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Somatic Embryogenesis from Leaf and Internode Explants of *Lathyrus sativus* L.

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ABSTRACT

Somatic embryos Development from Leaf and Internode explants of Lathyrus sativus L. Lathyrus sativus L. commonly known as grass pea. Here in the present study we developed an efficient micropropagation protocol through somatic embryogenesis from leaf and intermodal explants of Lathyrus sativus. Murashigae-Skoog medium supplemented with 2,4-D (1.0mg/l) and (BAP)benzylaminopurine (5 mg/l) resulted in the production of green embryogenenic callus with clearly differentiated somatic embryos with frequency of plant conversion rate from leaf and internode explants was50±0.8 and 53±0.5 respectivel y. The regenerated shoot were rooted on root induction medium, transplanted to pots and later transferred to field. The survival percentage of the transplanted plants was 33±0.1for Internode and 24± 0.6 for leaf explants. Therefore, this protocol could be useful for micropropagation of Lathyrus sativus.

Keywords*: Grass pea, Somatic Embryogenesis, Leaf and Internode explants, Micropropagation, MS Medium.*

INTRODUCTION

Grass pea *Lathyrus sativus* L. is well-known for its recalcitrance to *in vitro* approaches[1]. It is an annual cool-season legume crop grown for food and feed all over the world[2, 3]. The grass pea of the Fabaceae is a grain legume with lysine-rich protein. The legume is cultivated in many parts of India and several other developing countries including Bangladesh, Nepal, and Ethiopia. Although its cultivation requires the least crop management, the full potential of this crop has not been utilized because of the presence of the neurotoxic amino acid b-N-oxalyl-L-a,b-diaminopropionic acid which causes neurolathyrism in human beings on prolonged consumption. Conventional breeding practices and other agronomic approaches explored to date have not been successful in substantially reducing the toxin.

It can thrive well in adverse climatic conditions (drought, salinity and water-logging) and has substantially high protein content (25-30%) than other food legumes. The largest collections of grass pea genetic resources are in India followed by Syria and France. *In vitro* selection of low neurotoxin plants and gene transfer may offer genetic rectification in grass pea and make it suitable for consumption. An efficient *in vitro* culture system is a pre- requisite for success in isolation of somaclonal variants, or somatic mutants and also genetic transformation. In this context, somatic embryogenesis is gaining importance as the regenerates are derived from single somatic cells. But, legumes in general, are recalcitrant to regeneration *in vitro*[4,5]. Reported that Organogenesis and somatic embryogenesis are under separate genetic control and therefore, genotypes of the same crop species are most likely to respond differentially with regard to morphogenetic potential even in an optimum regeneration medium[6]. Studied inheritance of the ability to form somatic embryos using a diallel cross among six different pure lines of pea (*Pisum sativum*).

About 80% of the observed genotypic variation was due to additive effects. Analysis of the distribution of F3 family means from crosses between divergent lines indicated presence of a few major genes to control somatic embryogenesis in pea. Effect of genotype or genotype x medium interaction on callus induction and regeneration potential has been well documented in number of crop plants including grass pea[7,8,9,10]. Moreover, successful callus induction does not necessarily correlate with induction of somatic embryogenesis and regeneration ability of a genotype.

Although, *in vitro* propagation of grass pea has been reported using a variety of explants in various growth media [11,12,13,14,15]; there is limited information available on optimization of sucrose concentrations, which is commonly used as carbon source in plant tissue culture media. It is well known that exogenous sucrose affects photosynthesis and biomas formation positively[16,17]. Moreover, sucrose are also reported to have an osmotic role and act as source of energy to induce root and shoot regeneration in tissue cultured plants[18,19,20] , by increased nutrient function[21,22,23,24]. Previous studies further testify that sucrose also influence shoot regeneration and rooting during acclimatization. Rooting and root quality vary depending on genotype and sucrose levels[25]. The present study was carried out to standardize efficient protocols for micropropagation Medak genotype of *Lathyrus sativus* via direct somatic embryogenesis of leaf and internode explants.

MATERIALS AND METHODS

Plant Material

Seeds of the *Lathyrus sativus* were collected from wild plants located at Lingampally village, Pulkal Mandal, Medak district, Telangana, India. In addition to their use in *in vitro* studies, the seeds were also planted in the Botanical Garden of Department of Botany, Osmania University, Hyderabad and a plantation was raised in the form of a green hedge.

