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RESEARCH ARTICLE

A RAPID AND EFFICIENT PROTOCOL FOR *IN VITRO* PLANT REGENERATION OF *LATHYRUS SATIVUS* L. (GRASS PEA) THROUGH MULTIPLE SHOOTING

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ABSTRACT

A rapid, simple and reproducible protocol for in vitro multiple shoot induction and whole plant regeneration was developed from two cultivars of *Lathyrus sativus* L., viz. Nirmal B1 and a locally available variety. Cotyledonary node, shoot tip of five-day-old germinated seeds and nodal explant of ten-day-old seedlings were used as experimental material. The explants were cultured in Murashige and Skoog (MS) media fortified with different concentrations (0.125-2.5 mg/L) of 6-Benzylaminopurine (BAP) and Thidiazuron (TDZ) for multiple shoot proliferation. The maximum number of multiple shoot induction and proliferation (12 – 14 shoots / explant) was observed in cotyledonary node explant within 30 days using 1.5 mg/L BAP. The micro shoots were subcultured in the same media containing low concentration of IAA (0.03 mg/L) for further elongation. Up to 85% of shoots developed roots following their transfer to half strength of Murashige and Skoog MS media containing 0.25 mg/L Indole-3-butyric acid (IBA) within 25 days. Rooted plantlets were successfully hardened under culture conditions and were subsequently established in the greenhouse with survival rate of 85%. The entire *in vitro* regeneration process took relatively short period of time (90-100 days). Thus, the present study remained successful in developing a rapid and efficient in vitro regeneration through direct organogenesis of *Lathyrus sativus* for subsequent development of efficient transformation systems.

Abbreviations

BAP: 6-Benzylaminopurine, β-ODAP: β N-Oxalyl-L- alpha, beta-diaminopropanoic acid, IAA: Indole-3-acetic acid, IBA: Indole-3-butyric acid, MS: Murashige and Skoog media, TDZ: Thidiazuron.

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INTRODUCTION

Grasspea (*Lathyrus sativus* L.) remains an important annual grain legume and it is believed to be poor people's crop as it provides a cheap source of dietary proteins. It is cultivated in several tropical and sub-tropical countries all over the world especially in Asio-African regions. It is primarily used for human consumption, animal feed and fodder and several other purposes (Croft *et al.*, 1999; Hanbury *et al*., 2000). Seeds of *Lathyrus* are generally popular for higher protein content as compared to some commonly consumed grain legumes (Baudoin *et al.*, 1999; Hanbury *et al.,* 2000). It also serves as a rich source of essential amino acids like lysine and L-homoarginine (Querreshi *et al*., 1977). Moreover it has the ability to adapt to the saline, alkaline, water logged or clayey, drought or otherwise poor soil conditions and is reported to be resistant to a number of infectious diseases viz. Ascochyta blight, Powdery mildew attack, Thrips attack etc. (Tekele Haimanat *et al*., 1990; Campbell *et al*., 1994; Smartt *et al*., 1994; Hoque *et al*., 1996; White *et al*., 2002; Vaz Patto *et al*., 2014). These traits make grasspea an agronomically important legume. This is more so as its cultivation requires minimum crop management procedure. However, the limiting factor of its large scale cultivation and commercial use is the presence of the neurotoxin Beta-N-oxalyl-L-alpha, beta-diaminopropionic acid (ODAP).

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This neurotoxin causes neurolathyrism both in animals and humans and results paralysis in the lower limbs by affecting the central nervous system (Rao *et al*., 1964; Spencer *et al*., 1986; Spencer *et al*., 1995; Hanbury *et al*., 2000; Kuo *et al*., 2000; Lambien *et al*., 2009). Thus, development of β-ODAP free *lathyrus* variety remains a challenge for the plant breeders and biotechnologists. The conventional breeding techniques and agronomical approaches have failed to produce β-ODAP - free *Lathyrus* variety (Campbell *et al.*, 1994; Jiao *et al.*, 2006; Haque *et al.*, 2011) because this trait is highly influenced by climatic and odaphic environmental conditions (Girma and korbu, 2012). Development and application of suitable genetic engineering strategies for eliminating or reducing the neurotoxin in the plants remains a key approach for solving this problem and can supplement future plant breeding programmes to improve its agronomic importance. Development of an efficient transformation system remains a prerequisite for application of such genetic engineering strategies. To achieve the same, a rapid, efficient and reproducible *in vitro* plant regeneration procedure is required at the first place. Although there have been some past reports on *in vitro* shoot bud differentiation and plant regeneration through somatic embryogenesis from shoot tips (Gharyal *et al.*, 1980), decapitated axillary buds of embryo (Gharyal *et al.*, 1983), stem (Sinha *et al*., 1983), root explants (Roy *et al*.,1991; Roy *et al*., 1992), leaf (Sridhar *et al.*, 2015) as well as direct shoot regeneration from epicotyl explants (Malik *et al*., 1992), different types of cotyledonary node (Barik *et al*., 2004) and seed cultures (Malik *et al*., 1993) in different genotypes of *Lathyrus sativus* L., the efficiency of whole plant regeneration was low and time consuming.

