Development of transgenic groundnut with *Citrate synthase* gene through *Agrobacterium*-mediated transformation for improving phosphorous use efficiency

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Development of transgenic groundnut with *Citrate synthase* gene through *Agrobacterium*-mediated transformation for improving phosphorous use efficiency

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Affectionately Dedicated
to My Beloved Parents

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CERTIFICATE

This is to certify that the thesis entitled **'Development of transgenic groundnut with *Citrate synthase* gene through *Agrobacterium*-mediated transformation for improving phosphorous use efficiency'** submitted by Mr. AMIT KUMAR SINGH in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE (Agri.) in Agricultural Biotechnology to the University of Agricultural Sciences, Bangalore, is a record of research work carried out under my guidance and supervision and the thesis has not previously formed the basis of the award of any other degree, diploma, associateship, fellowship or other similar titles.

Bangalore
August, 2002

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INTRODUCTION

I INTRODUCTION

Despite significant increase in per capita agricultural production worldwide over last two decades, the challenge of producing sufficient food supply remains daunting due to increasing population growth, reduced availability of water, limit of agricultural land, expansion and pressure by biotic and abiotic stresses.

As it is not possible to reduce the population growth rate and we cannot increase water availability and arable land, it is better to look into other factors, which can be controlled.

Mineral nutrients play an important role in the plant nutrition. Phosphorous is the 'Master Key' element in crop production. It is the component of key molecules like nucleic acids, phospholipids, sugar phosphates and other biologically active molecules. Optimum phosphorous nutrition helps the plants in producing deeper and abundant roots, enabling uniform maturity of crop in time and also in providing protection against biotic and abiotic stresses. It is important in photosynthesis and it appears to be the modulator of gene transcription (Sadka *et al.*, 1994).

One of the major drawback with phosphorous is that it is highly reactive and immobile element, as it reacts with cations to form complexes and becomes unavailable for plant uptake. Phosphorous is available for plant uptake only at a narrow range of neutral soil pH values.

In acid soils, P forms insoluble complex with aluminium (Al), iron (Fe) and manganese (Mn) whereas in alkaline soils it combines efficiently with calcium (Ca) and magnesium (Mg). Therefore, although the total amount of

phosphorous in the soil may be high in most cases it is unavailable for plant uptake (Jose Lopez-Bucio, 2000).

So the development of plant varieties, which can scavenge phosphorous more efficiently from the soil, will be one of the major steps in increasing agricultural production. Development of novel varieties through classical breeding is slow and laborious process and some time unpredictable interms of durability. In such case the only alternative for developing novel varieties and plants seems to be by the application of genetic engineering.

To meet the P requirements by the plant nearly 30 million tonnes of P fertilizers have been used annually worldwide. Out of this, nearly 80 per cent is unavailable to plant because of adsorption, precipitation and conversion to inorganic forms. Moreover, in recent years increasing attention has been paid to the effect of excessive use of fertilizers, particularly P in environmental pollution. There are different methods to improve the phosphorous uptake by the plants from the soil. In response to P limiting conditions, some plants undergo physiological and developmental adaptations to scavenge limited phosphate from the environment, including, changes in the root architecture, induction of genes encoding high affinity P transporters, rhizosphere acidification and exudation of organic acids (Schachtman *et al.*, 1998) like succinic acid, malic acid and citric acid involved in solubilization of inorganic form to organic form of P (Dinkelaker *et al.*, 1989). Among these organic acids, citric acid has high potential because it acts as chelating agent. It is produced in plant system during TCA cycle and being an intermediate compound it does not have any adverse effect on plant growth or development. Citric acid has high affinity for divalent and trivalent cations, which displaces P from insoluble complexes, making it more available for plant uptake (Zhang *et al.*, 1997). Citric acid solubilizes the dicalcium phosphate to water soluble form due to decrease in pH.

Taking the idea of increased production of organic acid in the rhizosphere, which enhances the phosphorous solubilization and its uptake by plant, a gene encoding *Citrate synthase* enzyme is isolated from a bacterium *Pseudomonas aeruginosa* (Lynda *et al.*, 1989). *Citrate synthase* enzyme catalyses one of the important reactions in TCA cycle which leads to the production of citric acid. Citric acid produced in this way solubilizes insoluble phosphorous to soluble phosphorous.

So, overexpression of the gene encoding *Citrate synthase* enzyme synthesis will be a novel approach in enhancing phosphorous uptake by plant.

Groundnut (*Arachis hypogaea* L.) is an important and leading oilseed crop of tropical and subtropical regions of the world. The total area of the crop in the world is about 18.9 million hectares with an annual production of about 17.8 million tonnes of pods (Anon., 2001). Out of the nine oil seed crops grown in India, groundnut alone is claiming the largest area of about 8.5 million hectares with a production of about 8.2 million tonnes (Anon., 1999a). In Karnataka, it is grown over an area of about 1.326 million hectares with the production of 0.98 million tonnes of pods with a productivity of 922 kg per hectare (Anon., 1999b).

The edible oil requirement in the country is continuously increasing due to booming population growth and rise in the standard of living. Today the per capita consumption of oil in India is around 12g per day in contrast to the recommended in grams. At the present level of production it is needed to double the production to meet the growing population demand.

Groundnut has its unique characteristics of higher hydrogenated oil and protein content along with its soil fertility restorative ability. These properties

furnish the hopes in combating the present edible oil crisis and banishing the vegetable hunger, if the ultimate productivity of groundnut crop productivity is increased. So groundnut is recognized as one of the important cash crop making headway in Indian agriculture to meet the protein and oil requirements.

Oil seeds have high phosphorous requirements. Phosphorous is an essential nutrient for their growth, development and reproduction as it forms an integral part of key molecule such as nucleic acid, phospholipid, ATP and other biologically active compounds. Phosphorous also plays an important role in nodule formation thereby it improves the symbiotic nitrogen fixation efficiency in leguminous plants. Increasing cultivation of oilseeds create a higher demand for phosphatic fertilizers. Meanwhile, the reduction in available nutrients in arable and problematic soils have been also posing serious problem for groundnut production.

With the advent of gene transfer technology, it is now possible to get the transgenic plants which are more efficient to uptake P from the soil, which results in a better way to achieve more sustainable agriculture that could help and satisfy the growing world demand for food. *Agrobacterium* mediated gene transfer technique is the primary means of transformation and has provided a reliable means of creating transformants in a wide variety of species that are amenable to tissue culture and regeneration.

The present investigations were carried out with the following objectives

1. Production of transgenic groundnut plants using *Citrate synthase* gene following *Agrobacterium* mediated gene transfer and
2. Confirmation of the transformed plants by PCR technique.

REVIEW OF LITERATURE

II REVIEW OF LITERATURE

Phosphorous is one of the most important macronutrient required by plant for its growth and development. It is the component of key molecules such as nucleic acids, phospholipids, ATP and other biologically active compounds. After nitrogen P is considered to be the second most important nutrient limiting agricultural production.

2.1 Role of phosphorous in plant nutrition

Phosphorous is known to be taking part in vital functions in the plant growth and metabolism, and constitute an essential part of many sugar phosphates. It is also involved in photosynthesis, respiration and other metabolic processes like breakdown of sugars and nutrient transport within the plant (Tandom, 1987; Armstrong, 1988).

Phosphorous is an essential part of metabolites and phospholipids, which are present in the membrane. It plays an important role in energy metabolism because of its presence in AMP, ADP, ATP and pyrophosphate as well as in signal transduction. It is important in photosynthesis and it appears to be the modulator of gene transcription (Salisbury and Ross, 1992).

Optimum phosphorous content helps the plant in producing deeper and abundant roots, enabling uniform maturity in time, providing protection against biotic and abiotic stresses. Sufficient **P** nutrition results in high yield of superior quality produce (Tandom, 1987).

Presence of phosphorous around the root zone is essential for rapid root development, which helps, in better utilization of water and other nutrient by plants. **P** has pronounced effect on increasing the number of flowers. Adequate

amount of **P** increases the lime juice and ascorbic acid in tomato (Sharma and Manu, 1973). The **P** fertilizer will increase the total soluble sugar, ascorbic acid and reduce titratable acidity. Phosphorous and nitrogen interact closely in affecting maturity, excess nitrogen delays maturity and abundant phosphorous speedup the maturity (Salisbury and Ross, 1992).

2.1.1 Forms of phosphorous

In the soil, phosphorous is present in two forms, inorganic phosphate and organic form of phosphates. The organic phosphates containing compounds are derived from plants, animals and microorganisms and are composed of nucleic acids and phospholipids. The inorganic forms are compound of calcium, iron and aluminum (Salisbury and Ross, 1992).

In the soil, phosphorous is available in three forms *viz.*, H_2PO_4, HPO_4^{2-} and PO_4^{3-}. Among these forms H_2PO_4 and HPO_4^{2-} are preferred for uptake by the plants. The free inorganic **P** ions in the soil solution play an important role in **P** cycling and plant nutrition (Illmer and Schinner, 1992). Besides, fertilization the following two different pathways also increase this fraction.

1. The enzymatic decomposition of organic **P** compound (e.g. phosphomonoesters, phosphodiesters including phospholipids nucleicacid, phosphotriesters etc.,) and inorganic **P** compounds (e.g. pyro and metaphosphates).
2. The non-enzymatic solubilization of different rock phosphates and inorganic phosphorous sources. Therefore, free inorganic phosphates in soil solution play a central role in the cycling of P (Illmer and Schinner, 1992).

The two reactions namely fixation and immobilization result into forms of **P**, which are unavailable for plant uptake. The immobilized organic form

can account for 2 to 80 per cent of the **P** content of surface soils (Kucey *et al.*, 1989). These have to be mineralized into organic orthophosphates for plant consumption. Nearly, 90 per cent of the applied phosphatic fertilizers are fixed in the soil, rendering them unavailable for plant consumption (Larson, 1967; Stevenson, 1986). The phosphate that gets fixed by chemical reaction with mineral oxides and other soil components is an important sink that need to be trapped for phosphorous nutrition.

The major raw material for **P** fertilizer is rockphosphate (RP), since Indian RP are low graded (17-25% P_2O_5) it is not remunerative to use it for the manufacture of phosphate fertilizers. So India is importing 89.3 per cent of P_2O_5 requirement, which comes to 721.7 thousand tonnes during 1993-1994 alone (Anon, 1994), which is a big burden on foreign exchange.

