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In Vitro Culture and Genetic Transformation Studies on Glycine Max L. (Soya Bean)

Hareesh Tulasi, M.Sc, M.Phil,
Department of Bio-Technology,
Talla Padmavathi College of Pharmacy,
Orus, Warangal, Telangana.

Dr.M.Thiruvanganam, M.Sc, M.Phill, Ph,D.
Department of Bio-Technology,
Mardhu Pandiyar College, Vallam, Thanjavur, Tamil Nadhu.

Dr.T.Shastree, M.Sc, Ph.D,
Department of Bio-Technology,
Talla Padmavathi College of Pharmacy,
Orus, Warangal, Telangana.

Annam Sravan Kumar
M.Sc(Bio-Technology)
Chaitanya Degree And PG College,
Hanamkonda, Warangal, Telangana, India.

Abstract: ***In the development of plant biotechnology, we are witnessing a most dramatic evolution of new techniques, applicable to industry and agriculture. In the past three decades plant cell and tissue culture has emerged as a major tool in the study of an increasing number of applied and fundamental problems in the plant science and now it has become an important integral constituent of plant biotechnology. Our approach to problem had been empirical but we have now reached a stage where we can manipulate cell and tissues more precisely and in a target based manner, plant tissue culture is presently of great interest to molecular biologists, biotechnologists, plant breeders and industrialists. The process of cell and tissues culture plays a major role in producing pathogen free plants, synthetic seeds, secondary metabolites, somatic transformation. Tissue culture approaches are also being expanded to carry out investigations in cell and developmental biology, biotechnology, physiology, genetic and molecular biology that are intended to provided to provide insight into the fundamental mechanism of plant growth and differentiation. In view of its commercial value and used as vegetable , a protocol for large scale plantations raped clonal multiplication of disease free plant with other agronomically important traits of added value will be of advantage for commercialization of the species. In this paper we establish the protocol for callus induction from leaf and cotyledon explants. We also implemented the Direct regeneration in different explants by modulating the concentration and combinations of auxins and cytokinin and also plantlet establishment.***

Keywords: ***BioTechnology, Tissue Culture, Synthetic seeds, Disease free plants, Glycine max.***

Introduction:

Plant tissue culture was exploited for both basic and applied aspects such as haploidy, mutagenesis, somatic exbryogenesis, somaclonal variation, selection of cell lines resistant to antibiotics and anti metabolites, protoplast fusion, genetic manipulation and molecular biology (Dix and Street, 1975; Bajaj et al., 1977; Lindsey and Yeomann, 1983; Mantel et al., 1985).

Development of science of tissue culture is historically linked to the discovery of cell and subsequent propounding of the cell theory. Further tissue culture could be attributed to the cell doctrine, which implicitly admitted that a cell is capable of autonomy and even demonstrated the potential for totipotency-ability to regenerate an entire organism from a single plant cell. Hanning (1904) initiated a new line of investigation involving the culture of embryogenic tissue which later became an important applied area of investigation, using in vitro techniques. Most promising results were obtained in the year 1908 by Simon, who achieved success in the regeneration of bulky callus and buds and roots from popular stem segments. Therefore, Simon may be credited with having established callogenesis and to some extent even micropropagation. Plant tissue culture also offers

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a meaningful tool for isolating induced as well as spontaneously occurring variant cell lines for the increase of genetic diversity in crops. The use of this technique for mutant selection has many advantages over the use of other available methods. Mutation breeding in conjunction with tissue culture offers economy in space, time, labour and expenditure; Selection of mutants compared to other methods is also rapid.

The recent rapid progress in biology is due to development of new ideas and new methods of investigation. Modern biology attempts to elucidate progress which takes place at cellular level. The modern in vitro culture of protoplasts, cell, tissue and organ under aseptic conditions may be termed as tissue culture. The plant tissue culture was exploited both for basic and applied aspects of plants research, encompassing haploidy, mutagenesis, Somatic embryogenesis, Somaclonal variation, selection of cell lines for antibiotics metabolic analogue resistance, protoplast fusion, genetic manipulation and molecular biology.

The concept of cellular totipotency i.e. ability to regenerate and entire organism from a single cell and plant part (explant) proposed by Haberlandt (1902) emerged as corollay of the cell theory proposed by Schleiden (1838) and Schwann (1839). According to this concept, the genetic information necessary for the development of the entire organism is contained in all living cell. Consequently when the cells are released from the developmental controls, they are able to divide and differentiate into specific organs and whole organisms.

Direct regeneration

Organogenesis in legumes has been reported from several tissue types, including cotyledons, leaves hypocotyls, shoot tips, roots and anthers. Among different explants, leaves have been used most frequently in organogenic system, the age of the leaf and growth regulators used can effect the success of regeneration observed a gradient in regeneration potential, such that proximal regions of cotyledons gave rise to shoot regeneration.

As with cotyledons, the use of hypocotyls explants has been generally successful with few exceptions in Trifolium species (Webb et al., 1987). Various factors appears to effect regeneration from hypocotyls. High frequency of direct organogenesis from hypocotyl segments was reported in G. canescens (Kameya and Widholm, 1981). The frequency of organogenesis was dependent on seedling age and length and position of the hypocotyls explant.

Somatic embryogenesis

In tissue culture, plant regeneration is important. Regeneration is possible by two way i.e., organogenesis and embryogenesis. Plant regeneration by somatic embryogenesis from cultured cells was originally observed with carrot (Steward et al., 1958). In somatic embryogenesis, somatic cell divide to from complete analogous to zygotic embryos. The bipolar structure of somatic contains both shoot and root meristems.

Somatic embryogenesis is the process of a single cell or a group of a cells initiating the developmental pathway that leads to reproducible regeneration of non-zygotic embryos capable of germinating to form complete plants. Under natural conditions this pathway is not normally followed, but from tissue cultures somatic embryogenesis occurs most frequently and as an alter native to organogenesis for regeneration of whole plants. According to Sharp et al., (1982). Somatic embryogenesis is initiated either by 'Pre-Embryogenic Determined Cells (PEDCS) or by "Induced Embryogenic Determined Cells (IEDC). In PEDCS the embryogenic path way is predetermined and the cells appear to only wait for the synthesis of an inducer (or removal of an inhibitor) to resume independent mitotic divisions in order to express their potential. Such cells are found in embryonic tissues (including scutellum of cereals).