Somatic Embryogenesis

The explants comprised of leaf and internodes which were collected from forty days old plants growing in Botanical Garden, Deportment of Botany, Osmania University, Hyderabad. Explants were initially rinsed in 10 % (H_2O_2) hydrogen peroxide for 4 minutes and transferred to 50 % (C_2H_5OH) ethanol for 3 minutes, washed in (DH_2O) sterile distilled water and immersed in 0.1 % (w/v) ($Hgcl_2$) mercuric chloride containing two to three drops of Tween-20 for 5 minutes with intermittent shaking. The explants were then washed thrice with sterile distilled water and inoculated into sterile 25X150 mm culture tubes containing MS medium supplemented with 3 % (w/v) sucrose, 0.8 % (w/v) agar and the appropriate combinations of growth regulators. The pH was adjusted to 5.8 before autoclaving using 0.1 N NaOH and 0.1 N HCl. One hundred and fifty explants were inoculated for each experiment with three replicates amounting to a total of 450 explants. All the cultures were sub-cultured for every 10-15 days. The cultures were maintained in a sterile growth room with 16 hours photoperiod at 25±2° C with light intensity of 60 µ Em-2s-1 and 50 % relative humidity

MS media supplemented with 2, 4-D (0.5 mg/L), BAP l [0.5 mg/L], Glutamine [684.2 µM] and Citric acid [520.5 µM] (medium-A) was used as the medium for somatic embryogenesis. The explants with somatic embryos were transferred to plant conversion medium (MS medium supplemented with BA [2 mg/l], Glu [684.2 µM] and CA [520.5 µM]) (Medium-B) for conversion of somatic embryos to plantlets. Healthy, well developed and elongated shoots (3 cm) were transferred to root induction medium [Half strength MS supplemented with IBA (2 mg/l), Glu [684.2 µM] and CA [520.5 µM]] (Medium-C). Healthy, plantlets with well-developed roots were transferred to pots and gradually acclimatized in the glasshouse in the shade for two weeks by enclosing the pots in polythene bags. Subsequently, the plants were transferred to the sunny areas of the glasshouse and later to the field.

Scanning electron microscope (SEM)

For SEM observations, the embryogenic callus at different stages was used for the SEM studies. The callus samples were processed by fixing 4% (v/v) glutaraldehyde in 0.1 M phosphate buffer (Ph 6.8) for about 3 hrs of duration. The samples were washed thoroughly in distilled water 3 times.

Then the samples were dehydrated by passing through acetone series from low to high concentration [20, 40, 60, 80, 100% (w/v)] each lasting about 30 minutes of duration. The samples JFC-1100E/JEOL-100CX-BALZAR`S-4CD-Ion Sputtering device. After gold coating, the material was observed under JEOL-JEM-5200/JEOL-100CX-Scaning electron microscope and different stages of Somatic embryogenesis were photographed.

RESULTS AND DISCUSSION

In the present work, micropropagation through somatic embryogenesis was standardized from leaf and internode explants of the Medak genotype of *Lathyrus sativus*. The somatic embryos were observed to form on both types of explants i.e leaf and internode segment. The frequency of direct somatic embryo formation both in terms of explants forming embryos and number of somatic embryos formed per explants was higher in internode segments. Previous studies presented the micropropagation protocols from different accessions of *Lathyrus sativus*[13,26,,27]. Limited research was carried out on the micropropagation of *Lathyrus sativus* through somatic embryogenesis. In this study an attempt was made to develop efficient protocols for plant regeneration through somatic embryogenesis from leaf and internode explants.

MS medium was supplemented with BAP (1.0% mg/l) and 2, 4-D (1.0 mg/l) resulted in good response of shoot tip and leaf explants in the development of green embryogenic callus with clearly differentiated somatic embryos (Figure 1B & D). The response was genotype independent. The percentage production of somatic embryogenic callus from leaf explants was 64±0.4; Internode produced green compact callus with low percentage value of 35±0.6 (Table:-1).

The embryogenic callus was carefully cultured and by the 3rd week, somatic embryos were differentiated from it and which developed gradually into plantlets by passing through various developmental stages like globular and heart shaped embryos.