Also, the efficiency of plant regeneration was not very apparent from the existing literature. Thus, the objective of our present study was set to establish a rapid, efficient and reproducible *in vitro* plant regeneration protocol of *Lathyrus sativus* L. through multiple shooting. The results showed a multiple shoot induction frequency upto 91%, root induction frequency upto 85% and mature plant regeneration/survival frequency of upto 85%. Also, the plant regeneration was achieved in a relatively short time interval (90-100 days) as compared to existing protocols. Thus, the present study remains novel in offering a rapid and efficient *in vitro* plant regeneration protocol for *Lathyrus sp.*

MATERIALS AND METHODS

Seed source and surface sterilization

Seeds of *Lathyrus sativus* L. cv Nirmal B1 were obtained from ICAR, New Delhi. Seeds of locally available variety were procured from the local market. Prior to explant preparation, the mature seeds of both the variety were washed in 0.1% (v/v) Tween 20 for 15 min, followed by rigorous rinsing with sterile distilled water. Seeds were then surface disinfected with 0.1% (w/v) $HgCl_2$ for 5 min and rinsed five times with sterile distilled water. Seeds (12 seeds per plate) were germinated aseptically in petriplates (90 mm x 20 mm) in dark at 30 °C overnight on seed germination medium (SGM), which contained half strength MS (Murashige and Skoog, 1962) media with vitamins, 1% (v/v) sucrose and 0.8 % (w/v) agar as the gelling agent. Seeds were thereafter maintained at 26 ± 2°C with 16 h light/8 h dark in a growth chamber (150-200 $\mu E\ m^{-2}\ s^{-1}$).

Explant preparation

Aseptically excised 3.0 mm long apical meristem, cotyledonary node of 5-day-old *in vitro* germinated seeds and nodal segments (4.0 mm - 5.0 mm) of 10-day-old *in vitro* raised seedlings were selected as explants for direct shoot multiplication.

Organogenesis and plant regeneration

Disinfected explants viz. apical meristem, cotyledonary node and nodal segments were cultured on different strengths of MS basal media and B5 media (Gamborg *et al*., 1968) supplemented with 3.0% (w/v) sucrose for better regeneration of explant and multiple shoot initiation. The pH of the media was adjusted between 5.75-5.8 with 0.1 N NaOH or 0.1 N HCl and 0.8% agar was used as gelling component. Different concentrations of growth regulators like BAP (0.125-2.5 mg/L) and TDZ (0.125-2.5 mg/L) were used along with the basal media for multiple shooting. Twenty five explants each were used in three replicate experiments and they were cultured under 16 h / 8 h (light/ dark) photo period with light intensity of 150-200 $\mu E\ m^{-2}\ s^{-1}$ in a Percival plant growth chamber with periodic sub-culture every 2 weeks. Proliferating shootlets were excised and cultured in the multiple shooting media supplemented with different concentrations of IAA (0.01-0.05 mg/L), designated as elongation medium (EM) for further elongation of micro shootlets. Healthy elongated shootlets were transferred to liquid root inducing medium (RM) Containing different strength of MS media (½X, 1X and 2X) and B5 media, supplemented with different concentrations IBA (0.05-1.5 mg/L).

Hardening of the regenerated plants and acclimatization

Rooted plantlets were transferred to potting medium (PM) for hardening in mixture of soilrite (Keltech Energies ltd.) and sand (autoclaved) for about 20 days. Finally, the well grown plants were transferred to large pots containing soil mixture: sand (1:1) in greenhouse condition. Plants grown in these conditions were observed for their growth and survival rate.

Statistical analysis: Student's *t*-test was performed to compare the results following a Gaussian distribution.

RESULTS

Effect of different basal media composition on shoot and root regeneration

Different strengths of MS media (viz. ½X, 1X i.e. normal strength and 2X) and normal strength of B5 media were used for regeneration of shoots as well as roots. Of all different media used, 1X MS media was found to be most effective for shoot regeneration (Fig. 1) in both the genotypes tested. Shoot regeneration percentage varied between 80% to 87% in normal MS media and a better response of 87% was observed in local variety. ½MS, 2MS, B5 media resulted 71-75%, 31-37% and 53-63% shoot regeneration, respectively (Fig.1). On the other hand, it was observed that increasing strength of MS salts led to decrease in root regeneration efficiency in both the genotypes (Fig.2). Highest root regeneration percentage (55%) was observed in ½MS media. Relatively better root generation response (36%) was found in B5 media than normal MS (21-28 %) media (Fig.2).