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Protoplast isolation

The protoplast, also known as naked plant cell refers to all the components of plant cell excluding the cell wall, Hanstein introduced the term protoplast in 1880 to designate the living matter enclosed by plant cell membrane. The isolation of protoplasts from plant cells was first achieved by micro surgery on plasmolyzed cells by mechanical method (Klercker, 1892). Protoplast can be isolated from plant tissues (or) cultured cells by enzymatic digestion to remove the cell wall. Besides been useful for cell fusion studies, higher plant protoplasts can also take up. Through the naked plasma membrane, foreign DNA cell organelles, bacteria or virus particles these unique properties of protoplasts, combined with totipotent nature of plant cells, have opened up an entirely area of fundamental and applied search in experimental biology and somatic cell genetic (Gleddie et al., 1986).

Enzyme treatment

The release of protoplasts is very much dependent on the nature and concentrations of enzymes used. The two enzymes regarded essential to isolate protoplasts from plant cells are cellulose & macerozyme. Driselase, having and number of zymolytic activities such as cellulose, pectinase laminarinase and zylanase (Kao et al., 1974) has proved especially useful for isolating protoplasts from cultured cells. Increase in yield of mulberry proplasts by treatment with chemical substances has been reported earlier (Ohnishi and Kiyama, 1987).

Osmticum

A fundamental property of isolated protoplasts is their osmotic fragility and hence the need for suitable osmotic stabilizer to the enzyme solution, the protoplast washing medium and protoplast culture medium is necessary. A variety of solutes, ionic and non-ionic, have been tested for adjusting the osmotic pressure of the various solutions used in protoplast isolation and culture, but the most widely used osmotica are sorbitol and mannitol.

Legume transformation :

Legumes exhibit a diversity of responses when cultured in vitro. Depending upon several factors regeneration occurs via organogenesis and or embryogenesis either directly from explanted tissue or indirectly after an intervening callus phase. Methodologies leading to diversity of in vitro responses are a relatively new accomplishment with few exceptions, legumes were commonly described as recalcitrant species with regard to tissue culture a comment especially true of the large- seeded legumes. Today there are reports that at least 75 species from 25 genera in the leguminoseae, have undergone denovo regeneration todate, and limited contributions to crop improvement have been realized. Successful regeneration has been accomplished in large part by species-specific determination of parameters critical to regeneration, such as explant source, genotype and media constituents and advanced techniques.

In view of its commercial value and used as vegetable , a protocol for large scale plantations raped clonal multiplication of disease free plant with other agronomically important traits of added value will be of advantage for commercialization of the species.

Hence, the present investigation has bean carried out with the aim to achieve the following objectives

- To establish the protocol for callus induction from leaf and cotyledon explants.
- To achieve the Direct regeneration in different explants by modulating the concentration and combinations of auxins and cytokinin and also plantlet establishment.
- To induce the somatic embryo genesis and plantlet formation.
- To develop procedure for encapsulation of zygotic embryo of Glycine max.
- To isolate protoplast from leaves of Glycine max.
- To establish the protocol for Agrobacterium mediated genetic transformation using leaf explants.

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REVIEW OF LITERATURE

The degree of success in any technology employing plant cell tissue or organ culture is related to relatively few factors. A significant factor is the choice nutritional components and growth regulators. In the past two three decades a large number of reports have appeared on modification of about two dozen media composition (Street and Shillito 1977, Pieril 1987, Torres 1989).The most widely used media for plant tissue culture studies are those of Murashige and Skoog (1962) (MS) Linsmaier and Skoog (1965)(LS) and Gamborg's (1968) (B5).

Skoog and Miller (1957) demonstrated apparently simple relationship between –Auxin- Cytokinin balance of the nutrient medium, and the pattern of redifferentiation of un organized tobacco pith callus. High concentration of Auxin (IAA) promoted rooting where as proportionately more Cytokinin (Kinetin) initiated bud or shoot formation. Auxins proved to be essential for establishing successful culture of plant tissue due to their effects on nucleic acid and protein metabolism (Gauthert,1959, Rao and Swamy , 1972). Cytokinin influence cell growth by promoting nucleic acid and the synthesis of specific proteins required for cell division (Letham, 1968). Although the precise requirement for Auxins and Cytokinins may differ between species, this general approach, namely the manipulation of exogenous Auxin and Cytokinin level has formed the basis of regeneration techniques for large ranges species physical environment viz. state of medium, light, temperature, humidity besides source and size of explants are known to play an important role in in vitro organogenesis (Murashige, 1974).

Shoot number increases logarithmically with each subculture to give greatly enhanced multiplication rates. As this method involves only organized meristem, the plants obtained show minimum variability and allows recovery of genetically stable and true to type progeny (Murashige,1974 ; Hu and Wang,1983),A large number of plant species have been successfully propagated by this method includes several crop plants, ornamentals, fruit crops and woody trees (Hu and Wang, 1983 ;Pieril,1987;Mascarenhas and Muralidharan,1989;Chu, 1992; Dhawan,1993; Huett mann and Preece, 1993)

Micro propagated plants can be exploited commercially (Murashige, 1974; Mantell et al., 1985; Pieril, 1987; Chu, 1992) and as a means of germplasm storage for maintaining disease free stocks (Witherr,1989). Meristem culture technique has been used to produce species free from viral infections (Wang and Hu, 1982).
Morphogenesis can be obtained either directly from cultured explants or indirectly from callus or suspension culture (George and Sherington, 1984) in most cases, plant regeneration takes place by the formation of supplementary adventitious buds in the explants or de novo organization of shoot and root meristem in callus (Vasil et al., 1982).

Callus can be increased substantially by sub culture. The potential no of plants regenerated can thus be very high but callus is often subjected to genetic aberrations resulting in variability also loss of totipotency. Plant regeneration through adventitious shoot and root formation has been studied in a number of plants (Flick et al., 1983) and these studies shed light on several factors responsible for plant regeneration (Murashige, 1974; Flick et al., 1983).