2.1.2 Mechanism of phosphate solubilization

The mechanism, by which inorganic phosphate compounds are solubilized by microorganisms into more available form to plants, is not completely understood. Several workers have proposed various mechanisms. The increased production of organic acids by microorganisms and root exudation of plants seems to be the main cause.

2.1.3 Production of organic acids

A fall in the pH during the growth of phosphate solubilizing microorganisms in liquid medium containing insoluble phosphate has been often reported (Gaur, 1990; Yadav and Singh, 1991; Illmer and Schinner, 1992).

Studies have shown that during phosphate solubilization, there was a drop in pH (JagnuThakker, 1993; Reena, 1995). In certain cases relationship

between decrease in pH and phosphate solubilization could not be established (Mba Caroline, 1994; Illmer and Schinner, 1995). Many studies have reported that organic acids such as lactic, malonic, tartaric, succinic, gluconic and 2-ketogluconic acid in solubilization of inorganic phosphate sources either directly or by chelation of phosphate ions (Duff *et al.*, 1963; Pareek and Gaur, 1973; Rao *et al.*, 1982; Banik and Dey, 1981, 1982; Illmer and Schinner, 1992 and Babu-khan *et al.*, 1995).

Chandrashekaran (1969) concluded that rhizospheric microorganisms with legume produce more organic acids than those with non-legumes do and observed no quantitative difference in organic acid production.

Bajpai and Sundara Rao (1971) reported that, *Bacillus megathurium*, *B. circulans* and *Escherichia freundii* produced non-volatile acids such as citric acid, lactic acid from glucose in Pikovoskaya medium. Mishustin *et al.* (1972) studied in a model experiment solubilization of tricalcium phosphate in liquid media by citric acid, gluconic, glutaric, succinic and oxalic acids, which depended on pH and formation of soluble Ca-complexes.

Pareek and Gaur (1973) showed the release of phosphates from tricalcium phosphate and rock phosphate by organic acid and reported that aliphatic acids were more effective in solubilization of phosphate than phenolic acids.

The increased release of, citric acid may provide a mechanism by which a **P**-starved plant can enhance the availability of **P** from rhizosphere (Dong *et al.*, 1987).

Hoffland *et al.* (1989) reported that some species excrete citric acid and malic acid in response to **P** deficiency and Al-tolerance. Al-triggered exudation

of organic acid from the root apex. Citric acid is known to be an effective Al-detoxifier forming more stable Al-complexes than malate. It was reported that *Pencillin bilaji* increased phosphate solubilization by production of citric acid and oxalic acid under nitrogen starved conditions. Citric acid solubilizes the precipitated $AlPO_4$, which could help to increase the uptake (George *et al.*, 1991).

2.2 *In vitro* regeneration

Plant regeneration from somatic cells is essential for successful *in vitro* genetic manipulation. It also provides an alternative to vegetative propagation for crop plants that are not propagated by seeds.

The concept of totipotency, which is inherent in the cell theory put forth by Schleiden (1938) and Schwann (1939) is the basis for plant tissue culture. Recent progress in the field of plant cell and tissue culture has made this area of research one of the most dynamic and promising tools in experimental biology. Now *in vitro* cultures are also used as tools for the study of various basic problems in plant physiology, plant pathology, molecular biology, cell biology and genetics.

Totipotency is one of the important characters of plant, which eases genetic manipulations through tissue culture techniques. Tissue culture was started from pieces of whole plants. The small organs or pieces of tissue that are used are called as explants.

The part of the plant from which explants are obtained depends on,

1. The type of culture to be obtained, callus suspension, protoplast, anther, meristem or embryo culture
2. The purpose of the proposed culture
3. The plant species to be used

2.2.1 Factors influencing *in vitro* growth and morphogenesis

2.2.1.1 Effect of genotypes

The growth and morphogenesis of cultured tissue or organ in *in vitro* appears to be influenced by genotype. Thus the media and the cultured environment often need to be varied from one genus or species of plant to another and even closely related varieties of plant can differ in their cultural requirements.

2.2.1.2 Effect of environment

Most tissue cultures are maintained at the same diurnal temperature experienced by plants of the same species *in vivo*. The average temperature employed in a large sample of experimental reports to be 25^0C (with a range between 17 and 32^0C).

Moist atmosphere is important to prevent desiccation of cultures. The relative humidity in growth chambers used for culture is frequently given as 72 per cent but inside the culture vessel found to be greater. High humidity (98% RH) inside culture vessel appeared to assist in the formation of vitriscent shoots in carnation (Ziv *et al.*, 1983).

Since both photosynthesis and morphogenesis are dependent on light, it is an important factor in growth and development of plants. The three qualities of light, which most clearly influence the morphogeneisis *in vitro*, are wavelength, flux density and photoperiod. Effect of light on photosynthesis *in vitro* seems to be of less importance due to the heterotrophic nature of the culture (Dunstan and Turner, 1984).

Pentana *et al.* (1998) observed that direct organogenesis from cotyledons and development of organogenic callus from leaf explant of groundnut showed optimal growth at 35 ± 5^0C.

2.2.1.3 Plant growth regulators

The selection of appropriate plant growth regulator combination is the most important aspect in developing a successful protocol for micropropogation.

2.2.1.4 Gelling agent

Agar has been widely used as a gelling agent of the media for plant tissue culture. Agar concentration and agar types are greatly known to influence the growth response of micropropagated plants (Singha *et al.*, 1985). Physical and chemical properties of different types of gelling agents have been explored in order to explain the growth responses. Different brands or concentrations affect differently medium conducting and contaminating salts content (Debergh, 1983) but no correlation with growth has been found. Agar may contain growth-inhibiting substances and is prone to contain several impurities, which can be detrimental to tissue culture work.

Recently, Gelrite produced by *Pseudomonas elode* was developed as gelling agent for bacteriological and plant tissue culture media. The tissue discoloration has been avoided in banana callus culture by employing Gelrite as a gelling agent.

2.2.2 Regeneration of legumes

Malik and Saxena (1992) established the axenic seedlings culture of peas by culturing mature seeds on Murashige and Skoog medium (MS) supplemented with thiodiazuron (TDZ), various cytokinins or compounds with cytokinin like activity (Kinetin, TDZ, Zeatin) tested for inducing shoot formation in pea seed cultures, TDZ was found to be most effective.

Kumar *et al.* (1992) succeeded in efficient plant regeneration via somatic embryogenesis in chickpea *Cicer arietinum* cv (235). Leaf explants cultured on MS medium supplemented with 1.25 mg, 2,4-D and 0.25 mg kinetin/l yielded the highest number of somatic embryos during dark incubation. MS medium supplemented with B5 vitamin, 0.125 mg IBA and 2 mg BA/l was suitable for embryo maturation. The well formed somatic embryos germinated into plantlets on basal medium supplemented with 0.25 mg/l BA. Further development into healthy plantlets obtained on basal B5 medium.

Nalina Mallikarjuna *et al.* (1993) cultured the immature cotyledons from 10 elite cultivars on B5 medium supplemented with 0.7 per cent Agar, 3 per cent Sucrose, 14 µM Zeatin and 5µM IAA. Somatic proembryos intermixed with shoot buds were transferred to B5 medium supplemented with either 1 mg or 2 mg BA + 0.5 mg/l IAA, few of the embryoids germinated.

George and Eapen (1994) evaluated the seed and seedlings explant of pigeon pea for organogenesis and somatic embryogenesis. *Denovo* plant regeneration through organogenesis was obtained from mature cotyledons, primary leaves and roots of seedlings. Production of multiple shoots from the cotyledonary node was observed in culture of whole seeds on BA enriched medium. Somatic embryos were induced from immature cotyledons and

embryonal axes, but well developed plants could not be derived from these embryos.

Patel *et al.* (1994) obtained somatic embryogenesis in pigeonpea (*Cajanus cajan*) using distal halves of cotyledons from four genotypes. Formation of somatic embryos was dependent on concentration of specific cytokinins and mineral nutrient formulation. The cotyledons expanded rapidly and turned green and induction of embryogenesis occurred on a high cytokinin medium containing BA (22.2 µM), Kinetin (2.3 µM) and Adenine sulphate (271 µM). Reduction of cytokinins favoured maturation of somatic embryo. Complete withdrawal of cytokinins from the medium and addition of IBA (2.4 µM) and GA (2.9 µM) promoted germination of the embryos.

Suryaprakash *et al.* (1994) obtained embryogenic cell suspension cultures from 3 chickpea (*C. arietinum*) cultivars (C235, JG62 and E 100y) in a modified B5 medium containing 1.5 mg BA and 1.0 mg NAA/1 plantlets regenerated at a low frequency by serial subculture on solid agar medium. Shoot development was promoted on modified MS medium containing 20 mg NAA and 2.0 mg activated charcoal/ 1 improved root formation.

Ramana *et al.* (1996) reported the direct somatic embryogenesis in *C. arietinum*. Somatic embryos were directly induced from immature cotyledons on B5 medium supplemented with 2,4,5-T or 2,4-D in combination with BA/Kn medium containing 2 mg Zeatin/l induced germination of the cotyledonary stage embryos.

Vani and Reddy (1996) reported morphogenesis from callus cultures of chickpea (*C. arietinum*). The recalcitrant *C. arietinum* cv. JG-62, PGC1 and C-235 were studied for morphogenic response. The differentiation of shoots was achieved only from hypocotyl-derived callus of JG-62 on B5 supplemented

with BAP (1mg/l). Somatic embryogenesis was achieved from cotyledon-derived cultures of JG-62 on B5 medium with 2,4-D (2mg/l) and 3 per cent sucrose. Direct regeneration from the epicotyl explant of JG-62 was 100 per cent with 5-6 shoots/explant on B5 medium containing BAP (1mg/l) + Kn (1mg/l) + IAA (0.5 mg/l). Rooting was observed on B5 medium containing IAA (4 mg/l) and Kn (0.5 mg/l).

2.2.2.1 Media for legume culture

B_5 medium of Gamborg *et al.* (1968) L_2 and SL_2 media of Phillips and Collins (1979), Blaydes media have been especially developed for the culture of legumes. Qu *et al.* (1993) have evaluated the effects of various combinations of four media (B_5, MS, White and Norstog) and found MS and B_5 were the best media for plant regeneration for plant regeneration in groundnut.

