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Induction of somatic embryogenesis from embryonal axis, cotyledonary node and leaf explants of green gram [*Vigna radiata* (L.) Wilczek.]

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Abstract

Plant tissue culture is very useful to increase the production of secondary metabolites. India is still having large vegetarian people in dietary habit and heavily depends upon vegetative source to meet out its daily protein requirement. Green gram [*Vigna radiata* (L.) Wilczek.] is one of the important pulse crops grown in India. It belongs to the family Fabaceae. Embryonal axis (3day old seedlings), Cotyledonary node (4day old seedlings) and Leaf (5day old seedlings) explants were excised and cultured on MS medium supplemented with different plant hormones for callus induction. The highest percentage of calli formation was observed with 8 µM of 2,4-D (78±0.74) in 3 day old embryonal axis, and the lowest percentage of calli formation was observed with 4 µM of BAP (56±1.63) in 4 day old cotyledonary nodes. PEG concentration (1%, 2%, 3%), and its duration of incubation (o h,2 h, 4 h and 6 hours) in different explants on the frequency of somatic embryos was employed. Highest percentage of somatic embryos formed in 2 % (2 hours) globular stage (77±1.49), and the lowest percentage of somatic embryos in PEG 3 % (6 hours) cotyledonary stage (6±0.61) is formed. Finally, *in vitro* plantlets were transferred to pots containing a mixture of sterilized soil and sand: (3:1 Ratio) for two months to acclimate in greenhouse conditions. The present study aimed to produce somatic embryos and their subsequent conversion to plants offers potential to use in transformation studies.

Key words: Cotyledonary node, Embryonal axis, Green gram and Leaf.

Abbreviations: $HgCl_2$ - Mercuric Chloride; MS - Murashige and Skoog; PGRs - Plant Growth Regulators; 2,4 - D - 2,4 - Dichlorophenoxyacetic acid; IAA- Indole-3-acetic acid; Kn - Kinetin; BAP- 6benzylaminopurine; SE - Somatic Embryogenesis

Introduction

Pulses are referred to as the 'poor man's meat' and 'rich man's vegetable'. Legumes represent the third largest family of flowering plants with 730 genera and over 19,500 species. (Michael G. Simpson, 2010). Legumes are a particularly good source of 'soluble' fibre. Soluble fibre is known to play a significant role in reducing blood cholesterol levels which in turn reduces risk of heart disease (Truswell, 1994). Legumes are also a rich source of antioxidants, vitamins-B, iron, calcium, phosphorus, zinc and magnesium. It has good digestibility and flavor.

Green gram contains 51% carbohydrate, 26% protein, 10% moisture, 4% minerals and 3% vitamins (Kaul, 1982). *V. radiata* is also reported for the treatment of various ailments like hepatitis, gastritis, heat rash, etc. (Leung, 2007; Huijie *et al*., 2003) and Kumar and Singhal (2009) classified it as an anticancer. Finally, legumes are ideal for weight maintenance. All legumes, with the exception of soy beans, are low in fat and provide plenty of fibre and bulk, which may help control appetite by keeping you feeling fuller for longer.

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Plant tissue culture technique represents the simplest of the biotechnologies available to plant scientists today for crop improvement. Certain *in vitro* conditions could induce heritable changes in the genomes of plant cells. Hence, plant tissue culture opened an avenue for the selection of various desirable traits from *in vitro* cultures. Somatic embryogenesis (SE) is a process during which non-zygotic cells pass through cell dedifferentiation, without the gametes fusion, are able to produce embryos. Multiplication of green gram plants through *in vitro* micropropagation method mostly focuses on different explants including cotyledonary node multiplication, which is ideal for high clonal fidelity and efficiency (Khatun *et al.,* 2008). Standard protocols have been developed for multiplication from different green gram cultivars using cotyledonary node explants (Gulati and Jaiwal, 1994; Janaki and Manoharan, 2012; Vats *et al.,* 2014).

In vitro regeneration occurs in two main way including oraganogenesis and somatic embryogenesis. The first report on organogenesis in some tropical trees was by Rao *et al*., (1981). Somatic embryogenesis was first observed by Steward *et al*., (1958) in suspension cultures of carrot followed by Reinert (1958). Legume seeds have an exceptionally varied nutrient profile, including proteins, fibres, vitamins and minerals (Mitchell *et al*., 2009). The carbon energy supply that is needed upon germination is stored in grain legume seeds either mainly in the form of oil (soya bean, groundnut) or as starch (common bean, pea, faba bean, lentil, chickpea, cowpea, mung bean) (Hedley, 2001).