It is also reported, that plants regenerated for long term callus and cell suspension culture often associated with chromosomal variations(Evans et al., 1984: Evans and sharp, 1986) Larkin and Scowcroft (1981) have proposed a general term' " Somaclones" for plant variants obtained form tissue culture irrespective of their origin. Variation which occur in plants the regenerated from cultured cells and tissues has been reported for Morphological, Biochemical and genetic characters (Ahuja, 1986). Soma Clonal variation has been exploited in sugarcane per increase sucrose content and resistant to eye spot disease (Heinz et al., 1977; Krishnamurthy, 1982), in Potato for growth habit, tuber color and late blight resistance (Shephard et al., 1980; Thomas et al; 1982), in tomato for resistance to Fusarium wilt (Evans, 1989). The

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importance of somaclonal variations in crop improvement has been adequately reviewed by several authors (Evans et al; 1984; Evans and sharp, 1986; Scowcroft and larkin, 1988;).

Plant regeneration through somatic embryo genesis has several advantages over other routes to in vitro plant production and appears most promising for future large scale, rapid plant propagation. The totipotency of cells finds best expression in the formation of somatic embryo from single cells and their growth and development to form a complete plant (Ammirato 1983; 1987; Atree and fowke; 1993 Finer, 1994). Somatic embryogenesis has great implications in tissue culture technology. Firstly because somatic embryos have pre-formed root and shoot meristems their by reducing several of the labor intensive steps involved in the sub culture separation and rooting of individual shoots. Secondly, if the single cell origin of somatic embryos in universal, as on increasing number of recent reports confirm, them potentially all single cells in cell suspensions could be induced to form embryoids in a prodigious way (Steward et al., 1958; 1964; Jocobsen and kysely, 1984).

Somatic embryo genesis has now been observed in a wide range of plants including those of the major crops (Ammirato, 1983, Vasil and Vasil, 1984; Rangaswamy , 1986, Torres 1889). The literature contains hundreds of references describing the specific manipulations required to effect somatic embryo development from a verity of agronomically and horticulturally important plants (Zimmer man 1993) efficient regeneration of plantlets from embryogenic callus is important step towards the genetic manipulation (Lorze et al., 1988; Parrott et al., 1991; Mc Granahan et al., 1990).

The goal embryo culture is to real plants under aseptic conditions from isolated embryos grown in a defined nutrient medium. The fundamental and applied aspect of embryo culture was reviewed by several authors (Bhojwani and Razdan, 1983; Raghavan, 1986; Williams et al, 1987; Pieril 1987; Raghavan 1994). Several crosses between varieties of the same species generally yield viable hybrids. If sexual crosses are made between plants that belong to different species of the same genus or between plats that belong to different genera, hybrid production is difficult due to barriers at the level of pollination, fertilization and different stages of development of zygotic embryos (Raghavan, 1986, 1994;).

Another interesting area of research in plant tissue culture in protoplast isolation, culture and fusion, for the first time Cocking (1960) isolated the plant protoplasts using enzyme cellulose. The first report of plant regeneration from isolated protoplast was in Nicotina tabacum (Takaba et.al., 1971). Since then, the plant regeneration from isolated protoplasts has been reported in many species (Evans and Bravo, 1983; Roset and Gilsen, 1989). In order to overcome sexual barriers, advances in protoplast fusion technology have made somatic hybridization techniques to facilitate the introduction of agronomically important traits from wild relatives in to cultivated species. This approach has an immense potential in transferring desirable agronomic traits using chemical or electro fusion for crop improvement. (Razdon and Cocking, 1981; Jones, 1988; Parimrerd et.al., 1988; Kobayashi et.al., 1996).

CALLUS INDUCTION

Methodology:

Seeds of Glycin max cv KHST-2. were soaked in sterile distilled water for 24 hours. These were surface sterilized with 0.1% (W/V) Mercuric chloride (HgCl2) solution for 3-5 minutes followed by three rinses with sterile distilled water. These were germinated aseptically on MS basal medium (Murashige and Skoog ,1962) solidified with 0.8% (W/V) difco bacto agar at pH 5.8 ± 1.0 in 250 ml Ehrlen meyer flasks (50ml medium / flask).

For callus induction the explants viz, Cotyledon (0.6 - 0.8 cm2), from 3-week-old axenic seedlings and Leaf (0.8 – 1.0 cm2) explants from 5-week old seedlings were excised , these explants were inoculated to Ms medium supplemented with various concentrations of (0.5- 3.0 mg / l) of auxins such as 2,4 –Di chlorophenoxy acetic acid (2,4-D) , Indole 3- acetic acid (IAA) , Naphthalene acetic acid (NAA) all the

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explant growth regulators were used as Cytokinins and auxine alone in culture media . All media were adjusted to pH 5.8 before addition of 0.8% agar and autoclaved at 1210 C and 103 K pa for 20 minutes cultures in 25 x 150 mm cultures tubes.

Data Analysis:

At least 10 replicates were maintained for each treatment and data was recorded after 4 weeks of cultures. Each experiment was repeated at least twice with similar results and data presented are of one representative experiment. All the data were statically analyzed.

DIRECT REGENARATION

Regeneration from cotyledon

Seeds of Soyabean (Glycine max cv KHST-2) were collected from Agriculture Research station Warangal such seed were not damaged and uniform in size wear used. These selected seeds were rinsed in 70% alcohol for 1min then sterilized with 0.2% aqueous mercuric chloride (Hgcl2) for 5min and subsequently washed five times with sterile distilled water. The sterilized seeds were germinated in Petri plates having moistened filter paper for 48hrs. The cotyledon from four days old seedling were excised and cultured on MS medium containing different hormonal concentration and combination.

Pre conditioning the explants:

After appropriate sterilization the seeds were also germinated on MS medium containing 3% sucrose, 0.8% agar agar 1.0 mg/L BAP for 48hrs, The explants excised from these seedlings were considered as preconditioned explants .