2.3 Tissue culture of groundnut

2.3.1 Callus culture

Because of the ease with which callus can be established from almost any kind of whole plant tissue, the greatest number of callus explants has been made with species of broad leaf dicotyledonous plants. Morphogenetic callus is produced from a wide range of explants on MS medium. Mroginski *et al.* (1981) obtained shoot formation in the primary callus induced from leaves of groundnut seedlings by a wide range of NAA and BA combination. Optimum regeneration occurred with 1 mg/l of both compounds. Similarly Narashimhulu and Reddy (1983) and Pittman *et al.* (1983) reported sporadic shoot development from groundnut callus cultures on MS medium supplemented with varying concentrations of BA and NAA.

Ilahi (1993) obtained callus after one month from shoot apices of groundnut seedlings, grown on medium containing 1 mg/l BA and 0.01mg/l NAA. Better regeneration of callus was observed on MS medium supplemented with 0.5 mg/l of BA and inorganic biphosphate and 0.1mg/l IAA. Similarly callusing in leaflets derived from mature embryos of groundnut by supplementing MS medium with 4 mg NAA and 5 mg BAP/l was found optimum for inducing caulogenic buds (Chengalrayan *et al.*, 1994).

Ihsan-Ilahi *et al.* (1995) have induced callus from mature embryos on MS medium supplemented with 0.5 mg BA and 0.5mg 2,4–D/l. But regeneration occurred when this callus was sub cultured on MS medium containing 1 mg BA and 1 mg Kinetin/l. Cheng *et al.* (1998) reported callus and root formation on MS medium supplemented with 1 mg NAA + 1mg BA/l and 2 mg NAA + 2mg BA/l, respectively.

2.3.2 Somatic embryogenesis

Somatic embryogenesis is one of the most preferred regeneration methods in genetic transformation of crop plants. Organ such as shoots and roots can be induced from somatic embryos generated through *in vitro* culture that structurally similar to the embryos found in true seeds. The process leading to somatic embryo inception is termed as embryogenesis. Somatic embryos arise from a single cell or group of cells and they are induced by cultural conditions.

The factors affecting somatic embryogenesis were studied by Reddy and Reddy (1993). They could successfully induce embryos on MS medium containing 4 mg 2,4-D/l with 2 per cent sucrose.

Gill *et al.* (1992) have developed a procedure for inducing direct somatic embryogenesis, organogenesis and regeneration of plants from tissue cultures of groundnut, using a concentration of 1-25 mM thiodiazuron (TDZ).

George *et al.* (1993) have studied influence of genotype and explant source on somatic embryogenesis in peanut. They found that the frequency of embryo induction and the average number of somatic embryos per explant was highest for JLM (100 and 10.2 respectively), with immature embryogenic axis compared to other explants.

Eapen *et al.* (1993) have tested influence of different auxins, cytokinins and sugars on somatic embryogenesis from immature cotyledon explants. They found 2,4-D was most effective compared to other auxins in producing somatic embryos.

Chengalrayan *et al.* (1994) obtained embryo masses from embryo-derived leaflets of cv. JL 24 cultured on MS medium containing 20 mg 2,4-D/l. but development of somatic embryos was obtained by transferring on to 3mg 2,4-D/l.

It has been found that dark incubation favoured the formation of the somatic embryos. Somatic embryo maturation required MS medium supplemented with B_5 vitamins 0.125 mg IBA and 2mg BA/l (Kumar *et al.*, 1994). Suitably matured somatic embryos can be germinated *in vitro*, but only a small percentage as low as 30-40 per cent of them germinated in groundnut with a survival rate of 20 per cent.

In addition to the dark incubation, age of the seedlings from which explants were taken also found to affect somatic embryo production in groundnut (Murthy *et al.*, 1994). They found six day old seedlings produced

highest number of somatic embryos per seedling compare to seedlings of more than 21 days old on MS medium supplemented with 0.1 to 4 M of chlorofuran.

Venkatachalam *et al.* (2000) developed a plant regeneration method via direct somatic embryogenesis using mature cotyledon explant of groundnut without intervening callus on MS medium supplemented with 22.19 µM BAP and 2.68 µM NAA. They found the range of embryogenesis frequency was 10.7 to 80.2 per cent, depending on BAP and NAA concentrations. Similarly they obtained maximum embryo induction on MS medium containing 5mg/l BAP and 0.5mg NAA/l.

Ramanjini Gowda *et al.* (2001) developed regeneration protocol through somatic embryogenesis for the peanut cultivar JL-24. Mature embryos were cultured on MS medium supplemented with different concentrations of 2,4-D in dark for 12 days followed by exposure to light for five weeks. The highest number of somatic embryos was observed in 15mg/l 2,4-D. The embryos grown on different concentrations of 2,4-D and 12mg IBA/l gave the best results.

2.4 Transformation

2.4.1 Agrobacterium-mediated transformation studies

Approaches that rely on tissue culture for the regeneration of transgenic plants must take into account the type of culture (callus, suspension or protoplasts), the mode of regeneration (embryogenesis Vs organogenesis), the maintenance of regeneration potential over time and the ability to select stably transformed tissue.

Although several novel methods for the transformation of plants are available at present, methods based on the use of soil bacterium *Agrobacterium*

tumefaciens are still preferred in many instances. The primary event involves transfer of T-DNA from the tumour inducing (Ti) plasmid of *Agrobacterium* to the host plant genome. T-DNA carries the oncogenic (onc) genes that incite events, leading to tumourous growth in the plants. It further emerged that only the border region and the T-DNA are essential for the transfer and integration of DNA. Any exogenous DNA incorporated between the two ends of T-DNA is also transferred to the plant cells. To incorporate the desired DNA in the T-region of the large Ti plasmid, it required parallel development in gene cloning and DNA transfer methodologies. Understanding of conjugation, plasmid incompatability grouping, DNA cloning, mobilization and gene expression have made possible the development of very sophisticated intermediate and binary vectors.

2.4.2 Different methods for transformation of plant cells using *Agrobacterium*

1. Plant inoculation is the simplest method for obtaining the transformed cells when oncogenic strains of *Agrobacterium* are used. The desired transformants are identified on selective media.
2. Inoculation of *in vitro* derived tissues of *Vigna unguiculata* has been used to transform cell lines (Garcia *et al.*, 1986). Whole plants could not be regenerated from the transformed callus tissue.
3. Leaf disc method involves incubating the leaf discs with *Agrobacterium* followed by culture.
4. *In vitro* derived explants are cocultivated with *Agrobacterium*.

These techniques offer unique opportunities for introduction of desired genes into otherwise productive well-adapted genotypes both from species that cannot be hybridized as well as from easily crossable genotypes of the same species. The transformation technique can be expected to enhance the

efficiency of plant breeding programmes and may reduce the time required for the development of new genotypes but the time need for field-testing.

2.4.3 Transformation of groundnut

Lacoret *et al.* (1991) were able to transform the peanut calli for the first time using *Agrobacterium* and particle Bombardment.

Frankalin *et al.* (1993) cocultivated peanut callus with *Agrobacterium tumifaciens*, which resulted in kanamycin resistant, ß-glucuronidase (GUS) positive callus. But plant regeneration could not be achieved. Similarly Mc Kently *et al.* (1993) showed that the peanut embryo axes cocultivated with *Agrobacterium* were stably transformed.

Cheng *et al.* (1994) recovered primary transformants of Valencia-type peanut by *Agrobacterium* mediated transformation. The construct used by them had zein protein coding sequence and β-glucuronidase (GUS) as a reporter gene.

Eapen *et al.* (1994) were first to successfully develop transgenic plant containing GUS gene via *Agrobacterium*. Leaves cocultivated with *Agrobacterium* were regenerated on the media containing 1 mg/l NAA and 25 mg/l BAP.

Cheng *et al.* (1996) produced fertile transgenic groundnut plants with GUS gene using *Agrobacterium*. Molecular analysis of these plants confirmed stable integration of the GUS gene in the T_0 and T_1 generation plants.

Sowmya *et al.* (2001) developed transgenic groundnut plant containing *glucanase chitinase* gene using *Agrobacterium tumifaciens* mediated transformation technique.

Deng and Weizm (2001) developed transgenic groundnut plants using particle bombardment via somatic embryogenesis. They used zygotic embryo explants for transformation with plasmid carrying Hygromycin resistance gene and GUS gene.

2.4.4 Factors affecting *Agrobacterium* mediated transformation of groundnut

2.4.4.1 Genotype

It has been observed that some genotypes of the groundnut are more amenable for transformation compared to other genotypes.

Egnin *et al.* (1998) reported that the Valencia select market type cv. New Mexico was more amenable to *Agrobacterium* transformation than the runner market type cultivars.

2.4.4.2 Agrobacterium strain

Agrobacterium strains used in the transformation studies of the groundnut have been found to influence the transformation efficiency. *Agrobacterium* strain EHA101 was found superior in facilitating the transfer of *Uid* A in groundnut compared to strain C-58 (Egnin *et al.*, 1998).

2.4.4.3 Preculture of explants

Preculturing of the explants before cocultivation has been found to influence the transformation efficiency.

2.4.4.5 Other transgenic crops by *Agrobacterium* mediated transformation

Rotino *et al.* (1992) have transformed *Solanum* spp using a Bt gene effective against coleopteran insects via *Agrobacterium tumefaciens* mediated transformation. Metz *et al.* (1995) reported the *Agrobacterium* mediated transformation of broccoli (*Brassica oleracea* var *italica*) and cabbage (*Brassica oleracea* var *capitata*).

A reproducible system has been developed for the production of transgenic plants in Indica rice using *Agrobacterium* mediated gene transfer (Rashid *et al.*, 1996). Wenyu Yang and Xuerong Zhou (1997) transformed the *in vitro* grown potato using an *Agrobacterium tumefaciens* binary vector.

2.5 Transgenic plants with *citrate synthase* gene

The first *citrate synthase* transgenic plants were made by De la Fuente *et al.* (1997). They produced two transgenic plants (Tobacco and Papaya) containing *citrate synthase* gene to overcome the aluminium toxicity. These plants produce two to four-fold higher concentration of citric acid, which is involved in reducing the aluminium toxicity.