Role of plant growth regulators in somatic embryogenesis

Plant growth regulators (PGRs) are the most important factors involved in the regulation of these developmental switches under *in vitro* conditions (Fehér *et al*., 2003). Plant hormones (also known as phytohormones) are signal molecules produced within plants, that present in very low concentrations. Plant hormones control all process of growth and development, from embryogenesis, *viz*., the regulation of organ size, defense against pathogen, stress tolerance and also influence the reproductive development. Unlike in animals (in which hormone production is limited to specialized glands) each plant cell is capable of producing hormones. Naturally occurring plant hormones were auxins, cytokinins, gibberellins, abscisic acid and ethylene. Among these, auxins and cytokinins are mostly employed in plant tissue culture system to regulate cell division and differentiation in the explants. In modern agriculture practices, people have established the benefits of extending the utilization of plant hormones to regulate growth of other plants. When natural or synthetic substances are used in this manner, they are called Plant Growth Regulators (PGRs) which includes polyamines, jasmonates, brassinosteroids, oligosaccharins, sterols, phosphoinositosides and salicylic acid. PGRs also have significant role in regulatory function in plants which must not be ignored in culture systems (Gaspar *et al*., 1996; Jimenez, 2005).

Materials and methods

Collection and germination of seeds

Green gram seeds [*Vigna radiata* (L.) Wilczek] vareity CO 6 were obtained from Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India.

20-30 dry seeds were pre soaked for 30 minutes in running tap water in order to ensure uniform germination. Surface sterilization of seeds was done with 70% ethanol for 3 minutes, followed by treatment with of 0.1% $HgCl_2$ for 5 minutes. Finally, seeds were rinsed 5 times with sterile distilled water, put in sterilized filter paper to remove extra water, and then cultured in

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conical flask. These steps were carried out in laminar air flow chamber. The surface sterilized seeds were inoculated (12-15 seeds) on agar-water medium in 100 ml conical flasks. Seeds were uniformly germinated aseptically on semisolid 0.7% agar-water medium.

Preparation of explants

The explants were excised from 3-5 days old seedlings and were employed for callus induction. The explants were transferred to sterile petri dishes and the seed coat was carefully removed with forceps. For preparing various seedling derived explants, the seedlings were transferred to sterile petri dishes lined with two layers of moist sterile Whatman No.1 filter paper and excised to get different explants.

Embryonal axis, cotyledonary node and leaf explants were excised and cultured on MS medium supplemented with different plant hormones. In Borosil® test tube (25x150 mm size) 15 ml of medium was poured and then, 2 explants were inoculated and plugged tightly with non-absorbent cotton plug used in test tubes. Culture responses were routinely observed for 28 days. Seedlings and cultures were maintained at 25 ± 2°C under white fluorescent light (15 μmol m^{-2} s^{-1}) with a photoperiod of (16 h Light/8 h Dark) and Relative Humidity (RH) at 80%.

Preparation of culture media

MS (Murashige and Skoog 1962) inorganic salts, organic supplements, and vitamins were used as basal media for seed germination, callus induction, callus multiplication, shoot and root induction. The pH was adjusted within a range of 5.6 to 5.8 and agar was added to the solution at a rate of 7 g/L (0.7 % w/v). The conical flask was sterilized by autoclaving at 121 °C and 15 psi for 15 min.

Callus induction

Aseptically excised explants such as embryonal axis, cotyledonary node and leaf were inoculated on MS medium with specified concentration of plant hormones. 15ml of medium was used in Borosil® test tube (25x150 mm size) and then, 2 explants were inoculated and plugged tightly with non-absorbent cotton plug used in test tubes. Culture responses were routinely determined in 28-days old callus cultures.

Subculture of callus

After four weeks of inoculation, subculture was carried out with the same composition of fresh medium to maintain callus cultures. Repeated subculture on the same medium improves the friability of the callus. Callus formation from explant involves the progressive development of more random planes of cell division, less frequent specialization of cells and loss of organized structures. Calli formed were highly variable in colour and texture. Typical colours of callus were green, yellow, white and brown. Some cultures were rough surfaced and friable while others were smooth surfaced and hard. Calli, which were yellow to cream coloured and appeared friable, were visually selected and subcultured.