Rooting of In vitro regenerated shoots and Acclimatization:-

Well developed shoot were separated individually and planted on half strength Ms medium containing IBA (0.1- 1.omg1L) Rooting was observed within 4-5 weeks . The regenerated to sterile vermiculite .In order to ensure high humidity a glass beaker was inverted over. Each plantlet initially for few days. Subsequently, the plantlet was transferred to the field.

Data analysis:-

At least 20 replicates were maintained for each treatment and date was recorded after 4 weeks of culture. Each experiment was repeated at least twice with similar results and data presented are of one representative experiment. All the data where statistically analyzed.

SOMATIC EMBRIYOGENESIS

Plant material

Seeds of Glycin max cv KHST-2. were collected from Agriculture Research station Warangal. Such seeds were not damaged and uniform in size were used. These selected seeds were rinsed in 70% alcohol for 1 min. then sterilized with 0.2% Aqueous Mercuric chloride (HgCl 2) for 3 min and subsequently washed five times with sterilized distilled water. The sterilized seeds were germinated aseptically on MS (Murashige and Skoog 1962) Basal medium.

Culture media and culture conditions

Leaf (4 week old) of different sizes (0.5 - 10 mm) were cultured with the abaxial surface in contact with induction medium consisting of MS salts B5 vitamins 6% sucrose (0.5 – 5.0 mg/L) 2.4-D – chlorophenoxy acetic acid (2,4-D) and pH 7. the percentage of explants responding was evaluated after 4 weeks of culture. Responses scored were the percentage of explants with evidence of globular stage embryos.

All the cultures were incubated under 16/8 h. light/ dark photoperiod at 25 ± 2ºC a light intensity of 40 µ mol m-2 s-1 was provided by cool- white florescent tubes. The cultures were transferred to fresh medium after an interval of 4 weeks. For germination and plant let formation somatic embryos were transferred to MS medium supplemented with0.5 mg/L IAA + 0.5 – 5.0 mg/L BAP and incubated under the same culture conditions.

PROTOPLAST ISOLATION

Isolation of protoplasts from mesophll cell (Seeds of Glycine max cv KHST-2. was used in our study the

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seeds were obtained from ICRISA Hyderabad and the seeds presoaked over night and washed under tap water for 30 min to remove adherent particles. The seeds were immersed in 5% (v/v) Teepol for 10min rinsed with sterile double distilled water this was followed by surface sterilization with 0.1% (m/v) HgCl2 for 5 min and rinsed 5 times in sterile distilled water. The sterilized seeds were then placed on to basal Murashige and skoog (1962) medium for germination.

The leaves of 2-3 cms in length and 1- 1.5 cm in width were excised from 6 weeks old seedlings. The leaves were cut into pieces smaller than 1mm and incubated in filter sterilized enzyme solution. The enzyme solution consistsed of 20% cellulose "onuzuka" R-10 and 1% macerozyme R 10 prepared in MS salts at pH 5.5 with 0.6 m mannitol and osmoticum the sliced leaf pieces of all the cultivars were incubated in 10 ml of engume solution at 27oC and shaken at 40- 50 rpm for 4 5 hrs in dark.

Isolation of protoplasts from callus cultures

Seeds of Glycine max var cv KHST-2.were soaked for 24 hrs in sterilized water and surface sterilized with 0.1% HgCl2 for 3 to 5 minuts. Then these were washed 3 times with sterile distilled water for 5 minuts and germinated aseptically on MS basal medium. The cotyledon (0.5 – 0.8 cm2) from 4- weeks old axenic – seedlings were excised and inoculated to MS medium supplemented with 2.0 mg/L 2.4.D. alone and gelled with 0.8% agar cultures were maintained at 25 ± 2°C under a 16 hrs. photoperiod was provided by cool white fluorescent lamps.

15 – 20 days old. One – gram friable callus derived from cotyledon explants was gently broken into small pieces of callus. Incubation was carried out in 10ml of digestion solution containing 1% cellulose and 0.5% macerozyme with 0.6 m mannitol as osmoticum. Flasks were incubated at 27° C and shaken at 50 rpm for 3-4 hours in dark.

Purification of mesophyll and callus derived protoplasts.

The protoplasts were then purified by 60 µ m steel mesh. The filtrate was collected in screw cap centrifuge tube and centrifuged at 50 g for 5 minuted. The supernatant was discarded and the pellet containing protoplasts was loaded on 20% sucrose solution for purification and centrifuged at 100g for 10 minutes to get a distinct protoplast band. The band was taken in a screw cap centrifuge tube and washed with 5ml of 0.6M mannitol by centrifuging at 50g for 5-7 minutes. The pellet was suspended in culture medium containing MS +0.6 M mannitol + 2, 4-D and BAP at pH 5.7 and the protoplast yield was estimated using hemocytometer. Proto col is adopted for the purification of mesophyll derived protoplasts.

AGROBACTERIUM MEDIATERD GENETIC TRASFORMATION

In vitro leaf explants of Glycin max cv KHST-2 was used for the transformation study. Pods with immature seeds (3-5 mm) were harvested from field-grown plants and surface sterilized for 1 min in 70% ethanol followed by 15 min in a 50% commercial bleach containing Tween-20. After 4 rinses in sterile distilled water, immature seeds were excised and the cotyledons were removed as explants for culture. Cotyledon halves were placed MS medium (Murashige and Skoog, 1962), Cultures were placed at 25±2oC with 16/8 h light/dark at a light intensity of 22.5 □□E m-2s-1.

Bacterial Strain:

The Agrobacterium strain used was LBA 4404 harbon ring a binary plasmid PBIN 19 which has a npt II (Neomycin photo transferase II) gene and a vid A (gus gene). The Agrobacterium strain was grown on Lury and Bertani (LB) medium plates containing 5.0 gm/L NaCl, 10 gm/L Bactotryptone, 5 gm/L Yeast Extract and 100 mg/L Kanamycin and the pH was adjusted to 7.0 and solidified with 7 gm/L Difco/BactoAgar (Table 2)

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Transformation and Plant Regeneration:

For Co-cultivation two colonies from a freshly streaked plate were transferred to 10 ml of Liquid LB medium. Agrobacterium strain LBA 4404 was grown at 28 o C overnight in LB liquid medium containing 100 mg/L Kanamycin with shaking (approx. 250 rpm).

