+++	+	56%
1.5	**12**	**+**	**+++**	**+**	**74%**
2.0	10	+	+++	+	68%
BAP+NAA					
0.0	No response	No response	No response	No response	No shoot
4.0+0.5	**15**	**+++**	**+++**	**++**	**56%**
4.0+1.0	13	++	+++	++	49%
4.0+2.0	12	+	+	+	37%

'+' indicates the performance of shoot regeneration. +++ → Excellent, ++ →Average, + → Poor, the experiment was repeated thrice. Data were collected after 2weeks of culture. % shoot regeneration indicates the average regeneration rate of three different explants.

MS medium containing TDZ was more potent to enhance shoot regeneration percentage compare to BAP (Table 1). But the shoots developed on TDZ-containing medium failed to elongate. The formation of stunted shoots on medium supplemented with TDZ has been reported for several plant species, such as *Malus* sp. (Van Nieuwkerk *et al.*, 1986) and *Rhododendron* sp. (Preece & Imel, 1991). Nodal explants cultured on medium containing TDZ showed better shoot regeneration in this study too compare to other explants e.g. leaflet and inter node indicates that regeneration frequency depends on

explant type. As TDZ induced better shoot regeneration but without proper elongation therefore, future study should be needed to elongate shoots.

High frequency of shoot regeneration was recorded on MS medium containing different combinations of BAP and NAA with a range of 37 to 56% (Table 1). The combinations of BAP and NAA were found to be the most responsive for shoot formation from both nodal and leaflet explants in comparison to internode. Better shoot regeneration (%) was found optimum at the combination of 4.0 mg/l BAP and 0.5 mg/l NAA (56%) (Table 1). Addition of more NAA, 2.0 mg/l to BAP at 4.0 mg/l reduces the shoot regeneration frequency, however, rapid shoot formation was observed at this combination.

Root induction from *in vitro* regenerated shoots is an important step to get a complete plantlet. The regenerated shoots spontaneously rooted on regeneration medium. Shoots were further rooted in half strengths MS medium with various concentrations of IBA and NAA in *in vitro* culture conditions. Of the two auxins tested, NAA promoted better root formation (Fig. 1G) as compared to IBA. The best root initiation result was obtained in half strength MS medium supplemented with 1.0 mg/l NAA (Table 2). Almost 40% shoots were rooted vigorously at the above mentioned concentration of NAA (1.0mg/l) and less time required for rooting (14-17 days).

Table 2. Effect of different concentrations of NAA and IBA added to half-strength MS medium on root induction from regenerated shoots of grass pea

Growth regulators	Concentrations (mg/l)	Days required for root initiation	% rooted shoots
½ MS+ IBA	0.5	18-21	5.0
½ MS+ IBA	1.0	19-22	10.0
½ MS+ IBA	2.0	20-23	15.0
½ MS+ NAA	0.5	18-20	30.0
½ MS+ NAA	**1.0**	**14-17**	**40.0**
½ MS+ NAA	2.0	19-21	20.0

Rooted shoots were transferred to the pot with sterile soil and incubated for 7-10 days in growth chamber at 27□C and 70% relative humidity. The pots with the plantlets were covered with transparent polythene bags to prevent sudden desiccation. These plantlets were kept in a greenhouse which got acclimatized to the natural conditions and exhibited normal growth and development (Fig. 1H).

To exploit the potentiality of grass pea for general crop improvement to eliminate the toxic substances and to grow under different stress condition, modern approaches such as genetic modification is crucial. For this purpose a good *in vitro* regeneration protocol is required. Through this study, a protocol for high-frequency direct plant regeneration from leaflets, cotyledonary nodes and internodes of grass pea has been established. It is an efficient *in vitro* plant regeneration protocol without an intermediate callus phase. Direct shoot organogenesis from different parts of *in vitro* grown crop cultivars is amenable to

high frequency shoot regeneration and may be used for future genetic transformation study.

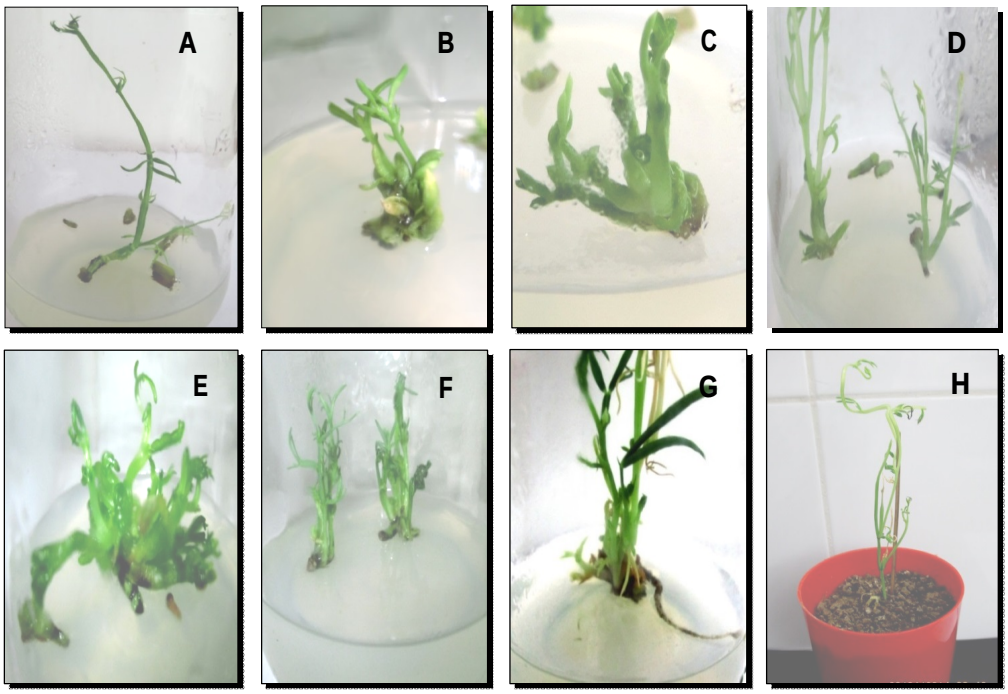

Figure 1. Shoot Regeneration of Grass pea. A-Direct shoot regenerated from nodal explant, B-Shoots regenerated from leaflet, C to D-Multiple shoots regenerated at high concentrations of BAP, E to F-Multiple shoots obtained on MS medium containing TDZ, G-Regenerated shoots rooted, H-Plantlets acclimatized and potted

Acknowledgement: The authors gratefully acknowledged the financial support provided by the Ministry of Science and Technology, Government of the People's Republic of Bangladesh and Jahangirnagar University research grant. Authors are also thankful to Dr. Nathu Ram Sarkar of BLRI for providing grass pea seeds to continue this research.

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