Callus cultures are extremely important in plant biotechnology. Manipulation of the plant hormones *i.e.,* auxin to cytokinin ratio in the medium can lead to the development of shoots, roots or somatic embryos from which whole plants can subsequently be produced. Callus cultures can be used for regeneration of plantlets, preparation of single cells, suspension cultures, for protoplasts preparation and genetic transformation studies. In some instances, it is essential to go through a callus phase prior to regeneration *via* somatic embryogenesis or organogenesis.

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Stress-factor incubation of explants and callus

For stress incubation, abiotic stress factors, such as PEG-6000 w/v (1-3%) were used. Different explants or callus derived from different explants were incubated in stress factor containing medium for various durations (0h, 2h, 4h and 6 hours). Stress factor incubation was carried out in hormone free MS medium containing the stress factor. Culture responses were routinely observed after 28 days from the time of inoculation.

In present study, explants prepared from 3day old seedlings such as embryonal axis, cotyledonary node (4day old) and leaf (5day old) were inoculated in hormone free stress factor PEG-6000 w/v (1-3%), containing MS medium at specified concentration and incubated for specified durations (*i.e.,* 0 h,2 h,4 h,6 h).

Stress factor incubated callus (*ca*.15 g) along with incubation medium was transferred to tubes and centrifuged at 500 × g for 3 min. Washing by centrifugation was repeated 3 times by using the washing medium. The supernatant was withdrawn by using pasteur pipette. The pelleted callus tissue was cultured on the embryogenesis induction medium with *ca*. 500 mg inoculum.

The explants and callus were inoculated into 100 ml MS medium supplemented with plant hormones was added in Borosil 250 ml Erlenmeyer flasks containing stress-factor incubation and subsequently incubated for specified time in an orbital shaker (New Brunswick, USA) at 100 rpm at 24 °C. The stress-factor incubated callus was cultured on semi-solid embryogenesis induction medium and microscopic observations were carried out in 28-days old callus.

Suspension cultures

Suspension cultures can be obtained from callus cultures by transferring a piece of callus tissue into liquid medium and subjecting it to continuous shaking. In general, the growth rate of the suspension-cultured cells is generally higher than that of the solid culture. The former is more desirable, mainly for the production of functional metabolites on a large scale. A piece of the callus is transferred to a liquid medium in a vessel such as an Erlenmeyer flask and the vessel placed on a gyratory shaker. The suspension cultures of conical flask was shaken on gyratory shaker (Excella E24 Incubator Shaker Series) 100 rpm for 25^0C maintaining the cultures.

Fine suspension cultures were subcultured weekly to monthly depending on subculture inoculum density. Weekly subculture was necessary if 50 ml of an old culture were removed and reloaded with 50 ml of fresh medium was added. A monthly subculture or longer was validated if a single clump of early staged embryogenic tissue was used to inoculate 70 ml of fresh medium was added.

Microscopic observation

After one month, the total number of somatic embryos at each developmental stages was observed and recorded. Different stages such as globular, heart, torpedo and cotyledonary shaped embryos were observed. Calli were observed routinely for scoring the frequency of occurrence of different stage embryoids by using phase contrast cum fluorescent microscope (Nikon, Japan).

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Embryo induction

The callus was inoculated directly in the suspension culture for the somatic embryo induction, whereas, explants were inoculated into semisolid embryogenic callus induction medium and the callus (28 days) derived from this were subsequently inoculated into suspension cultures. For embryo development, embryogenic tissues were washed with hormone free proliferation medium and transferred to semisolid medium.

The semisolid medium was the same as that used for embryo proliferation. Washed, embryogenic suspension culture tissue was pipetted onto the semisolid medium, the tissue was allowed to settle and the liquid medium was decanted and discarded. Mature embryos, which were obtained in 28 days on the semisolid medium, were then placed on germination medium.

Development and maturation of somatic embryos

Globular embryos formed after 30 days of culture were separated and transferred to MS basal medium augmented with 30 g/L sucrose, 7 g/L agar and different growth regulators and incubated for two weeks under the conditions. MS basal medium supplemented with auxin and cytokinin concentrations were tested for further development and maturation of somatic embryos, while MS basal medium lacking growth regulators served as the control. The desiccated embryos were inoculated individually on MS basal, full and half strength MS semisolid medium supplemented with different PGRs of auxin and cytokinin was used.