Kanamycin was added since the binary vectors are not completely stable in Agrobacterium in the absence of antibiotic selection (Klee et al., 1987). For transformation, the cotyledon and leaf explants were submerged and gently shaken in the Agrobacterium tumefaciens suspension for about 10 minutes and blotted dry on a sterile filter paper. Afterwards, they were transferred to shoot regeneration (SR) medium containing MS salts (0.5 mg/L) IAA+(30. mg/L) BAP for leaf explants and co cultivated under 16 hr. photo period of 50-60 µmol m-2 S-1 For 3 days at 25± 2 oC. After co- culture, the explants were washed in the MS liquid medium blotted dry on a sterile filter paper and transferred to the freshly prepared selective SR medium (MS1) supplemented with antibiotics 200 mg/L cefotoxime and 100 mg/L Kanamycin. Simultaneously a control was also maintained. After 4 weeks, the growing shoots were excised from the primary explant and sub cultured in fresh proliferation selective medium containing 100 mg/L KM (MS2). The green healthy shoots from explants were subjected to 2-3 passages of selection by repeated excision of branches and their exposure to selective elongation medium (MS2).The green shoots were transferred to MS medium containing 0.1 mg/L with Kanamycin (100 mg/L) for root induction (MS3).

Culture conditions and Data Analysis:

All the cultures were incubated at 25± 2 oC and 16 hr. /8 hr. photoperiod under 50-60 µmol m-2 S-1 white fluorescent light. All the experiments were carried out in 10 replicates. The experiments were replaced at least 3 times, keeping all the Parameters unchanged.

Histochemical GUS Assay:

The histochemical GUS assay was carried out according to Jefferson (1987). Staining was done by placing the tissue into X-gluc, staining buffer in a small vessel, X-Gluc stock was prepared by dissolving X-Gluc 20 mg/ml in Dimethyl Sulphoxide (DMSO). To make 1 ml of staining buffer 0.85 µl sterile distilled water was mixed with 100 µl monosodium PO4 (pH-7), 5 ml of X-Gluc stock and 5 µl Triton X-100 in an effendorff tubes. The sample was incubated overnight at 37 oC. Later these explants were treated with aceto alcohol (1:3 v/v) mixture to remove chlorophyll and then fixed in 70 % ethanol. The tissues were examined under sterio micro scope for the evidence of blue cells. X-glucuronide (5-bor bromo-4- chloro-3-indolyl glucuronide) is color less but the indoxy product derived after glucoronidase activity undergoes oxidative dimerisation to from an insoluble indigo blue.

We have standardization the plant regeneration in Glycin max cv KHST-2 from cotyledon and leaf explants using. MS medium supplemented with 0.5 mg/L IAA+3.0 mg/L BAP. The combination was routinely used for the present transformation experiments. The explants (cotyledon discs) co-cultivated with Agrobacterium tumefaciens formed shoots 6 weeks of culture on selective shoot regeneration medium (MS1). The control explants which were not co-cultivated did not produce when cultured on MS1 medium indicating the effective level of Kanamycin (100 mg/L).

Kanamycin sensitivity of cotyledon and leaf explants was assessed prior to Agrobacterium transformation to determine the concentration of Kanamycin needed for effective growth of transgenic plants. At 50 mg/L Kanamycin caused chlorosis and eventual necrosis in all explants by the end of the fourth week. Whereas concentrations of 75 mg/L and 100 mg/L Kanamycin completely inhibited the formation of shoot buds. In the present study higher concentration of Kanamycin (100 mg/L) was used for selection of transformants to prevent possible escapes.

Particle bombardment

The Particle Gun or Particle Bombardment or Gene Gun or Biolistic method of genetic transformations is one among the commonly used methods of gene

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transfer the steps involved in the techniques are as follows.

Preparation of Gold particle:

Gold particle of 0.6µl acts as micro carriers. 35µl of the prepared gold particles from the stock solution were aliquoted in to a micro centrifuge tube. The tube was centrifuged and supernatant was discarded. The following competent were then added sequentially, 200µl sterile water + 25µl DNA + 250µl of CaCl2 + 50µl of spermidine. The mixture was then incubated for 20min at 4°C on a vortex shaker.

Particle were then pelleted by centrifuged at 14000 rpm for 1min. The particle were then washed twice with 600µl of 100% ethanol and is finally suspended in 72 µl of ethanol. The required quantities of micro projectiles are loaded on to the center of a macro carrier.

Working Method of the Device:

The Biolistic Particle system Gene Pro 2000Hc Device Indigenous) was used of bombardment. Bombardment parameters were ser as given below 6cm distance was maintained between macro carrier and the target tissue, Helium Pressure of 30 psi and a chamber vacuum of 25 inches of Hg.

Transformation with Gus, NPT II Construct:

In Vitro raised fresh callus of Glycine served as target tissue for bombardment. By using the above parameters, target tissue was bombarded with PGus NPT II construct. Where Gus and NPT II acts as reporter and selectable marker gene used to test the transformation efficiency. NPT II gene (Neomycin phospho tranferase) confers resistance to kanamycin.

The transformation efficiency of regenerants can be tested by culturing the bombarded tissue on kanamycin containing medium stable transforments grow on the above medium by detoxifying kanamycin.

Gus staining:

The X-Gluc stock was 20mg/ µl in DMSO. To make 1ml staining buffer, 850 µl sterile water was mixed with 100 µl sodium phosphate, 50 µl X-Gluc and 5 µl triton-X. Leafe tissue tissues were incubated at 37C over night. Explant de colourised when placed in 1: 3 ratio of acetic acid and alcohol. Presence of blue spots on the target tissue indicated stable transformation.

RESULTS

CALLUS INDUCTION

Callus induction ability of different explants such as cotyledon and leaf was investigated by using varying concentrations of different auxins individually .Callus proliferation was initiated at the cut surfaces of the explants studied and later it covered the entire surface. Both colour and texture of the callus also varied with growth regulators supplemented. The results are presented in (Table-3-4) and shown in (Plate-II and III.) The explants viz. cotyledon and leaf cultured on MS medium supplemented with different concentrations (0.5- 3.0mg/L) of auxin such as NAA, 2,4-D and IAA individually exhibited initiation of callus after 15 days of incubation while it took 12-15 days in cotyledons.