Table 1. Effect of different concentration of TDZ and BAP on multiple shoot regeneration frequency per explant in two cultivars of *L. sativus* L.

Concentration of hormone (mg/L)	Mean shoots per explant			
	Nirmal B1		Loc al	
	TDZ	BAP	TDZ	BAP
0.125	2.49±0.08	-	3.98±0.15	-
0.25	5.66±0.19	3.86±0.17	6.32±0.18	5.13±0.17
0.5	8.04±0.20	6.79±0.19	9.24±0.23	7.63±0.20
1.0	-	8.30±0.15	-	9.27±0.15
1.5	Callus	12.80±0.26	Callus	14.23±0.18
2.0	Callus	9.02±0.16	Callus	8.97±0.21
2.5	Callus	4.46±0.21	Callus	2.42±0.09

Table 2. Comparative account of multiple shoot induction (MS+1.5 mg/L BAP) from shoot tip, cotyledonary node and nodal explants of two cultivars of *L. sativus* L.

Variety	Type of explants	Percentage of multiple shoot induction (Mean) %	No of shoots /explant
Nirmal B1	Shoot tip	71	8.23±0.18
	Cotyledonary node	88	12.77±0.27
	Nodal explant	65	6.24±0.14
Local	Shoot tip	77	8.95±0.12
	Cotyledonary node	91	14.21±0.18
	Nodal explant	68	6.93±0.17

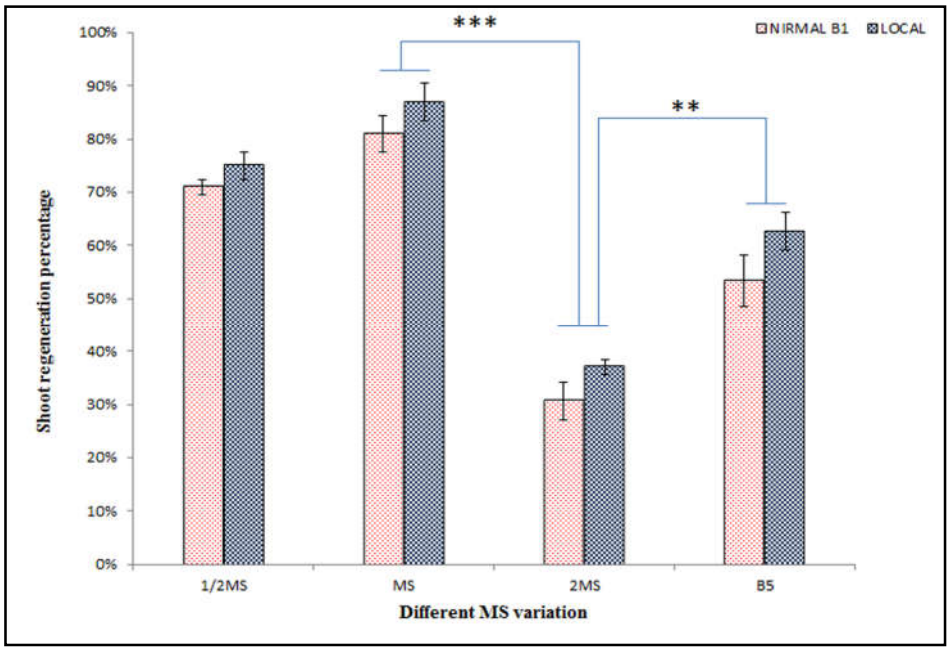

Fig. 1. Effect of different types of basal media on shoot regeneration percentage. . Results are expressed as mean ± SE for triplicate experiments. ** indicates P<0.01, * indicates P<0.005**