The developmental process pertaining to embryogenesis (somatic embryos) was study by Scaning electron microscope. The different stages of somatic embryos viz: globular and heart shaped stages were identified (Figure 1E). The Figure 1 demonstrates the fact that response of plant regeneration explained above in terms of somatic embryogenesis occurred normally in accordance with the pattern of development of zygotic embryo of angiosperms. Hence, the results and discussion regarding organogenesis and embryogenesis presented above stand validated in accordance with[26,27].

Somatic embryos have the potential to develop into plantlets. Hence, these somatic embryos were further sub-cultured on a different culture medium to induce their germination and conversion into plantlets. The somatic embryos were cultured on MS medium supplemented with 1.5 mg/l benzylaminopurine (BAP) responded well by efficient shoot development with good frequencies (Table:-1). The shoots regenerated from different explants were rooted efficiently on ½ strength MS medium supplemented with different concentrations of indole-3-butaric acid (IBA) (1.0, 1.5 and 2.0 mg/l). The highest frequency of rooting (65%) was recorded on the culture medium containing 1.5 mg/l IBA[26,13,27] reported good rooting with various concentration of IBA similar to the present observation. Root induction was reported by addition of 1-Naphthaleneacetic acid (NAA) to the ½ strength basal medium and also on MS medium without growth regulators[27,28]. A total of 30% and 35% of the plantlets developed from leaf explants internode were transplanted to pots and acclimatized in the glass house for 15 days. The actual number of plants transplanted to Botanical Garden Dept of Botany was 31 and 22 of leaf and internode explants and the percentage survival was 33 and 24 (Table:-1). Thirteen plants of "Medak" *Lathyrus sativus* genotype are now growing in the Botanical Garden.

Table-1. Frequency of germination or conversion of somatic embryos on (MS medium) Murashigae-Skoog medium supplemented with BAP(1.5 mg/l) and frequency of profuse rooting on root induction medium (½ strengh MS+IBA 1.5 mg/l) with survival percentage of transplanted plantlets of Medak genotype of *Lathyrus sativus*.

Genotype	Explants	No. of inoculated explants bearing somatic embryos*	**Frequency of plant conversion Mean±S.E	No.of plantlets transferred to root induction medium ½ MS+BA 1.5 mg/l	***%.of plants with profuse rooting Mean±S.E	No.of plats transferred to pots	****%.of survival of the transplanted plants in glasshouse Mean±S.E	Actual No.of plants transplanted to botanical garden	*****%.of survival of the transplanted plants in botanical Garden Mean±S.E	No.of established plants in botanical Garden
"Medak"	Leaf	300(100/repliate)	50±0.8	162	66±0.6	114	29±0.7	31	24±0.6	7
	Internode	300(100/repliate)	53±0.5	149	55±0.1	83	24±0.5	22	33±0.4	6

*The internode or leaf explants bearing eight somatic embryos approximately.
**Percentage of explants conversion of the total inoculated explants bearing somatic embryos.
*** Percentage rooting (development of profuse roots) of the total transferred plantlets recorded 2 weeks after transfer to the root induction medium.
**** Percentage survival of the transplanted plants in glasshouse after three weeks.
***** Percentage survival of the transplanted plants in Botanical Garden after three weeks