Bucio *et al.* (2000) stably transformed the tobacco plants with *citrate synthase* gene by *Agrobacterium* mediated transformation. Using the plasmid containing chimeric genes Nos, CaMV 35S promoter, *npt-II* and *hpt-I*.

Resultant transgenic plants were efficient in absorbing P by converting the insoluble form of P to soluble form by producing citric acid.

Southern blot and PCR analysis were used to confirm the integration of *citrate synthase* gene of *Pseudomonas aeruginosa* in transgenic tobacco plants (Bucio *et al.*, 2000). Pichia (1987) employed the HPLC technique to know the expression of *Citrate synthase* gene activity results in increased citrate solution.

Dalhaize *et al.* (2001] reported that overexpression of *citrate synthase* in tobacco of *Pseudomonas aeruginosa* is not associated with either enhanced citrate accumulation or efflux.

2.6 *In Planta* transformation

In Planta transformation is a new approach for development of transgenic plant. This strategy of transformation is tissue culture independent. The advantage of using this method for transformation study is that recalcitrant plant species can be easily transformed and problem of somaclonal variation associated with tissue cultured plant can be overcome. *In Planta* transformation was first reported in *Arabidopsis thaliana* by Feldman and Marks (1987). They applied *Agrobacterium* to *Arabidopsis* seeds, then collected progeny seeds and germinated them on antibiotic containing media to identify transformed plants.

Bechtold *et al.* (1993) reported *In planta* transformation of *Arabidopsis* by another method known as "Vacuum infiltration". In this method *Arabidopsis* plants are uprooted and placed into a bell jar in a solution of *Agrobacterium*. A vacuum was applied and then released, causing air trapped within the plant to bubble off and be replaced by with the *Agrobacterium* solution. Plants were transplanted back to soil, grown to seed, and in next generation stably

transformed line were selected on the media containing antibiotic or herbicide depending on marker gene present in the construct used for transformation.

Cheng *et al.* (1994) later succeeded in generating transformed *Arabidopsis* lines by using its flower. Reproductive inflorescence were clipped off, *Agrobacterium* was applied to the center of the plant rosette, new inflorescence formed a few days later were again removed, *Agrobacterium* was reapplied and plants were then allowed to develop and set seed. Plants obtained from these seeds were confirmed as transgenic.

2.6.1 *In planta* transformation studies on groundnut

Mc Kently *et al.* (1993) developed transgenic groundnut plants by employing *In planta* transformation. They used zygote embryo axes of mature seeds as explant. Gene integration in the transgenic plants was confirmed by using PCR, Southern blotting and GUS assay.

Brar *et al.* (1994) obtained transgenic peanut plant via the particle bombardment of shoot meristem excised from mature embryonic axes. This system also did not utilize tissue culture and resulted in the direct development of transgenic shoots from bombarded meristems.

Rohini and Sankara Rao (2001) developed fertile transgenic peanut plant containing tobacco chitinase gene using *In planta* transformation technique. Zygotic embryos obtained from matured seeds were immersed in the bacterial suspension. Plants germinated from these embryos were analysed for gene integration by PCR and Southern blotting.

MATERIAL AND METHODS

III MATERIAL AND METHODS

The present investigation "Development of transgenic groundnut with *Citrate synthase* gene for improving phosphorous use efficiency was carried out at the Department of Biotechnology, UAS, GKVK, Bangalore-560065. The details of the work done are given below.

The following experiments were conducted during the investigations

3.1 Standardization of regeneration protocol for groundnut cv. JL-24

This experiment was conducted with the objective to reduce the time required for the regeneration of the groundnut cv. JL-24 through somatic embryogenesis.

3.1.1 Material

1. Seeds: Seeds of the groundnut cv. JL-24 was obtained from National Seed Project, G.K.V.K, Bangalore.
2. Sterile forceps, scalpels
3. 0.1 per cent $HgCl_2$
4. Sterile blotting papers
5. Sterile double distilled water
6. Glass bead sterilizers
7. Laminar flow chamber
8. MS medium
9. Hormones (2,4-D and TDZ)

3.1.2 Procedure

1. Zygotic embryos from groundnut seeds were removed without damaging them.
2. These embryos were soaked under running water overnight and washed with double distilled water for 4-5 times.
3. Then they were treated with 0.1% $HgCl_2$ for 1 minutes and washed with sterile double distilled water for 4-5 times and blotted dry on sterile blotting papers
4. Then these explants were inoculated on to the medium containing 15 mg/l 2,4-D (Krishnamurthy, 2000) and incubated in the dark condition for different periods like 0,5,8,10,12 and 15 days for callus induction.
5. After dark incubation explants were placed on MS medium containing 15 mg/l 2,4-D and different concentrations of TDZ for callus growth and somatic embryogenesis.
6. Subculturing of callus was done at every 2 weeks until it produced somatic embryos.
7. Somatic embryos were excised from callus and subcultured on MS basal medium for germination
8. Once the shootlets were formed they were shifted to medium containing 12 mg/l IBA for rooting (Krishnamurty, 2000).

Following hormone concentrations were tried for callus growth and somatic embryo production.

Treatments

T_1=MS +15mg/l 2,4D

T_2=MS +15mg/l 2,4-D + 0.2mg/l TDZ

T_3= MS+15mg/l 2,4-D + 0.5mg/l TDZ

T_4= MS+15mg/l 2,4-D + 0.75mg/l TDZ

T_5= MS+15mg/l 2,4-D +1.0mg/l TDZ

Note: Each treatment was given with 5 replication and 8 explants in each replication.

Dark experiment

This experiment was conducted to know the influence of dark period on callus production.

Zygotic embryos were inoculated on to MS medium containing 2,4-D (15mg/l) and they were incubated for 0,5, 8,10, 12 and 15 days under darkness.

After the dark incubation calli were obtained which were shifted to another medium containing 15mg/l 2,4-D with different concentrations of TDZ and cultured under light in culture room at 25^0C for 16 hrs photoperiod for callus growth and somatic embryo production Subculturing was done at every 15 days.

The observations recorded were as follows

a. Callus growth
b. Number of somatic embryos per explant
c. Number of days taken for somatic embryo production

3.2 Transformation of groundnut with *Citrate synthase* encoding gene

Transformation of the groundnut cv. JL-24 was carried out using above standardized protocol as well as with a tissue culture independent

transformation method to make a comparison on their transformation efficiency.

Bacterial strain

The *Agrobacterium tumefaciens* strain EHA 105 was used for the transformation studies. It contained a binary vector pVM-CS with *npt-II* and *hpt-1* selection makers, CaMV 35 S promoter for constitutive expression and ocs terminator (Fig.1). It was kindly provided by Dr. P.M. Reddy, IRRI, Philippines.

Maintenance of bacterial culture

The *Agrobacterium* culture was grown in YEP broth containing 100mg/l kanamycin at 28^{0}C over night. An equal volume of glycerol was added and was stored at –20^{0}C as a mother culture. For routine use, solid YEP medium containing 100 mg/l of kanamycin was streaked with the mother culture once in a month and stored at 4^{0}C.

Preparation of YEP medium (per litre)

Tryptone	10 g
Yeast extract	10g
NaCl	5 g
Agar	15g

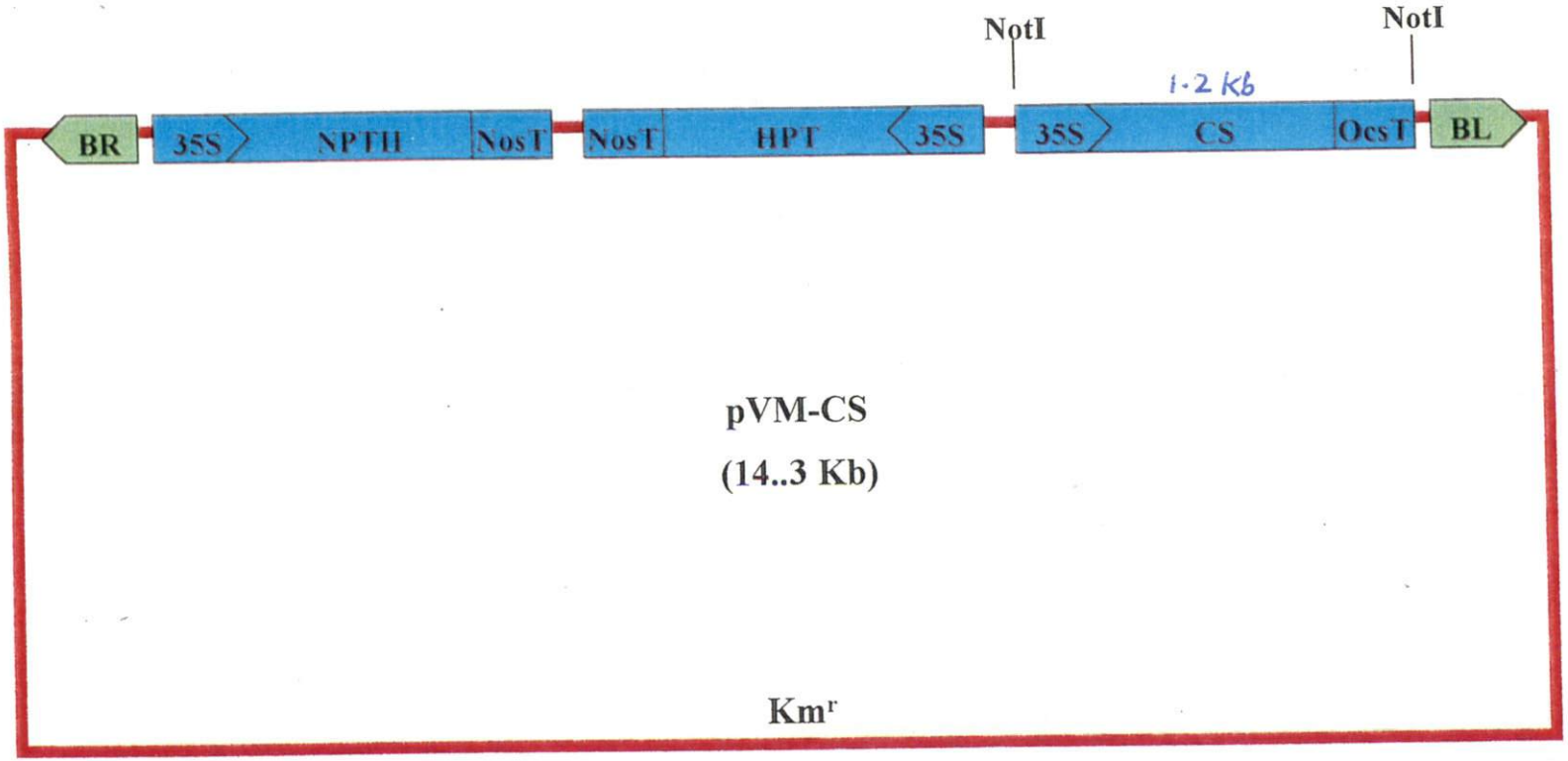

Fig 1. Map of Ti plasmid (p VM-CS) carrying *citrate synthase* gene

Preparation of *Agrobacterium* culture for cocultivation

Agrobacterium culture was maintained in the solid YEP medium containing 100 mg/l kanamycin. Subculturing was done at every month in fresh medium containing kanamycin

- For transformation, single colony was taken from the YEP plate and inoculated to a bottle containing 20 ml YEP with 100 mg/l kanamycin and was kept in an incubator in shaking condition overnight at 27^0C.