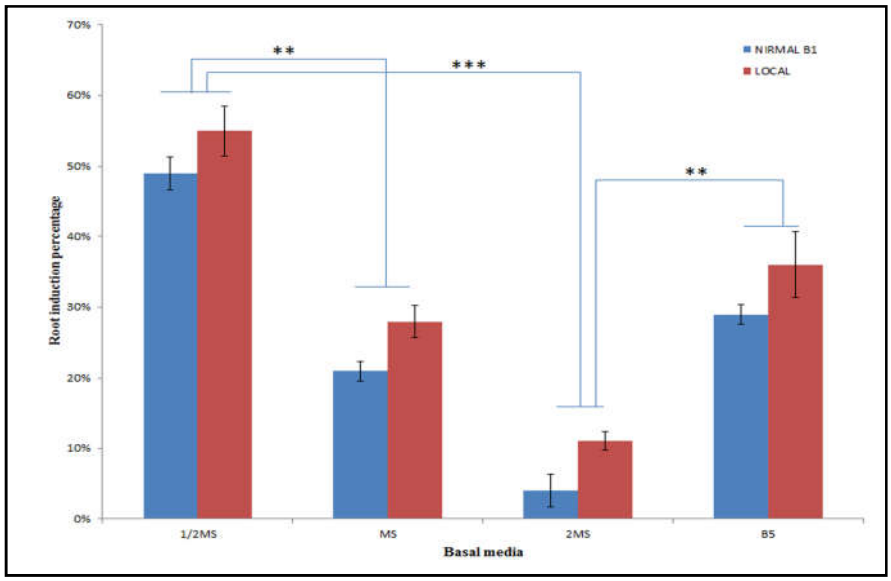

Fig. 2. Effect of different types of basal media on root induction percentage. Results are expressed as mean ± SE for triplicate experiments. ** indicates P<0.01, * indicates P<0.005**