Cotyledon explants:

For callus induction the explant viz. cotyledon (0.6-0.8 cm^2) from 4 weeks old axenic seedling were excised and inoculated to MS medium supplemented with various concentrations of NAA,2,4-D and IAA (0.5-3.0 mg/L). Callus proliferation was initiated at the cut surface of the cotyledon explants .The results are presented in (Table-3) and shown in (Plate-II.)

Effect of NAA:

On NAA supplemented medium early induction was observed in all concentration of NAA. High amount of callus was induced at 1.5 mg/L NAA. 100% callusing response was recorded in cotyledon explants at all the concentrations of NAA. Morphology of callus was also found to be varied at different levels of NAA. White compact callus was found at 1.0 ,1.5 and 2.0 mg/L NAA. At 2.5 and 3.0 mg/L NAA induced cream

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friable callus. Low amount of callus was induced at high concentrations of NAA. (Plate-II).

Effect of 2, 4-D:

On 2,4-D supplemented medium very high amount of callus was observed at 0.5 and 1.0 mg/L 2,4-D.High amount of callus was observed at 2.0 mg/L 2,4-D. Morphology of callus was also found to be varied at different levels of 2,4-D (Table-3). Brown compact friable callus was induced at 2.0, 2.5 and 3.0 mg/L 2, 4-D .100 % callusing response was recorded in cotyledon explants at all concentration of 2,4-D.

Effect of IAA:

Effect of IAA on callusing ability of cotyledon explants is shown in (Table -3). High percentage 98% of response was observed at 0.5 mg/L IAA. Responding callus was also varied at different levels of IAA. Whereas moderate amount of callus was observed at 2.0 and 3.0mg/L IAA. High amount of callus was induced at 2.0 mg/L IAA. White friable types of callus was induced at 1.0 , 1.5 and 2.0 mg/L IAA. At high concentration 2.5 and 3.0 mg/L IAA also induced white friable callus.

Leaf explants

Callusing efficiency of leaf explants cultured on MS medium containing various concentrations of NAA, 2,4-D and IAA is presented in Table-4. Callus proliferation was initiated at the cut surfaces of the explants studied and later it covered the entire surface.

Effect of NAA:

On NAA supplemented medium early induction of callus was observed in all the concentrations of NAA.98% callusing response was recorded in leaf explants at o.5 mg/L NAA. Callusing response was decrease from 1.0 to 3.0 mg/L when NAA was increased .Morphology of callus was also found to be similar at different levels of NAA. White compact and White friable callus was induced at all concentrations of NAA .Very high amount of callus was induced at 2.0 mg/L NAA. Low amount of callus was induced at 3.0 mg/L NAA. (Plate- III Fig-a).

Effect of 2,4-D

On 2,4-D supplemented medium also induction of callus was observed at all the concentration of 2,4-D in leaf explant culture High percentage of response was observed at low level of auxins used in leaf explants but at 3.0 mg/L 2,4-D the callus induction was inhibited in leaf explants. 100 of callusing response was recorded at 1.0 ,4.0 mg/L 2,4-D .High amount of callus was induced at 0.5 -3.0 mg/L 2,4-D .Morphology of callus was also found to be varied at different levels of 2,4-D.At 2.0 and 3.0 mg/L 2,4-D induced white friable callus.(Plate-III Fig-b).

Effect of IAA:

Effect of IAA on callusing ability Leaf explants is shown in Table-4.Highest percentage (100%) of response was observed at 0.5.and 1.0 mg/L IAA in leaf explant culture. High amount of callus was induced at 1.5 -2.5 mg/L 2,4-D The callus induction was moderate at low concentrations of IAA. Induction of white friable callus was observed at 0.5-3.0 mg/L IAA, (Plate-III).

DIRECT REGENARATION

The role of cytokinins and auxin- cytokinin combination on direct plant regeneration adventitious bud induction from different explants viz. cotyledon and leaf explants was studied in order to find out the efficient protocol and potential explants in *Glycine max* cv KHST-2. All the ex plants were cultured on ms medium fortified with different concentrations of cytokinins alone and combination with auxins. These explants were enlarged 3-4 fold with in one week of culture initiation. Morphogenic changes were apparent after 6 weeks culture. the explants viz cotyledon and

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leaf developed shoot primordia in large numbers directly from all cut surfaces in contact with medium in all the concentrations and combinations of phyto hormones used. The result are presented in tables 2-5 and shown in (Plates IV-V)

Cotyledon explants :-

Effect of various concentrations of cytokinins such as BAP and Kn alone and in combination with various concentrations of auxin IAA was studied on direct multiple shoot bud induction in cotyledon explants of *Glycine max cv KHST-2*) (Tables 6 - 7) (Plate IV) Direct adventitious shoot regeneration on MS medium containing various concentrations of BAP (1.0-3.0 mg/L) observed with varied results. Highest respond cultures with maximum frequency of multiple shoot bud induction was observed at 2.0 mg/L BAP (10.3 ± 0.47 shoots/explants) followed by 2.5mg/L BAP (6.5 ± 0.37) shoots/explants. At high concentrations of BAP the percentage responding cultures and shoot bud proliferation were reduced (Table-6).

Morphogenetic response of cotyledon explant culture on various concentrations of cytokinin such as Kn (1.0-3.0 mg/L) was observed. High percentage (60) of responding cultures was found at 1.5mg/L Kn compared to all other concentrations tested. where as more number of shoots were regenerated from cotyledon explantsat1.5mg/L Kn(7.5± 012shoots/explants) followed by 2.0mg/L Kn (6.0 ± 0.38shoots/explants). At high concentrations of Kn less frequency of shoot organogenesis was observed on the whole, the response of BAP as sole growth regulator was found to be maximum in inducing shoot organogenesis of all the concentrations tested in comparison to Kn (Plate IV).