- The culture that read 1.0 OD at 600nm was chosen for transformation

- The overnight grown culture was centrifuged at 10,000 rpm for 5 min at 4^0C. Supernatant was discarded and pellet was resuspended in ½ MS media.

3.2.1 Transformation through somatic embryogenesis

3.2.1.1 Hygromycin sensitivity test

Hygromycin is one of the most popular selection marker gene used in plant transformation. Hygromycin sensitivity test was performed to find out the concentration of hygromycin required for selection of transformed plants. Twelve days old untransformed calli were placed on the MS medium containing15mg/l 2,4-D and 0.75mg/l TDZ with different concentrations of the hygromycin (20,23,25, 28 and 30 mg/l) and after that plates were kept in growth chamber.

Observations were recorded for the survival of explants at different days intervals.

3.2.1.2 Cocultivation: Twelve to fourteen days old calli obtained from the zygotic embryo explants were used for cocultivation.

To optimize the transformation different parameters like cocultivation period, cocultivation incubation period and acetosyringone concentration, experiments were set up.

3.2.1.3 Cocultivation period

Twelve day old calli were dipped in the bacterial culture and cocutivated for different periods as the treatments are given below.

Treatments

T_1 = 5 minutes

T_2 = 8 minutes

T_3 = 10 minutes

T_4 = 12 minutes

T_5 = 15 minutes

Note : Three replications each with 8 explants.

After the cocultivation calli were blotted dry on sterile blotting paper and kept for different cocultivation incubation periods like 1, 2, 3 and 4 days on MS basal medium. Later they were shifted to medium containing cefotaxime (350 mg/l).

Observations were recorded for the healthy or dead calli to identify the best cocultivation period.

3.2.1.4 Concentration of acetosyringone

Calli were dipped in the bacterial culture with different concentrations of acetosyringone and cocultivated for 8 minutes.

Treatments

T_0=Control

T_1=75 µM

T_2=100 µM

T_3=120 µM

T_4=150 µM

T_5=180 µM

Note: Four replication each with 7 explants.

Observations were recorded for the number of the cocultivated calli surviving on the selection medium at 7^{th} day.

Cocultivation was done using the treatments giving the best result from above experiments

3.2.1.5 Selection of transformants

- After 3 days of cocultivation, calli were transferred on the MS basal medium containing hormones (15mg/l 2,4-D and 0.75 mg/l TDZ) with hygromycin (25 mg/l) and cefotaxime (350 mg/l).

- Calli kept on the selection plate were kept at 25^0C with 16 hrs photoperiod until it produced somatic embryo

- Subculturing was done at every 15 days interval with same media with higher concentration of hygromycin (28 mg/l) and lower concentration of cefotaxime (100 mg/l).

- Only transformed calli could survive on the selection plate and untransformed were dead on the same.

- Once somatic embryos were formed, they were shifted to MS basal medium without hygromycin for germination. Subculturing was done at every 15 days on same MS basal medium. Once shootlets were formed rooting was done.

3.2.1.6 Root induction of transformants

Shootlets were shifted aseptically on to MS basal medium containing IBA (12 mg/l) for rooting. Rooted plants were then hardened.

3.2.1.7 Hardening of putative transformants

When the rooted shoots produced 2-3 leaves they were transferred to a hardening media after thoroughly washing the agar away from the roots. The hardening media consisted of autoclaved peat and sand mixture in the ratio of 3:1. After the plants were planted in this media, 5 ml of ½ MS diluted 100 times was added to the media whenever it turned dry. The bottles were sealed and placed in the growth room. When the plants grew to 6 cm the cap was removed and mouth of the bottle was covered by tying the polyethylene bags.

3.2.2 Transformation through tissue culture independent method (*In planta* transformation)

Because transformation efficiency through somatic embryogenesis was not found to be efficient this method of transformation was also followed.

3.2.2.1 Procedure

1. Groundnut seeds were taken out gently from pods.
2. These seeds were kept in 2 per cent Bavistin for 1 hr to kill the fungal spores and afterward they were washed thrice with sterile distilled water.
3. Then they were treated with 0.1 per cent sterile $HgCl_2$ for 2 minutes and washed with sterile double distilled water for 4-5 times and blotted dry on sterile blotting paper.
4. Then seeds were cut into two parts. The portion, which contains zygotic embryo, was used for cocultivation.

3.2.2.1.1 Cocultivation

Half seed containing zygotic embryo was immersed in the bacterial suspensions containing 150 µM acetosyringone for different periods as treatments given below.

Treatments

T_1 = 20 minutes

T_2 = 30 minutes

T_3 = 35 minutes

T_4= 45 minutes

T_5 = 55 minutes

T_6 = 60 minutes

After cocultivation using above treatments these explants were blotted dry on sterile blotting paper and cocultivated on the MS basal medium for different incubation periods i.e., 1, 2, 3, 4 and 5 days. After this they were shifted to MS basal medium with cefotaxime (400mg/l).

Observations were recorded for the survival of the explants at 7^{th} day.

Cocultivation of the half seed explant was done using the best cocultivation period (30 minutes for four days) obtained from the above study. After the cocultivation explants were blotted dry on the MS basal medium with 400mg/l cefotaxime.

3.2.2.1.2 Hardening of putative transformants

When the plants reached 8cm in height they were hardened by the similar procedure as it was done for plants obtained through somatic embryogenesis.

Later these plants were transferred to pot in culture room containing peat soil and sand (2:1:1) and transferred to greenhouse.

3.3 Confirmation of gene integration

To confirm the transformation, the control and the putatively transformed groundnut plants were tested for the presence of *npt* II gene by using PCR. For this purpose both the plasmid and plant genomic DNA were isolated as follows:

3.3.1 Isolation of plasmid DNA from *Agrobacterium*

3.3.1.1 Procedure

1. A single *Agrobacterium* colony was picked up aseptically using sterile inoculation needle and was grown overnight in 1 ml YEP medium containing kanamycin (50mg/l) in a sterile microfuge tube.
2. Overnight grown 1 ml culture was added to 50ml Yep medium containing kanamycin and again grown overnight.
3. One ml of culture was taken and centrifuged at 5000 rpm for 2 minutes.
4. The supernatant was poured off and the cell pellet was resuspended in 100 ml of Solution I + 25 µl of Lysozyme and incubated for 10 minutes at 37°C.
5. 200µl of Solution-II was added mixed by gentle shaking and incubated for 10 minutes at room temperature.
6. 150µl 3M sodium acetate (pH 4.8) was added to the viscous solution and mixed by gentle shaking and kept in ice for 30minutes. After that it was centrifuged at 12000rpm for 5 minutes and supernatant was transferred into another tube.
7. 30µl of phenol was added and vortexed.
8. The suspension was centrifuged for 3 min at 5,000rpm.
9. The supernatant was poured out into a new tube.
10. The tube was filled with 2 volume of chilled absolute alcohol and mixed by inversion.
11. The tube was stored overnight at 0°C in freezer.

12. DNA was pelleted by centrifuging the tubes at 13,000 rpm for 10 min. The supernatant was removed and the pellet was washed with 0.5 ml of 70 per cent ethanol.
13. The DNA was pelleted again in a microfuge tube and the supernatant aspirated off.
14. The pellet was dried at 36°C for 1 hr.
15. The pellet was suspended in 50µl TE.

3.3.1.2 Purification of plasmid DNA

1. Equal volume of phenol was added and centrifuged at 5,000rpm for 2 minutes.
2. The supernatant was collected and treated with equal volume of chloroform: isoamylalcohol (24:1).
3. After centrifugation at 5,000 rpm for 2 minutes the supernatant collected.
4. To the supernatant equal volume of chilled ether was added and mixed well.
5. The sediment was collected and treated with 2 volume of chilled absolute alcohol and kept at –20°C for 30 min.
6. To the pellet 1 ml of 70 per cent alcohol was add and the pellet was dislodged by gentle tapping.
7. The solution was centrifuged at 5,000 rpm for 2 min and to the pellet 1-2 drops of absolute alcohol was added.
8. Then it was vacuum dried and stored in sterile water (Sambrook *et al.*, 1993).

3.3.2 Isolation of plant genomic DNA

CTAB method

Cetyl trimethyl ammonium bromide is a detergent and is used along with other reagents to liberate nucleic acids from the cell. This is an efficient

method for isolating plant genomic DNA from leaf tissues. The high molecular weight DNA obtained is purified by phenol : chloroform method to remove the proteins and other plant debris.

Procedure

1. The leaf tissue was washed in water and the excess water was blotted with blotting paper and air dried briefly.
2. 2g leaf tissue was weighed from each transformed plant and control plant
3. The leaves from individual plants were cut into pieces, placed in –20°C freezer overnight and ground well using pestle and mortar with 6 ml of hot extraction buffer.
4. The extract was poured into 50 ml polypropylene tubes were incubated at 65°C in a water bath for 15-20 min with gentle shaking.
5. Equal volume of chloroform: isoamylalcohol mix was mixed well by inverting the tubes.
6. The contents were centrifuged at 13,000 rpm for 10 min at room temp
7. The supernatant was taken and to this 0.6 volume of chilled Isopropanol was added.
8. Centrifugation at 10,000rpm for 5 min.
9. The pellet was dissolved in 600µl of TE
10. The DNA was purified by Phenol : chloroform cleaning as described in the earlier experiments and further recovered by ethanol precipitation.
11. The resulting DNA pellet was dissolved in TE buffer treated with RNase and stored at –20°C.