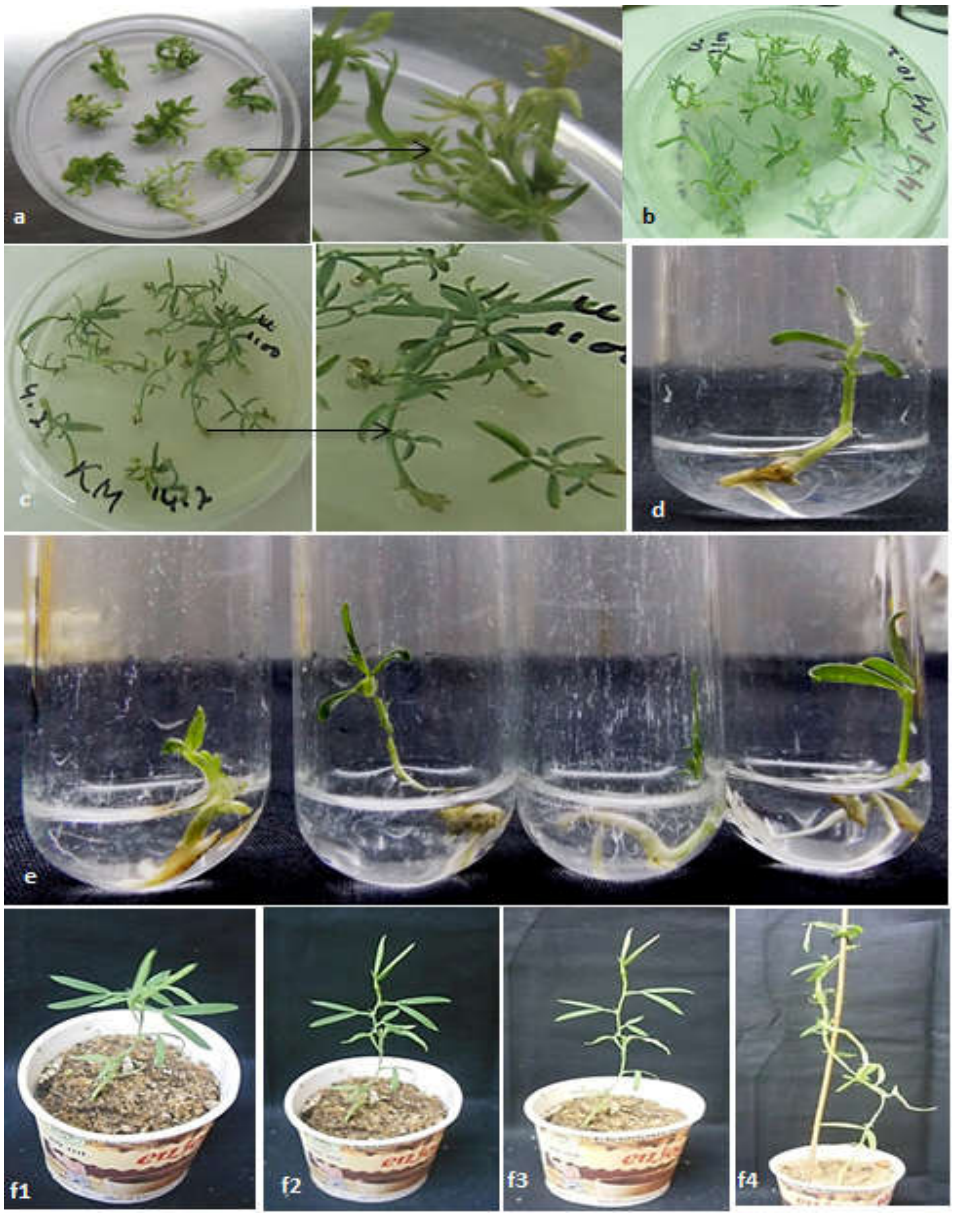

Fig. 3. Different stages of whole plant regeneration of grass pea. (a): Multiple shooting of *L. sativus* L. (b): Separated shootlets from multiple shoot. (c): Elongation of microshoots. (d): Root initiation in liquid RM. (e): Healthy and slender root response in liquid RM. (f1-f4): Different stages of Acclimatization of rooted plantlets in green house condition. Black arrow indicates magnified version of shoots

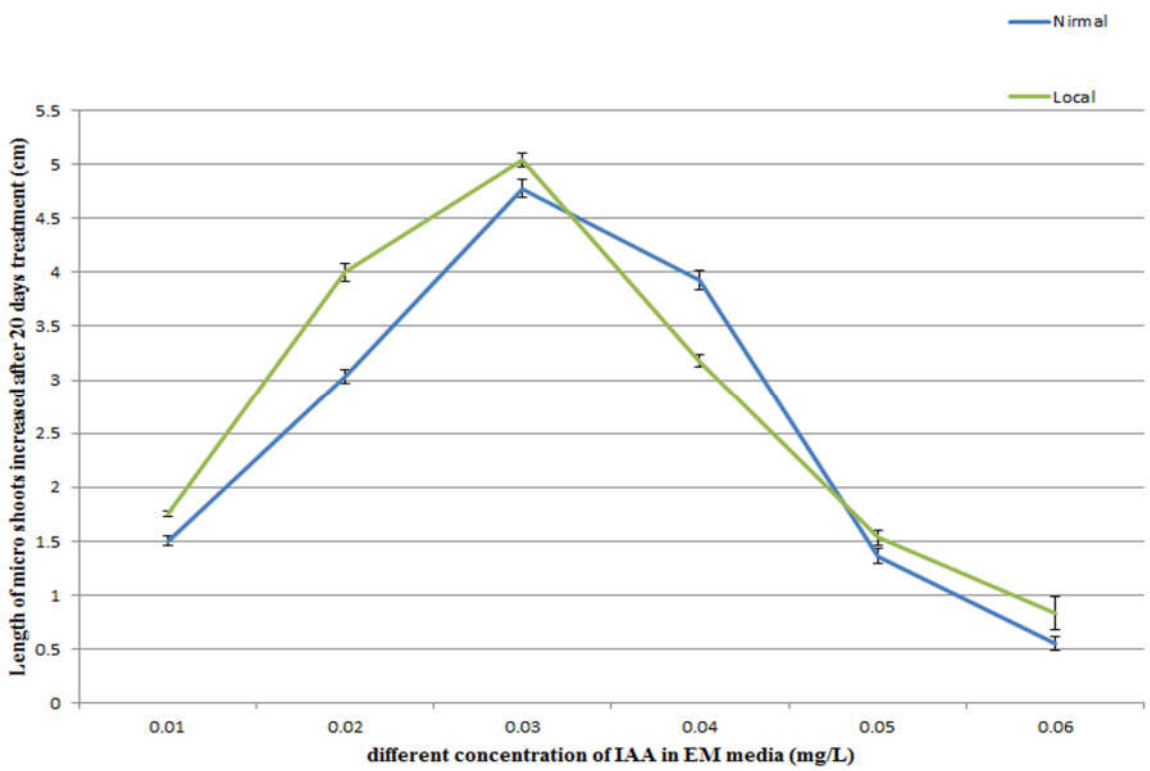

Fig. 4. Effect of different concentration of IAA on elongation of microshoots of two different genotypes (Nirmal B1 and Local). Results are expressed as mean ± SE for triplicate experiments