Influence of auxin – cytokinin combination such as IAA (0.5mg/L) + BAP (1.0- 3.0 mg/L) and IAA (0.5mg/L) + Kn (1.0 - 3.0 mg/L) in cotyledon explants showed variable response (Table-7). Auxin (0.5 mg/L) IAA was taken in combination with cytokinin BAP (1.0- 4.0 mg/L) showed maximum percentage (59%) responding cultures and high frequency of shoot regeneration (7.8 ± 0.32) shoots/explants) at 0.5 mg/L IAA + 2.0 mg/L BAP. As the concentration was increased to 3.0 mg/L gradually the induction of shoot organogenesis was found to be reduced. The cotyledon explants when cultured on MS medium fortified with 0.5 mg/L IAA in combination with 1.0 and 1.5 mg/L BAP (2.2 ± 0.32 and 4.3 ± 0.42 shoots/explants with 52 and 55 percentage of responding.

Similarly cotyledon explants were cultured on MS medium supplemented with (0.5mg/L) IAA in combination with various concentrations of Kn (1.0-3.0mg/L) . The result showed the direct shoot regeneration in all the concentrations and combination tested. Cotyledons cultured on 0.5mg/L IAA in combination with different levels of Kn showed highest number of shoots (5.0 ± 0.35) 2.0 mg/L Kn + 0.5 mg/L IAA, and more percentage (53) of responding cultures. But at high level of Kn shoot bud induction was reduced along with the response too. (Table – 7).

Leaf explants

Seeds of Soyabean (*Glycine max* cv KHST-2) were rinsed in 70 % alcohol for 1 minute then sterilized with 0.2% aqueous mercuric chloride (HgCl2) for 5 min and subsequently washed five times with sterile distilled water for 5 minutes and germinated aseptically on MS basal medium Leaf explants (0.8 - 1.0 cm2) from 8 week old seedling were excised and cultured on MS medium containing 3 % (w/v) sucrose with various concentrations of cytokinins (BAP and Kn) alone and also in combination with auxin IAA (0.5 mg/L). the results are presented in Tables (8-9) and shown in (Plates V)

Leaf explants cultured on MS medium fortified with BAP/Kn as sole growth regulator showed the direct organogenesis / shoot bud induction (Table-8). All the cultures were responded (100%) at 2.0 mg/L BAP but gradually the percentage of response was decreased up to 75 % More number of shoots per explants (7.3 ± 0.43) was observed at 2.0 mg/L BAP and the number of shoot bud induction was found to be decreased as the concentration of BAP increased (Table-8).

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Leaf explants were cultured on MS medium amended with various concentrations of Kn (1.0 – 3.0 mg/L) (Table -8) to find out the difference between BAP and Kn inducing the direct plant regeneration from leaf explants in *Glycine max* cv KHST-2. Maximum number of shoot bud proliferation (6.0± 0.4) was found at 2.0 mg/L Kn . compared to all other concentrations of Kn. Number of shoots/ explants was reduced at high concentrations of Kn. 2.5,and 3.0, mg/L Kn. Percentage of response was gradually increased up to 2.0 mg/L Kn. And afterwards decrease the percentage of responding cultures. Like wise the average number of shoots/ explants development was increased gradually from 1.0 mg/L to 2.0 mg/L Kn but later it was decreased from 2.5 mg/L to 3.0 mg/L Kn .

To find out the influence of auxin- cytokinin combination on direct regeneration the leaf explants were cultured on MS medium fortified with 0.5 mg/L IAA and different concentrations of cytokinins such as BAP/Kn in *Glycine max* cv KHST-2. Leaf explants cultured on MS medium containing 0.5 mg/L IAA in combination with various concentration of BAP, showed variable results (Table -9). Highest percentage of response was observed at 0.5 mg/L IAA + 2.0 mg/L BAP (Plate –V). The percentage of response was increased up to 2.0 mg/L BAP and later gradually decreased at high concentrations. High frequency of shoots (8.0 ± 0.43) were induced per explants at 2.0 mg/L BAP and the induction ability was decreased as the concentration of BAP increased.

To observe the difference in the direct organogenesis efficiency between BAP and Kn, the leaf explants were also cultured on 0.5 mg/L IAA in combination with various concentrations of Kn (Table-9). Leaf explants cultured on MS medium augmented with 0.5 mg/L IAA and different concentrations of Kn showed maximum shoot bud induction (8.4±0.32) shoots/ explants at 2.0 mg/L Kn with high percentage of responding cultures was 75 (Table-9). When the concentration of Kn has been increased beyond 2.0 mg/L the average number of shoot/explant was gradually reduced. At high concentration of Kn (3.0 mg/L) only 4.0 ± 0.32 shoots/ explant were induced. Like wise, the percentage of responding cultures was also found to be high (75) at 2.0 mg/L Kn and gradually it was decreased as the concentration of Kn was increased

SOMATIC EMBRIYOGENESIS

Results on somatic embryogenesis in *Glycin max* cv KHST-2.are presented in Tables 10, leaf cultured on various concentrations of NAA in combination with 0.5 mg/L BAP become Swollen, and generally de differentiated and developed friable callus after 15- 20 days of culture. Within 25-30 days of culture, globular embryos had formed directly on the surface or primary embryos were cut in to fragments and cultured on the same induction medium secondary somatic embryos were induced within three weeks.

Thus proliferation of somatic embryos occurred in two ways

1. multiplication of somatic embryos from the explant through primary somatic embryo genesis and
2. proliferation of secondary somatic embryos from already formed somatic embryos through repetitive embryogenesis.

Among the various concentrations of NAA tested in combination with 0.5 mg/L BAP the percentage of somatic embryo formation was found to be higher at 3.0 mg/L NAA + 0.5 mg/L BAP in cotyledon explant (Table – 10). There was generally increased tendency of somatic embryos formation with the increasing concentration of NAA in combination with BAP was observed. However when the concentration of NAA increased to 5.0 mg/L + 0.5 mg/L BAP somatic embryo genesis was reduced. Maximum number of somatic embryos /expalnt and higher percentage of response for somatic embryo formation have been found at 3.0 mg/L NAA + 0.5 mg/L BAP in Leaf explants of *Glycin max* cv KHST-2**.** *(*Plate – VI). with the increase of NAA concentration up to 5.0 mg/L with 0.5 mg/L BAP there is gradual enhanced somatic embryos induction was recorded. Percentage of response and somatic embryo induction were decreased when the concentration of NAA was increased above 5.0 mg/L. In the present investigation

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Leaf explants showed maximum percentage of somatic embryogenesis and high frequency of somatic embryo induction / explants (27.0 ± 0.36).