3.3.3 Spectrophotometric assay of DNA concentration

It is critical to know exactly how much DNA is present in the solution before carrying out any experiments with it. DNA concentration can be accurately measured by ultraviolet absorbance spectrophotometry. Absorption

at 260 of DNA solution is directly proportional to the amount DNA present in the sample. Usually the absorbance is measured at wavelengths 260 and 280nm. An absorbance of 1 at 260nm corresponds to 50µg/ml of double stranded DNA. The purity of DNA preparation was checked by calculating the ratio of absorbance A260/A280, which is 1.8 if pure. A ratio less than 1.8 indicates that the preparation is contaminated either with phenols or proteins. Values higher than this indicates the presence of RNA in the preparation.

3.3.4 PCR amplification and examination of amplifed DNA fragments

The *hpt-I* (Hygromycin phosphotransferase-I) and *npt-II* (Neomycin phosphotransferase-II) genes confer the resistance to hygromycin and kanamycin respectively. These genes were present as selection marker at upstream of the *Citrate synthase* gene. By the presence of *hpt-I* gene the transformants can be selected on the media containing hygromycin. The presence of the *npt-II* gene indicates the possible integration of *Citrate synthase* gene in the plants. So by amplifying *npt-II* gene by using *npt-II* specific forward and backward primers the integration of *Citrate synthase* gene in to the plant can be ascertained.

3.3.4.1 PCR amplification

The following components were sequentially added into a 0.5 µl PCR tube.

1. 2 µl of sample DNA (as template).
2. 0.5 µl each of forward and reverse *npt-II* primers (20 mM concentration).
3. 3 µl of 2.5 mM dNTP mix solution.
4. 5 µl of 10X *Taq* polymerase assay buffer with 15mM $MgCl_2$.
5. 38.0 µl autoclaved double distilled water.
6. And 1 µl of *Taq* DNA polymerase (3 units/µl).

PCR was performed initially for 4 minutes at 94^{0}C; followed by 35 cycles of melting at 94^{0}C for 1 minute, annealing at 56^{0}C for 40 seconds and synthesis at 72^{0}C for 1 minute; and final extension at 72^{0}C for 7 minutes, after all the cycles were completed.

Table 1. Reagents used in PCR amplification

Reagents	Concentration	Volume
DNA template	250 ng	2 µl
Primers (forward & backward)	20 mM	0.5 µl each
dNTPs	2.5 mM	3 µl
10X *Taq* polymerase buffer	-	5 µl
Double distilled water	-	38.0 µl
Taq DNA polymerase	3 units/µl	1 µl

3.3.4.2 Agarose gel electrophoresis of DNA

After PCR 20µl of the mixture was gel electrophoresed on 1.0 per cent agarose containing ethidium bromide (5ng/ml) with 1 X TBE (pH 8.5) buffer. The amplified DNA band was visualized as a fluorescing band on a trasnsilluminator.

EXPERIMENTAL RESULTS

IV EXPERIMENTAL RESULTS

Experiments were carried out to standardize the regeneration protocol for groundnut cultivar JL- 24 and development of transgenic groundnut with *Citrate synthase* gene to improve the phosphorous use efficiency by employing the *Agrobacterium* mediated transformation technique. The data obtained from these studies are presented here under.

4.1 Standardization of regeneration protocol for groundnut cv. JL- 24

4.1.1 Effect of dark incubation on callusing

Experiments were carried out using zygotic embryo as explant. They were cultured on 15 mg/l 2,4-D and incubated in the dark condition for different periods. Data obtained from this study is given in Table-2.

We observed that 12 days dark incubation of the zygotic embryo results in the good size of callus production compared to less or more number of days of dark incubation. Zygotic embryos, which were not given dark incubation, did not convert into callus.

4.1.2 Effect of hormones on the callus growth and somatic embryogenesis

Twelve days old calli obtained after the dark incubation were transferred on to 15 mg/l 2,4-D and varying concentration of the TDZ (0.0, 0.2, 0.5, 0.75 and 1.0 mg/l) and incubated under light. Data obtained from the study is given in the Table-3.

Table 2: Effect of dark incubation of groundnut cv. JL-24 zygotic embryo on callus formation

Sl. No.	Treatments (days)	Callus formation
1	0	-
2	5	+
3	8	++
4	10	+++
5	12	++++
6	15	+++

- No callus formation

+ Very less callus formation

++ Less callus formation

+++ Medium callus formation

++++ More callus formation

Plate 1: **Zygotic embryo explants kept on the MS medium containing 15 mg/l 2,4-D for callus induction**

Plate 2: **Calli formed after 12 days dark incubation on medium containing 15 mg/l 2,4-D**

Plate 1

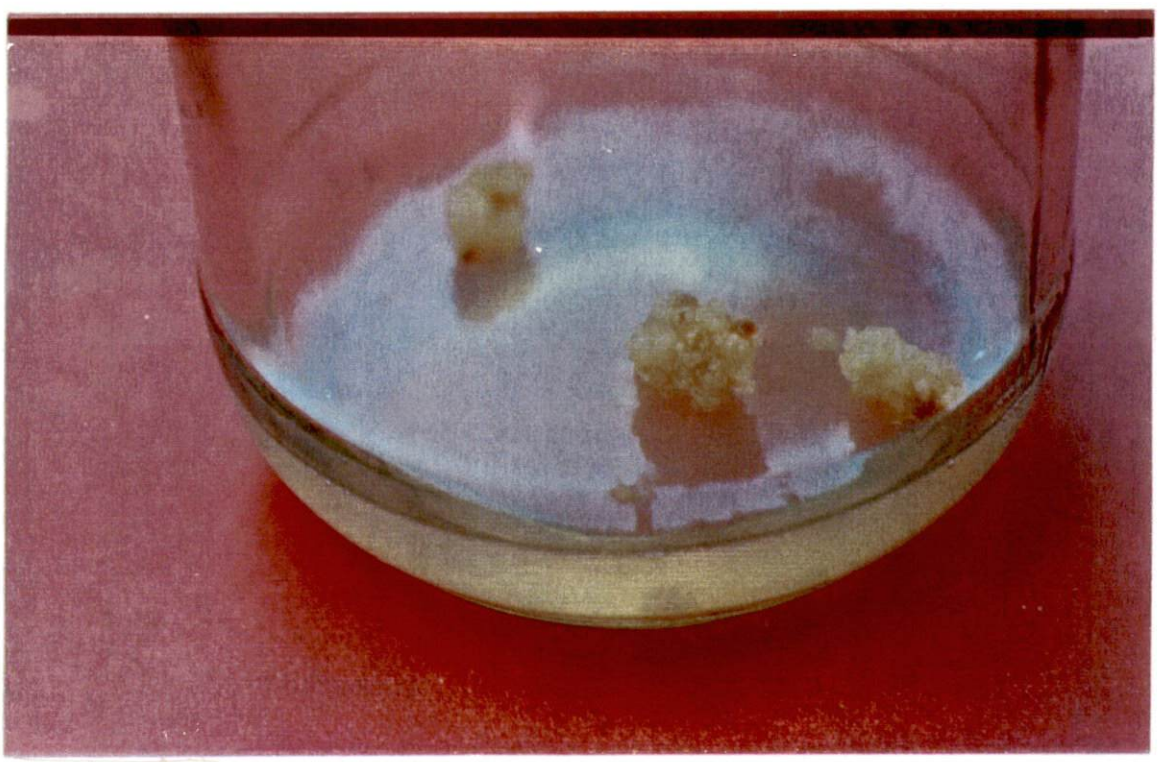

Plate 2

Plate 3: Difference in callus growth of groundnut cv. JL- 24 on the medium containing 15 mg/l 2,4-D with different concentrations of TDZ

T_1= Callus placed on medium containing only 15 mg/l 2,4-D

T_2= Callus placed on medium containing 15 mg/l 2,4-D + 0.2 mg/l TDZ

T_3= Callus placed on medium containing 15 mg/l 2,4-D + 0.5 mg/l TDZ

T_4= Callus placed on medium containing 15 mg/l 2,4-D + 0.75 mg/l TDZ

Plate 3

Plate 4: **Somatic embryo formed on the medium containing 15 mg/l 2,4-D and 0.75 mg/l TDZ**