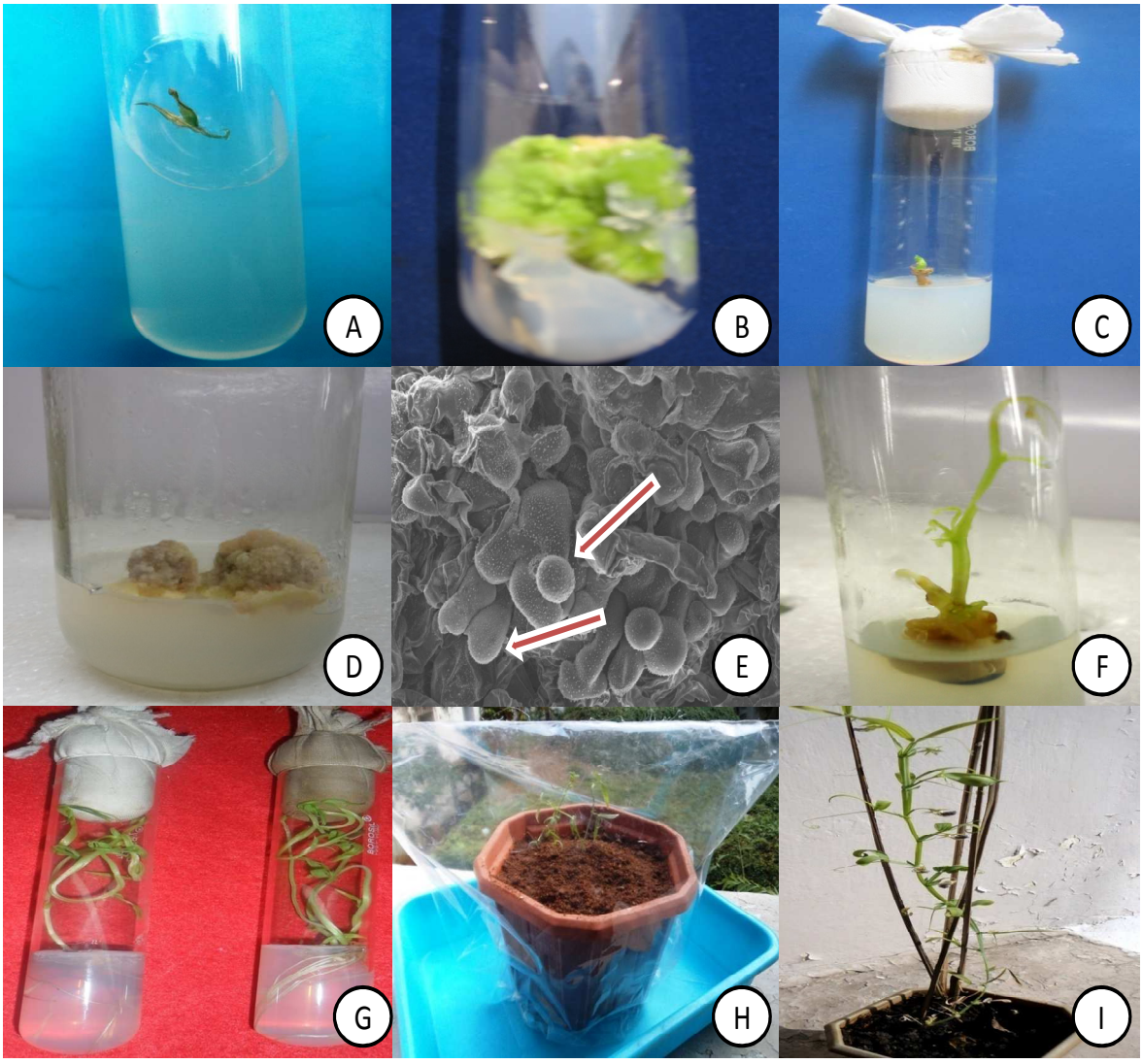

Figure:-1. Somatic embryogenesis and plant regeneration from leaf and internode explants of *Lathyrus sativus*. (A) Leaf explant on medium-A. (B) Proembryos from the embryogenic callus produced from the leaf explants. (C) internode on medium–A. (D) Proembryos from the embryogenic callus that was developed from internode explants. (E) SEM section showing heart shaped and oval shaped somatic embryos. (F) Plantlets of *Lathyrus sativus* converted from somatic embryos on medium-B. (G) Well developed roots from the plantlets inoculated on root induction medium-C. (H) A plantlet transferred to pot and gradually acclimatized in the glasshouse before transfer to the Botanical garden. (I) Well established plantlet in the glasshouse before it transferred to the Botanical garden.

CONCLUSION

Global warming, constantly diminishing water resources around the world and a growing human population mean that introduction of new strategies for sustainable agriculture becomes necessary. Consequently, scientists, breeders and farmers turned their attention to the forgotten and underestimated crops that would improve food security of people. One of such plant is grasspea *Lathyrus sativus*. The present study a standardized protocol for efficient plantlet regeneration was developed. Somatic embryogenesis from the leaf and internode explants of *Lathyrus sativus* is a suitable method for micropropagation. Initiation and proliferation of grasspea callus was efficient. Proliferation of tissue was dependent on the media composition, especially in terms of applied growth regulators and supplements. This protocol was applicable to biotechnological improvement of *L. sativus* an important legume crop.

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