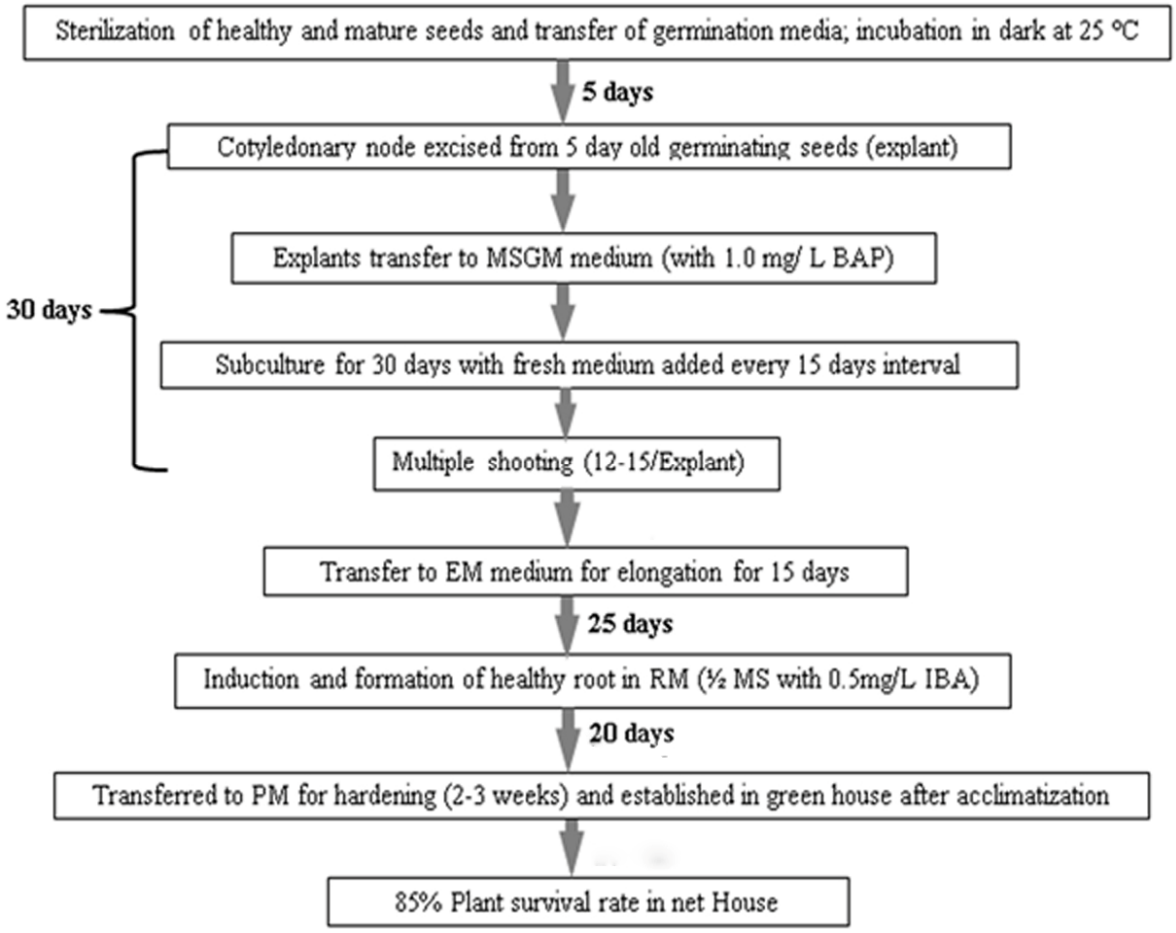

Fig. 5. Flow chart for *Lathyrus sativus* L. plant regeneration protocol

Table 3. Effect of different concentration of IBA on *in vitro* rooting of two different cultivars of *L. sativus* L.

Variety	IBA(mg/L)	Percentage of root induction (Mean) %	Number of roots/microshoots (mean ± SE)	Days required(mean ± SE)
Nirmal B1	0.062	49	1.02±0.14	38.89±0.57
	0.125	57	1.28±0.16	31.21±0.36
	0.25	76	3.85±0.27	25.11±0.53
	0.50	63	2.05±0.21	34.72±0.37
	1.0	53	1.63±0.21	44.69±1.18
Local	0.062	57	1.11±0.13	41.65±0.59
	0.125	64	1.40±0.15	33.56±0.39
	0.25	85	4.03±0.21	25.98±0.43
	0.50	71	2.23±0.20	38.79±0.37
	1.0	60	1.85±0.19	48.31±0.55

Table 4. Different media composition for *in vitro* regeneration of *Lathyrus sativus* L.