The calli developed form leaf explants containing globular embryo were transferred to maturation medium containing MS medium supplemented with 3.0 mg/L / 3.5 mg/L NAA + 0.5 mg/L BAP respectively.

Hence the somatic embryos with various developmental stages (heart and Globular) were further sub cultured on fresh. MS medium containing various concentration of BAP (0.5- 5.0 mg/L) in combination with 0.5 mg/L IAA for germination of somatic embryos induced form Leaf explants. Of these media tested MS + 0.5 mg/L IAA + 3.0 mg/L BAP Proved to be the best for somatic embryos germination and plantlet formation after 4 weeks of culture.

Individual embryos analyzed into distinct bipolar Structures and passed through each the typical developmental stages (Globular, Heart torpedo and cotyledonay) (Plate VIII) after 6 weeks of culture. When these embryos with different developmental stages transferred to the same medium further germination of embryos was not observed. Differentiated somatic embryos were of diverse morphogenies. Any structure with at least one distinct cotyledon, hypocotyls and root axis after 28 days on MS medium. Germination percentages at different relative humidities. The vast majority of somatic embryos enlarged considerably and turned green although dead. Responses of surviving somatic embryos were categorized as follows.

a) No roots or shoots
b) Roots only
c) Shoots only
d) Roots and shoots, shoots were defined as pubescent apical structure because their morphology and size varied widely. Somatic embryos with no roots or shoots or with roots-only after 30 days on MS medium (half strength MS medium) rarely progressed to seeding growth of sub cultured in to fresh medium. In contrast, structures with shoots only casually developed vigorous root system upon sub culture to fresh medium and sub squinty to sterile vermin culture.

Thus germination was scored as those structures with shoots only and those with shoots. Germination frequency was different in various concentrations of BAP in combination with IAA. At 0.5 mg/L 2.4-D in combination with (0.5–5.0mg/L) BAP Showed maximum responding cultures and high frequency of germination (30.0 ± 0.32) at 3.0 mg/L BAP + 0.5 mg/L IAA. Somatic embryos germination frequency was gradually decreased at high concentrations of BAP.(Table - 11).

PROTOPLAST ISOLATION

A mixture of 2% cellulose and 1% macerozyme was suitable for isolation of viable protoplasts from mesophyll tissues of leaf cultures of *Glycine max* cv. PKST-5 were investigated each enzyme was in effective by itself but when used in combination it resulted satisfactorily (Evans and Bravo 1983). For *in vitro* leaf explants above combination of macerozyme and cellulose gave an optimum yield.

In case of *Glycine max* cv. KHST-5 cultivars, prolonged incubation periods i.e 10-12hrs were observed to be unfavorable shrinkage of protoplasts in these cultivars. Cell digestion was taisly good when above mentioned conditions were applied for protoplast isolation. The number of protoplasts showed increase during shorter treatment time and reached a peak at 4-5 hours of incubation in dark. Beyond 5 hours of incubation the protoplast yield gradually decreased and further resulted in complete shrinkage of protoplasts at 10-12 hours of incubation from these result it was estimate that the adequate time for enzyme treatment to isolate maximum number of protoplasts from mesophyll cells of *Glycine max* cv. KHST-5 (Plate IX).

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AGROBACTERIUM MEDIATERD GENETIC TRASFORMATION

To assess the efficiency of soybean genetic transformation by the integrated bombardment we performed independent experiments combining the effect of tungsten particle bombardment by a low pressure helium gun (Finer *et al.*, 1992) and T-DNA transfer via *A. tumefaciens* LBA4404 K233 (Hiei *et al.*, 1994) infection. Microwounds caused by the tungsten particles would enhance *Agrobacterium* attachment to cells and gene transfer due to chemical signals from the wounded tissues (Hooykaas *et al.*, 1991). The "super binary" vector used in the experiments contains a *gus* A-intron gene, certifying that any GUS activity detected should be from eukaryotic cells. Additionally, the T-DNA contains a hygromycin resistance gene (*hpt*) for selection of transformed tissues (Hiei *et al.*, 1994). Embryogenic clumps of soybean cv. BRAGG and cv. IAS5 cultivars were evaluated in the experiments. These cultivars are known to be susceptible to infection by armed *A. tumefaciens* strains (Droste *et al.*, 1994).

CONCLUSION

Plant cell and tissue culture has emerged as major tool in the study of an increasing number of applied and fundamental problems in the plant science and now it has become an important integral constituent of plant biotechnology. Plant cell and culture technology could serve as a supplemented to the conventional methods of plant breeding for production of lite plant lets in vitro which is an important propagation technology to agriculture during the last decade. Significant progress has been made in the propagation of plants. Which novel features through tissue culture technology. To meet the ever growing commercial requirements the realization of in vitro multiplication of a large number of clonal plants with the improved charters has been giving significance.

The genetic transformation protocol developed during the present investigation can be used to transfer genes of interest for a better utilization of the species as a vegetable and in medicine to produce or to enhance the secondary metabolities in *G.max*.

Based on the experimental data the following conclusions can be made:

- Callusing efficiency from different explants in *G.max*..was studied and the protocols have been established.
- Among the auxins tested NAA induced the high quantity of callus in all the explants used.
- Leaf explants were proved to be better in inducing high yield of callus.
- The combination of IAA+BAP induced more number of shoots than Kn.
- Protocols have been established for efficient regeneration from different explants via direct organogenesis.
- High frequency of shoots were formed directly on medium containing IAA + BAP.
- Leaf explants were found to be efficient in producing maximum number of shoots per explants.
- High percentage of protoplast were formed from cotyledon explant.
- Somatic embryo formation and plantlet development was established.

Protocols were established for *Agrobacterium* mediated genetic transformation using cotyledon and leaf explants.

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