Conversion of somatic embryos and plant regeneration

Matured somatic embryos were transferred to regeneration media to promote somatic embryo conversion and plant development. The regeneration medium consisted of half strength of MS medium. The established plants with expanded primary leaves and roots were detached from the culture tubes and the roots, specifically, were given delicate wash with tap water in order to remove any medium attached with them. Complete plantlets with well developed roots in the tubes were subjected to the following hardening procedure. After loosening the cotton plugs, the plantlets were removed from the test tubes and washed in running tap water. Subsequently, plantlets were transferred to pots containing sterilized soil and sand: (3:1) mixture. Greenhouse and field trials indicated that the best revegetation occurred only when the plants were grown in containers in a nursery and then transplanted back into their natural environment.

Statistical analysis

In present investigation, 150 explants were employed in each experiment. For callus samples, 30 test tubes were inoculated with specified callus tissue either with or without PEG incubation. For, suspension cultures 15 flasks were employed for each experiment. Each experiment was repeated thrice. A completely randomized design (CRD) was used in all the experiments and analysis of variance and mean separations were carried out using Duncan's Multiple Range Test (DMRT) (Duncan, 1955). Significance was determined at 5% ($P \leq 0.05$) level.

Results and discussion

Response of various explant types in the development of proliferative callus was optimized as a function of age of source-seedlings from which explants were prepared. Seedlings at the age of 3 day embryonal axis, 4day cotyledonary node and 5 day leaf were employed and their results were presented in **Table 1**. The explants were cultured on MS medium supplemented with various auxin concentrations (4 µM, 6 µM, 8 µM) of 2,4-D and IAA. The highest percentage of

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calli formation was observed with 8 µM of 2,4-D (78±0.74) in 3 day old embryonal axis, 8 µM of 2,4-D (76±0.54) in 4 day old cotyledonary nodes and 4 µM of IAA (78±0.58) in 5 day old leaves. The lowest percentage of calli formation was observed with 6 µM of IAA (66±0.53) in 3 day old embryonal axis, 4 µM of IAA (68±0.58) in 4 day old cotyledonary nodes and 6 µM of 2,4-D (64±0.81) in 5 day old leaves.

The explants were cultured on MS medium supplemented with varying cytokinin concentrations (4 µM, 6 µM, 8 µM) of Kn and BAP. The highest percentage of calli formation was observed with 8 µM of Kn and 8 µM of Kn (76±0.74) in 3 day old embryonal axis, 4 µM of Kn (78±0.50) in 4 day old cotyledonary nodes and 4 µM of BAP (76±0.58) in 5 day old leaves. The lowest percentage of calli formation was observed with 8 µM of BAP (60±1.09) in 3 day old embryonal axis, 4 µM of BAP (56±1.63) in 4 day old cotyledonary nodes and 6 µM of Kn (62±0.81) in 5 day old leaves.

Many authors stated that *In vitro* regeneration *via* somatic embryogenesis is important for clonal propagation and is usually an integral part of genetic modification (Varisai Mohamed *et al*., 2004; Huo *et al*., 2005). Leaf segments from young seedlings have been shown to be suitable explants for somatic embryogenesis in green legumes (Shekhawat and Galston, 1983; Kumar *et al*., 1988).

PEG concentration (1%, 2%, 3%), and its duration of incubation (o h,2 h, 4 h and 6 hours) in different explants on the frequency of somatic embryos was employed. Highest percentage of somatic embryos formed for following stages **(Table 2).** PEG 2 % (2hours) globular stage (77±1.49), PEG 2 % (6hours) heart stage (74±2.97), PEG 3% (6hours) torpedo stage (71±1.71), PEG 2 % (2hours) cotyledonary stage (15±0.95) is formed. Lowest percentage of somatic embryos formed for following stages. PEG 1 % (6hours) globular stage (28±1.91), PEG 1 % (4hours) heart stage (23±1.71), PEG 3% (2hours) torpedo stage (16±1.49), PEG 3 % (6hours) cotyledonary stage (6±0.61) is formed **(Figure 1).**

Conclusion

In the present study, cotyledonary stage somatic embryos developed into plantlets in hormone-free half strength MS semisolid medium at a germination frequency of 75% **(Figure 1q).** Also, high frequency of somatic embryogenesis and plantlet regeneration of green gram has been achieved. The *in vitro* developed plantlets were further grown in soil and sand at a survival rate of 100% **(Figure 1r).** Finally, 55 plantlets were developed for harvesting. Somatic embryos could possibly be encapsulated to use as artificial seeds for mass propagation of elite germplasm during afforestation program.