Medium	Composition	Application
Seed germination media (SGM)	½ Strength MS Salts + Vitamins + 1% Sucrose + Agar 0.8% (w/v.)	Seed Germination
MSGM (Multiple shoot generation media)	MS (Macro + Micro salts) + Vitamins +1.5 mg/L BAP + Sucrose 3 % + Agar 0.8% (w/v).	Multiple Shoot formation
EM (Elongation Medium)	MS (Macro + Micro salts) + Vitamins + 1.5 mg/L BAP + 0.03 mg/L IAA + Sucrose 3 % + Agar 0.8% (w/v).	Shoot Elongation
RM (Root Inducing medium)	½ Strength MS Salts + Vitamins +0.25 mg/L IBA + Sucrose 3%, Agar 0.8% (w/v)	Rooting
PM (Potting Medium)	autoclaved Soilrite and sand (1:1)	Transplantation of regenerated plants in pots for hardening

Multiple shoot induction and elongation of microshoots

MS media supplemented with BAP and TDZ (0.125-2.5 mg/L) were used for multiple shoot induction. Of all the different concentrations of growth regulators tested, 1.5 mg/L BAP was found to be most effective for inducing multiple shoots (Table 1) within 30 days. It was observed that cotyledonary node explants produced the maximum number of shoots (12-14/ explant) (Fig. 3. a) as compared to shoot tip and nodal explants (Table 2). Moreover, high percentage (88-91%) of multiple shoot induction was observed for cotyledonary node explant (Table 2). Among the two genotypes tested local variety produced higher number of shoots per explant as well as higher percentage of multiple shoot induction (Table 2).

Same media supplemented with low concentration of IAA (0.01-0.06 mg/L) resulted further elongation of microshoots (Fig.3. c). The IAA concentration of 0.03 mg/L was found to be most effective for elongation of microshoots upto 5 cm (Fig.4).

Root development and hardening of the regenerated plants

The individual elongated shoots were isolated and transferred to liquid ½ MS media fortified with IBA (0.062-1.0 mg/L) for root development. The IBA concentration, 0.25 mg/L was found to be most effective (Table 3) for rooting with of maximum percentage of root induction higher number of root

per shootlet (Fig.3 e) and the minimum time requirement for root development in both the genotypes. In this particular IBA concentration Nirmal B1 showed 76% root induction with 3.85±0.27 roots per shootlet within 25 days whereas local variety showed 85% root induction with 4.03±0.21 roots per shootlet in same time period. Rooted plantlets were initially hardened in soilrite:sand (1:1) and subsequently established in the greenhouse on the mixture of soil: sand (1:1). The survival rate of the plants under the field conditions was recorded as 85% (data not shown). All the media used for *in vitro* culture of *L. sativus* L. are listed in table 4 and the entire process of plant regeneration through multiple shooting is summarized in the flow chart (Fig. 5).

DISCUSSION

In the present study, we reported an efficient and reproducible protocol for *in vitro* regeneration of *Lathyrus sativus* L. through multiple shooting using nodes from 10-day-old seedlings, cotyledonary node and shoot tips from 5-day-old germinating seeds as explants within a very short period of time (90-100 days). We have tested three types of explants on four types of basal media (½MS, MS, 2MS and B5), fortified with different concentrations of growth regulators (BAP and TDZ) in two *Lathyrus* varieties (Nirmal-B1 and local) for plant regeneration and multiple shooting. Previous study on grasspea (*Lathyrus sativus* L.) showed the shoot formation in calli from shoot apex and leaf explants without any subsequent root development (Mukhopadhyay and Bhojwani, 1978; Gharyal and Maheshwari 1983). Both BAP and TDZ are essential for induction of shoot regeneration on immature zygotic embryos (Sahin-Demirbag *et al*. 2010), but some reports stated that TDZ was more efficient to proliferate shoot buds rather than BAP in case of grasspea (Malik *et.al.*, 1992). Our result showed that MS salts supplemented with BAP is more effective for high number of shoot regeneration under *in vitro* condition and this is consistent with previous reports (Sinha *et al.*, 1983; Barik *et al.*, 2004). It is found that 1.5 mg/L BAP resulted in highest shoot regeneration frequency (88-91%) and maximum number of shoots (12-14) per explant in two varieties of *Lathyrus sativus* L. (Table 2). However, increase in the concentration of BAP above 2.0 mg/L reduced shoot bud proliferation frequency (Table 1). On the other hand, MS medium supplemented with 0.5 mg/L TDZ led to generation of highest number of shoots/explant for Nirmal-B1 and local variety among different concentrations of TDZ tested (Table 1). Lower concentration of TDZ showed better response in number of multiple shoot proliferation, whereas increased concentration of TDZ failed to elongate microshoots. Formation of stunted shoots on MS medium supplemented with TDZ had been reported earlier in several plant species like *Malus* sp. (Van Nienwkerk *et al*., 1986) and *Rhododendron* sp. (Preece and Imel, 1991). This inhibition of shoot elongation is a common problem with TDZ probably due to its super-optimal cytokinin activity (Barik *et al.,* 2004). The present study revealed that TDZ containing media produced a small amount of callus at the base of the explants which ultimately hampered the shoot regeneration process and this is also supported by previous report (Barik *et al*., 2004). As the response to cytokinin treatment varied among different genotypes, it can be stated that *in vitro* regeneration and multiple shooting efficiency was genotype dependent. Several previous reports suggested the efficiency of epicotyl meristematic tissue for higher number of shoot bud proliferation in case of different grain legume (Pickardt *et al*., 1991; Amitha and Reddy, 1996) and also in *Lathyrus* (Barik *et al*., 2005-a). In the present study, of all the three types of explants tested, the cotyledonary node showed maximum response in terms of percentage of multiple shoot induction (88%-91%) and number of shoots per explant (12.77 ± 0.27-14.21± 0.18) in both the genotypes. MS media containing BAP along with different concentrations of IAA (0.01 mg/L- 0.06 mg/L) were tested to improve further elongation of microshoots. IAA at 0.03 mg/L induced the elongation of microshoots upto 4.7 cm in Nirmal-B1 and 5.04 cm in local variety (Fig. 4).