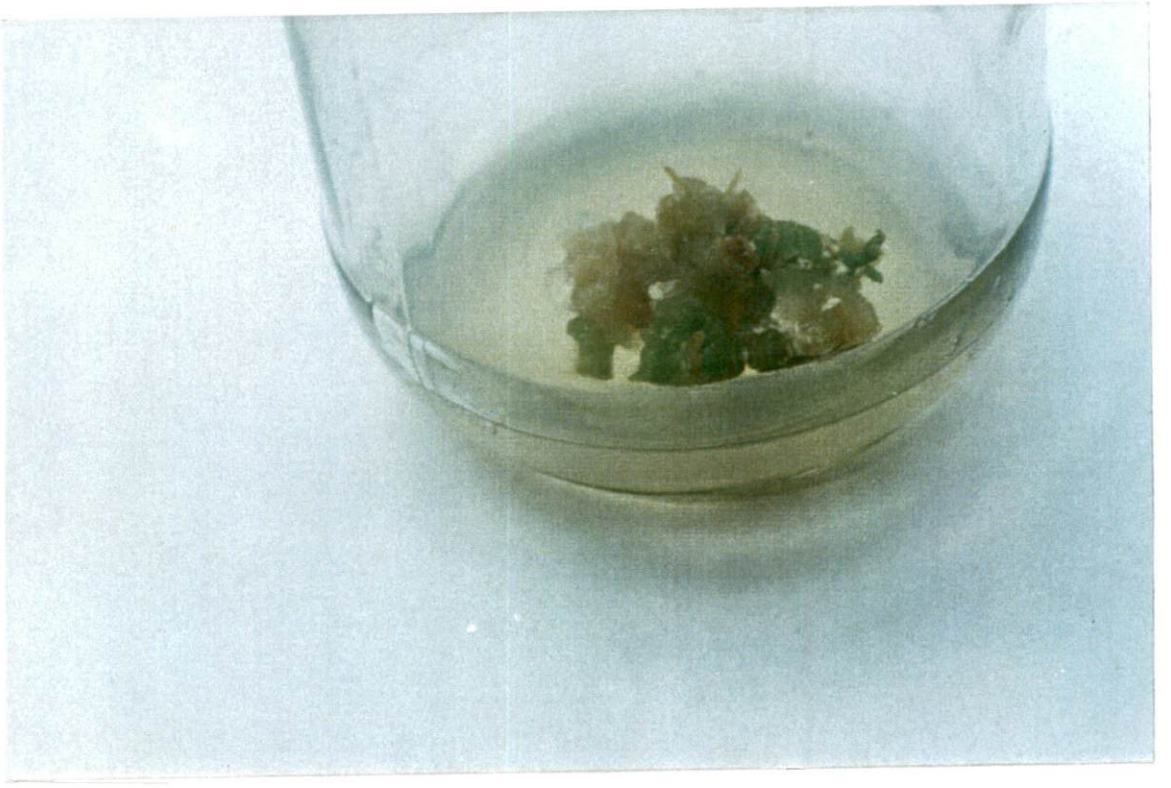

Plate 4

It was observed that calli cultured on the medium containing 15 mg/l 2,4-D with TDZ showed better callus growth (Plate 3) compared to the calli allowed to grow on medium supplied only with 2,4-D (15 mg/l). Growth of the calli were maximum in the treatment containing 0.75mg/l TDZ with 15 mg/l 2,4-D.

The effect of TDZ on the somatic embryo formation was also documented. It was observed that the presence of TDZ along with 2,4-D enhances the process of somatic embryogenesis. Calli cultured on the medium supplied only with 2,4-D (15 mg/l) took longer time for somatic embryo formation than calli cultured on the medium containing TDZ with 2,4-D (Table-3).

It took only on an average of 38 days to form somatic embryo in the medium containing 0.75 mg/l TDZ and 15 mg/l 2,4-D, whereas calli kept on the medium containing only with 2,4-D took 55 days. Number of embryo formed also varied among the treatments. Number of somatic embryo formed on an average in the treatments having TDZ along with 2,4- D were more than those cultured only on 2,4-D. On an average four somatic embryos were formed in the treatment containing 0.75 mg/l TDZ with 15 mg/l 2,4-D. Increase in the concentration of TDZ beyond 0.75 mg/l did not show any improvement in terms of number of somatic embryos formed and number of days taken for somatic embryo formation.

4.2 Development of transgenic plant containing *Citrate synthase* gene with *Agrobacterium*

Transformation studies were carried out using the protocol, which was standardized by us, and also by following *In planta* transformation technique (Feldman *et al.*, 1987).

Table 3: Effect of 2,4-D and TDZ on the callus growth and somatic embryogenesis of groundnut cv. JL-24

Sl. No.	Hormone concentration (mg/l)	Callus growth	Avg. no. of days taken for somatic embryo formation	Avg. no. of somatic embryos formed per explant
T_1	2,4-D (15)	+	55	3
T_2	2,4-D (15)+TDZ (0.2)	+ +	48	3.5
T_3	2,4-D (15)+TDZ (0.5)	+++	43	3.75
T_4	2,4-D (15)+TDZ(0.75)	++++	38	4
T_5	2,4-D(15)+TDZ(1.0)	++++	38	4

+ Very less callus growth

++ Less callus growth

+++ Medium callus growth

++++ More callus growth

4.2.1 Transformation through somatic embryogenesis

4.2.1.1 Hygromycin sensitivity test

In order to determine the lethal concentration of hygromycin, calli were kept on the different concentrations of the hygromycin (20 to 30 mg/l). Data obtained from this study is given in the Table-4.

It was observed that upto 20 mg /l of hygromycin calli were growing well (Plate-7) but when the concentration was increased further growth stopped and were killed at 25 to 28 mg/l concentration. Hence, this lethal concentration was chosen for the selection of the transformants in further studies.

4.2.1.2 Standardization of cocultivation period

Twelve to fourteen day old calli derived from zygotic embryo cultured on the medium containing 15 mg/l 2,4-D were used for cocultivation. These calli were immersed in the bacterial suspension that read 1 OD at 660nm and they were allowed for different period ranging from 5 to 20 minutes and then these explants were incubated on MS agar medium for 1 to 4 days. After this these explants were washed with sterile distilled water followed by cefotaxime 350 mg/l, and then they were kept on the media containing cefotaxime (350 mg/l). Observations were recorded at different periods for healthy explants to identity the best cocultivation period. The data obtained from the study is given in Table-5.

It was observed that dipping of calli for eight minutes followed by three days cocultivation and then washing with cefotaxime solution found to be optimum for the survival of explant i.e., free from growth of bacteria.

Table 4: Effect of hygromycin on the callus growth and somatic embryogenesis of groundnut cv. JL-24

SL. NO	Concentration of hygromycin(mg/l)	Survivable at different intervals (Days)					Callus growth and somatic embryogenesis
		1	3	5	7	15	
T_1	20	H	H	H	H	H	Yes
T_2	23	H	H	H	Y	D	No
T_3	25	H	H	Y	D	D	No
T_4	28	H	Y	D	D	D	No
T_5	30	Y	D	D	D	D	No

H: Healthy
Y: Yellowing
D: Dead

Table 5: Survival or death of calli cocultivated with *Agrobacterium* after 7 days incubation on medium containing cefotaxime (400 mg/l)

Treatments*	Survival of explants at 7th day			
	1day cocultivation	2days cocultivation	3days cocultivation	4days cocultivation
T_1	H	H	H	H
T_2	H	H	H	D
T_3	H	H	D	D
T_4	H	D	D	D
T_5	D	D	D	D

*Treatments: Period for which calli were dipped in bacterial culture

T_1 = 5 minutes

T_2 = 8 minutes

T_3 = 10 minutes

T_4 = 12 minutes

T_5 = 15 minutes

H = Healthy

D = Dead

Plate 5: **Shoot formation from somatic embryo on MS basal medium**

Plate 5

Plate 6: **Rooting of somatic embryo on MS medium containing 12 mg/l IBA**

Plate 6

Plate 7: Sensitivity of the calli to different concentrations of hygromycin

T_1= Calli placed on the medium containing 20 mg/l hygromycin

T_2= Calli placed on the medium containing 23 mg/l hygromycin

T_3= Calli placed on the medium containing 25 mg/l hygromycin

T_4= Calli placed on the medium containing 28 mg/l hygromycin

T_5= Calli placed on the medium containing 30 mg/l hygromycin

Plate 8: Control and transformed calli on the medium containing hygromycin

1= Control

2= Control placed on the medium containing hygromycin (25 mg/l)

3= Cocultivated placed on the medium containing hygromycin (25 mg/l) + cefotaxime (350 mg/l)

Plate 7

Plate 8

4.2.1.3 Effect of acetosyringone concentration on infection of calli with *Agrobacterium*

An experiment was conducted with different concentration of acetosyringone (75, 100, 120, 150 and 180 µM) added to bacterial suspension before 60 minutes of cocultivation. A treatment without acetosyringone was also maintained as control. Among different treatments tried, it was observed that in the treatment with 150 µM concentration of acetosyringone maximum number of cocultivated calli were found to survive when kept on the selection medium. Beyond this concentration not much increase in the survival of calli on the selection plate was observed. At 150 µM concentration 44 per cent of the total cocultivated calli were found to be surviving. The treatment in which acetosyringone was not added not a single callus survived (Table-6).

4.2.1.4 Selection of transformants

Transformed calli were selected on the medium containing 25 mg/l hygromycin and 350 mg/l cefotaxime for one and half month. It was observed that a large number of calli were found to survive on the selection plate (Plate 8). But they were not able to regenerate as complete plant with root and shoot. Only one plant was found to regenerate with good number of roots out of several surviving calli.

4.3 Transformation of groundnut with tissue culture independent technique (*In planta* transformation)

Because transformation through somatic embryogenesis was not found efficient, this technique of transformation was also followed.

Table 6: Effect of different concentrations of acetosyringone on the level of infection of groundnut cv. JL-24 calli cocultivated with *Agrobacterium*

Sl. No.	Acetosyringone concentration (µM)	No. of calli cocultivated	No. of calli surviving at 7th day on selection medium	Calli surviving on selection medium (%)
T_0	Control	80	0	0
T_1	75	80	9	11
T_2	100	80	19	24
T_3	120	80	26	33
T_4	150	80	35	44
T_5	180	80	34	43

4.3.1 Standardization of cocultivation period

Half seed explant containing zygotic embryo was used for cocultivation (Plate 10). *Agrobactreium tumefaciens* containing *Citrate synthase* gene was grown overnight at 27°C was adjusted to OD 1 at 660nm wave length. Explants were dipped and allowed for different period of time in the above solution ranging from 20 to 60 minutes and then explants were further incubated on MS agar medium for one to five days. Then these explants were washed with sterile double distilled water followed by cefotaxime at the rate of 400 mg/l and placed on the medium containing cefotaxime. The observations identified the best cocultivation period. The data obtained from the study are given in the Table-7.

It was observed that the cocultivation of half seed explant for 30 minutes with the *Agrobacterium* followed by four days incubation period was found to be optimum for the explants to be healthy and free from bacterial growth.

4.4 Confirmation of gene integration

Large number of plants obtained through half seed method and the one plant obtained through somatic embryogenesis were analysed by PCR. DNA from the putative transgenic plants and control plants was isolated and PCR was carried out using the *npt-II* primers. In the T-DNA region of gene construct both *hpt-I* and *npt-II* are present upstream of *Citrate synthase* gene. So by using *npt- II* primers integration of the gene was confirmed. PCR amplification product analysis showed the presence of a 800 base pair size band in more than 50 per cent of the putative plants obtained through half seed and also the one plant obtained through somatic embryogenesis (Fig. 2).