The application of plant tissue culture for improving and propagating important agricultural crops is based on the ability of the cells to regenerate plants. SE induced *in vitro* results in efficient plant regeneration and provides a valuable system in plant biotechnology applied in micropropagation and genetic transformation of different plant species. Embryonal axis, Cotyledonary node, Leaf explants of green gram is a suitable method for SE. Initiation and proliferation of green gram callus was efficient. Tissue proliferation was mainly depends on the media composition, especially in terms of applied growth regulators and supplements. This protocol was applicable to biotechnological improvement of green gram is an important legume crop.

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Table 1. Effect of seedling age and explant types of callus induction on auxins and cytokinins

Plant growth regulators	Concentration (µM)	Callus induction (%)		
		Embryonal axis (3day old)	Cotyledonary node (4day old)	Leaf (5day old)
Auxin (2,4-D)	4	70±0.89	72±0.80	74±0.67
	6	74±0.50	74±0.92	**64±0.81**
	8	**78±0.74**	**76±0.54**	72±0.37
Auxin (IAA)	4	70±0.83	**68±0.58**	**78±0.58**
	6	**66±0.53**	72±0.86	72±0.66
	8	76±0.50	74±0.56	70±0.72
Cytokinin (Kn)	4	72±0.73	**78±0.50**	64±0.81
	6	74±0.92	66±0.83	**62±0.81**
	8	**76±0.74**	60±0.94	70±0.54
Cytokinin (BAP)	4	64±0.50	**56±1.63**	**76±0.58**
	6	74±0.50	68±0.86	68±0.86
	8	**60±1.09**	70±0.89	74±0.67

*Auxin supplemented with MS + 2, 4-D, MS + IAA at various concentrations specific to different explants. *Determined on 28d old callus
*Cytokinin supplemented with MS + Kn, MS + BAP at various concentrations specific to different explants. *Determined on 28d old callus
Number of different explants tested = 30. Values are means ± SD of 5 replication of 3 repeated experiments; means within a column followed by the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5 % level

Table 2. Effect of PEG concentration, and its duration of incubation in different explants on the frequency of somatic embryos

Stress concentration	Incubation duration (Hours)	Somatic embryos (500mg of fresh weight of calli)	Different stages of somatic embryos (%)			
			Globular	Heart	Torpedo	Cotyledonary
Control	0	n.d	n.d	n.d	n.d	n.d
(PEG 1%)	2	124±1.07	70±2.39	50±1.67	0	0
	4	95±2.88	45±1.57	**23±1.71**	0	0
	6	118±1.17	**28±1.91**	35±0.77	0	0
Control	0	n.d	n.d	n.d	n.d	n.d
(PEG 2%)	2	145±0.97	**77±1.49**	38±1.41	45±1.97	**15±0.95**
	4	127±0.93	56±2.25	37±2.34	55±1.88	0
	6	105±2.05	47±1.60	**74±2.97**	70±1.67	8±0.16
Control	0	n.d	n.d	n.d	n.d	n.d
(PEG 3%)	2	111±0.94	66±1.41	68±2.00	**16±1.49**	13±0.97
	4	88±1.00	74±1.90	57±1.67	53±1.71	10±0.67
	6	130±2.98	35±1.67	63±1.71	**71±1.71**	**6±0.61**

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Number of different explants tested = 50. Values are means ± SD at 5 replication of 3 repeated experiments; means within a column followed by the same letters are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5 % level. [n.d- not detected].