Although shoots were longer with BAP in combination with IAA, no multiple shoot was formed in media supplemented with IAA alone. According to the earlier findings, gradual increase in concentration of IAA (0.5-1.0 mg/L) resulted in reduced percentage of shoot elongation and it became nil when IAA concentration was raised to 2.0 mg/L (Chakraborty *et al.*, 2006). Poor frequency of *in vitro* root development is considered to be a major constraint in grasspea (*Lathyrus sativus* L.) tissue culture and this significantly reduces the whole plant regeneration potential. It is observed that use of different cytokinins played a crucial role in root developement. Present study showed that shoots regenerated on BAP supplemented media led to efficient root development rather than the shoot from TDZ containing media, which was also previously exhibited in case protein pea (Ochatt *et al*. 2000) and *L. sativus* L (Ochatt *et al.* 2002). Strength of basal media plays a vital role in *in vitro* rooting. It is well known that, rooting in certain plant species may occur ideally when the overall salt strength of the medium is reduced. In some cases, the salt strength reduction may eliminate the need of using auxin (s) for rooting (Verma, 2012), which is the case for even *Lathyrus* also (Ochatt *et al*. 2001). Though the use of auxins was reported to enhance root induction capability from reduced strength of basal media (Shridhar *et al*., 2015). The highest frequency of rooting (65%), recorded so far, was in the culture medium containing 1.5 mg/L IBA (Barik *et.al.*, 2005-b; Tripathy *et.al*., 2013; Piwowarczyk and Pindel, 2014). Optimum root induction on ½ strength B5 media supplemented with low dose of IBA was exhibited by Roy *et al.* 1992 and he also underlined specific and narrow utility of growth regulators for rooting of *Lathyrus sp*. Our study also remained successful in inducing high root generation frequency in comparison to existing literature. In our present study, ½ MS media augmented with 0.25 mg/L IBA resulted the highest roots induction frequency and maximum number of roots/shootlets (76% and 3.85 ±0.27 in Nirmal-B1 variety and 85% and 4.03±0.21 in local variety, respectively) within a short period of time (25 days) (Table 3). Healthy rooted plantlets were transferred to potting medium (PM) (Fig.3. f1-f4) filled with sand: soilrite (1:1) and were covered with plastic bags to retain 80% humidity. After one week, plantlets were maintained in reduced humidity (50-60%) and gradually, the plastic bags were removed and the plants were transferred to green house. 85% of the plants survived under this condition. The present study thus successfully demonstrated that *Lathyrus sativus* L. cultivars can be effectively regenerated from cotyledonary nodes within a short span of time (90-100 days). The present study thus bears immense significance in terms of high frequency of multiple shooting, root development and survival of the regenerated plantlets within a relatively short time span. In conclusion, the comprehensive method reported here is efficient and reproducible. The rate of multiple shoot induction and plant regeneration using two different genotypes has

broadened the practical scope for development of an effective transformation system in desirable genotypes of *Lathyrus* sp. The efficient rooting method proposed here not only increased the whole plant regeneration frequency but also resulted in very high recovery of plants after transplantation to the glasshouse. Thus this protocol provides an efficient means for *in vitro* manipulation of grasspea within a short time of 90-100 days.

Author contribution

Experiments were designed by SC with valuable scientific inputs from JB and BRP. Experiments were performed by SC, AM, NS and BRP provided valuable suggestions for troubleshooting throughout the study. SC, JB, AM analysed the data. The manuscript was prepared by SC and AM in consultation with AC, JB and BRP. All the authors read and approved the MS.

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Conflict of Interest

The authors declare no financial or commercial conflict of interest.

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