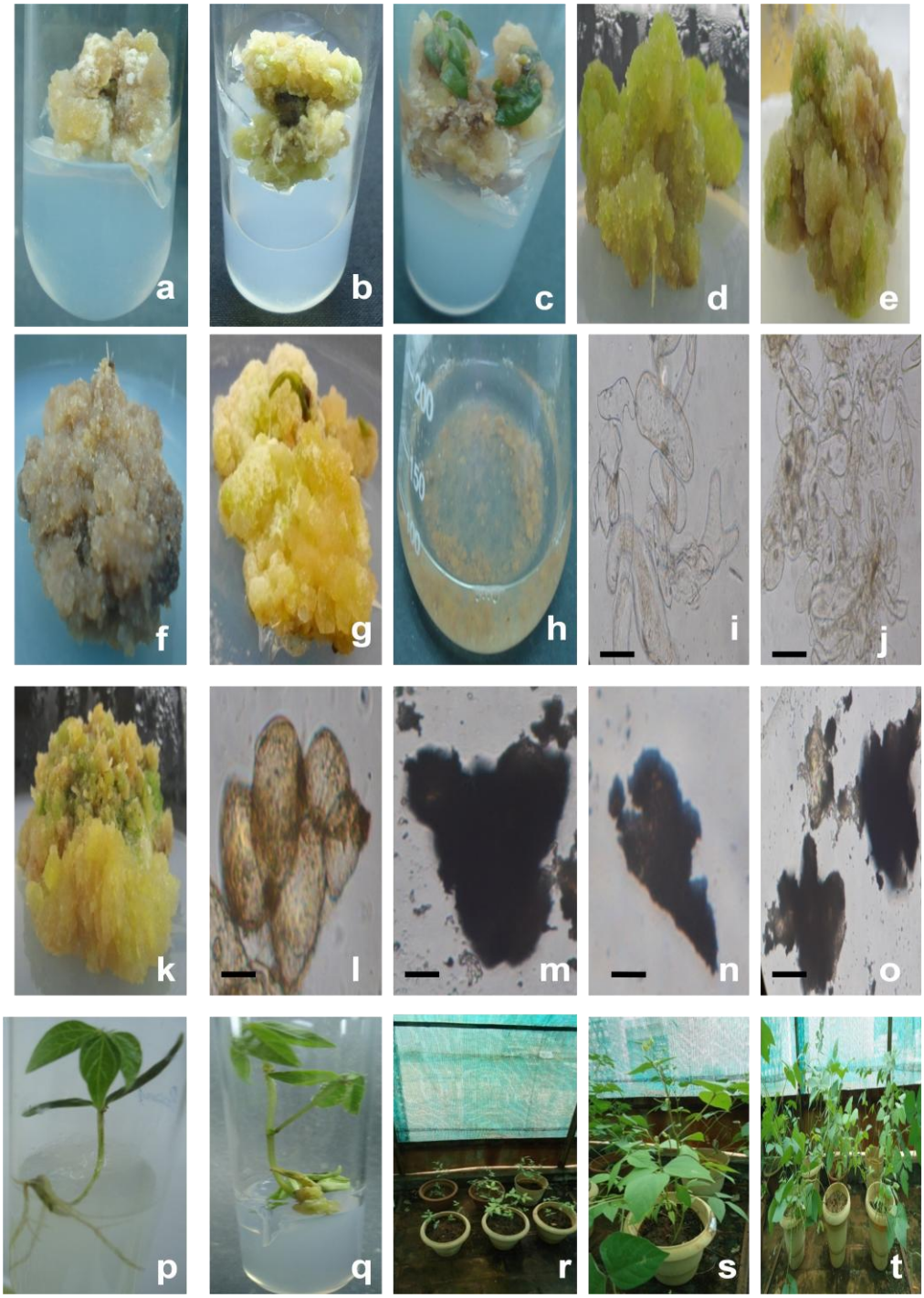

Figure.1: Somatic embroyogenesis of green gram

[(a) Vitrified soft callus from embryonal axis (3 days old seedlings) explants raised in MS + 2,4-D (8µM), (b) Yellowish friable callus from cotyledonary node (4 days old seedlings) explants raised in MS + 2,4-D (8µM), (b) Glossy and translucent soft callus from leaf (5 days old seedlings) explants raised in MS + IAA (4µM), (d) Greenish friable calli from embryonal axis explants raised in MS + 2,4-D (8µM) + Kn (8µM), (e) Greenish yellow friable calli from embryonal axis explants raised in MS + IAA (6µM) + BAP (6µM), (f) Brownish friable calli from cotyledonary node explants raised in MS + 2,4-D (6µM), + Kn (6µM), (g) Yellowish friable calli from leaf explants raised in MS + IAA (6µM) + BAP (6µM), (h) Suspension culture, (i) Embryogenic cells in primary calli from embryonal axis explants raised in MS + 2,4-D (8µM), (j) Non- Embryogenic cells in primary calli from embryonal axis explants raised in MS + 2,4-D (6µM) [bar in (i,j): 50 µm], (k) Pro-embryogenic mass in calli from cotyledonary node raised in MS + 2,4-D (8µM), (l) Globular stage somatic embryo, (m) Heart stage somatic embryo, (n) Torpedo stage somatic embryo, (o) Cotyledonary stage somatic embryo [bar in (l-o): 1 mm], (p,q) Plantlet regenerate for 20 days (r) 20days plants are transfer to pots (s) Flowering for 35-37 days (t) Harvesting for 65-70 days].

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