Table 7. Survival or death of the half seed explants cocultivated with *Agrobacterium* after 7days incubation on medium containing cefotaxime (400 mg/l)

Treatments*	Survival of explants at 7th day				
	1day cocultivation	2days cocultivation	3days cocultivation	4days cocultivation	5 days cocultivation
T_1	H	H	H	H	H
T_2	H	H	H	H	D
T_3	H	H	H	D	D
T_4	H	H	D	D	D
T_5	H	H	D	D	D
T_6	H	D	D	D	D

*Treatment: Period for which half seeds were dipped in bacterial culture

T_1 = 20 minutes

T_2 = 30 minutes

T_3 = 35 minutes

T_4 = 45 minutes

T_5 = 55 minutes

T_6 = 60 minutes

H = Healthy

D = Dead

Plate 9: **Transgenic plant obtained through somatic embryogenesis**

Plate 9

Plate 10: **Half seed explant used in *In planta* transformation**

Plate 11: **Hardening of transgenic plant obtained through *In planta* transformation**

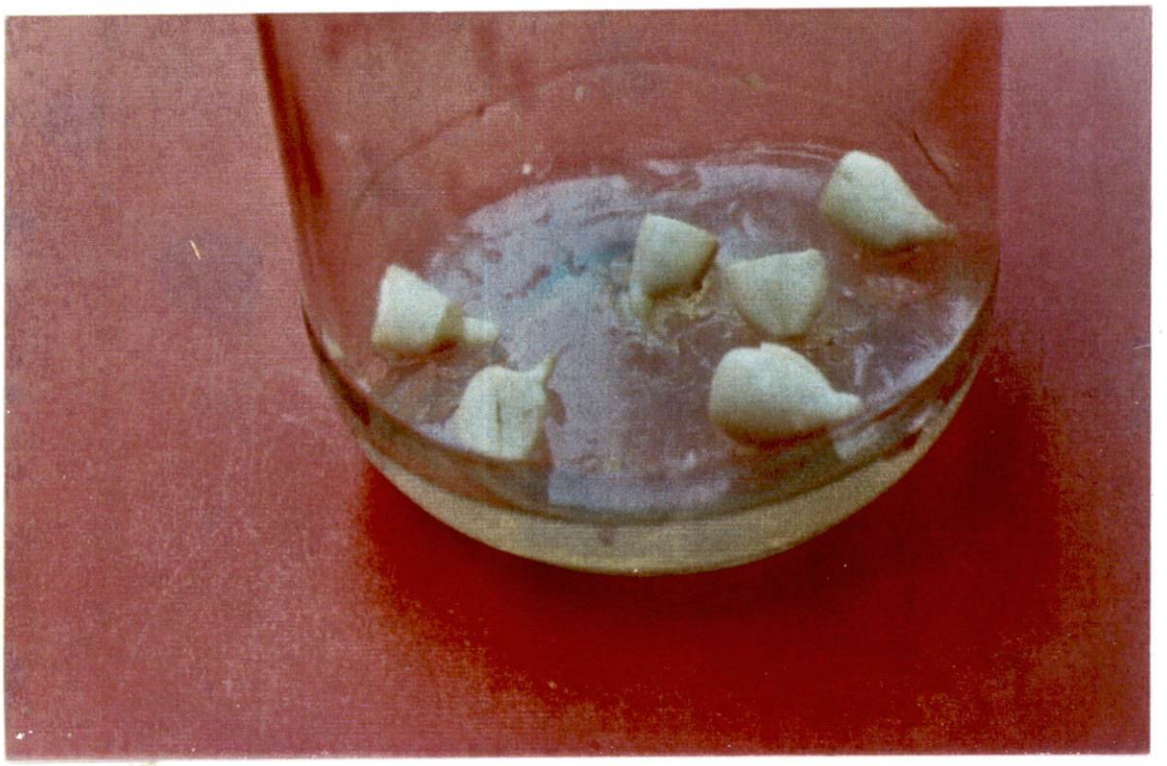

Plate 10

Plate 12: Transgenic plants in the growth chamber

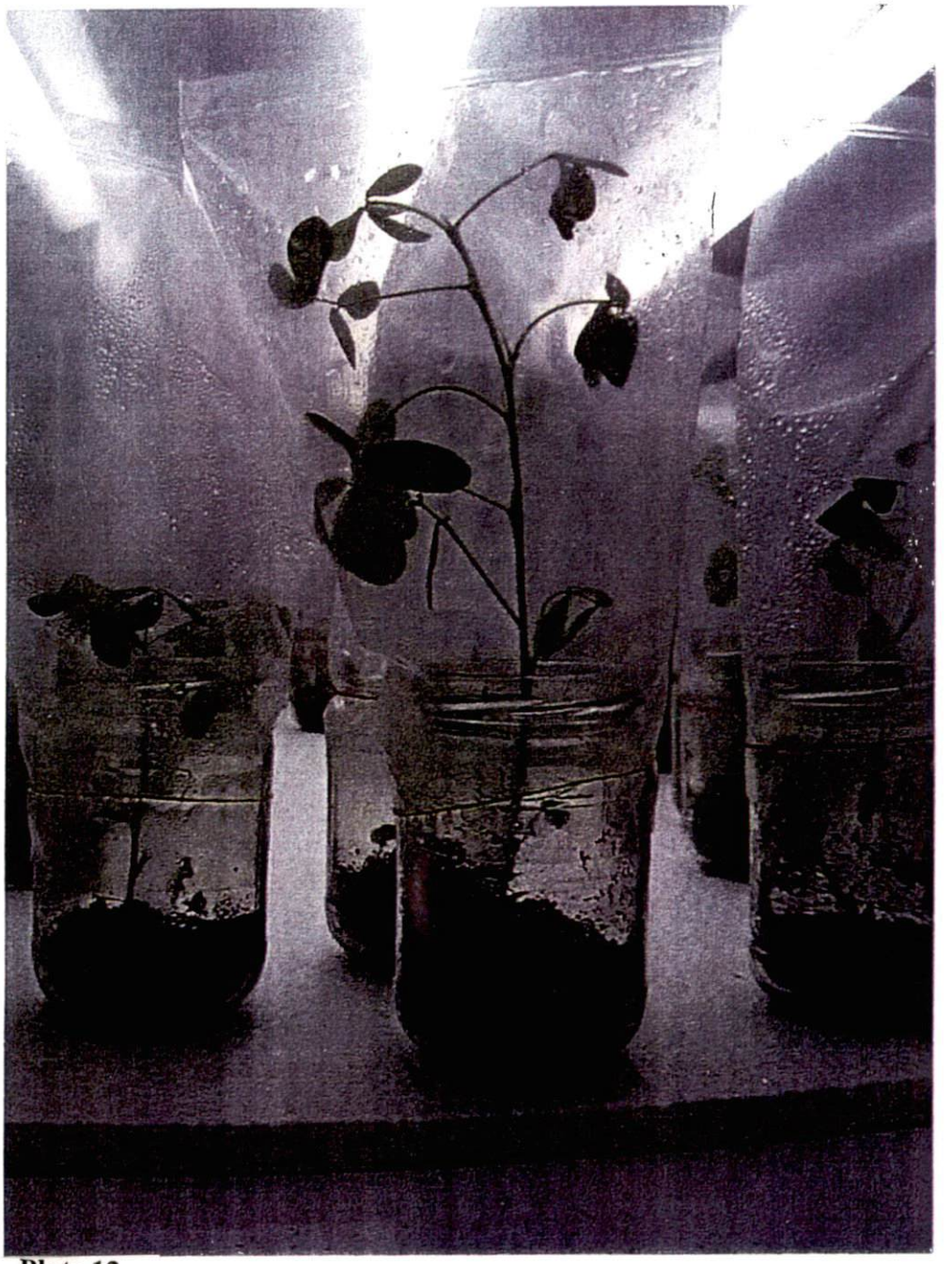

Plate 12

Plate 13: **Transformed groundnut plants in the greenhouse condition**

Plate 13

Fig.2 Gel photograph showing PCR amplification of *npt-II* gene in transgenic groundnut plant.

M – Marker

Lane 1 – Plasmid DNA (Positive control)

Lane 2,3,4,7 and 8 – Genomic DNA of transgenic plants obtained through half seed method

Lane 5 – Genomic DNA of transgenic plant (without primer)

Lane 6 - Genomic DNA of transgenic plant obtained through somatic embryogenesis

Lane 9 and 10 - Genomic DNA of control plant

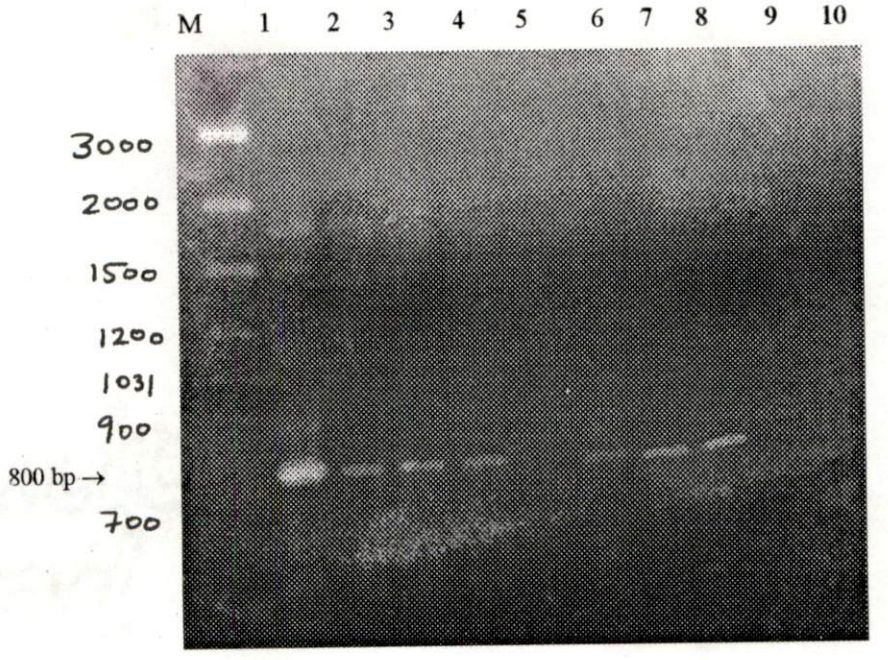

Fig. 2

DISCUSSION

V DISCUSSION

Soils all over the world are deficient in phosphorus and they require application of phosphatic fertilizers to supply the phosphorus nutrient to the crop plants. Since India is not having required phosphorus deposits for production of phosphatic fertilizers, large quantity of Phosphatic fertilizers interms of Rock phosphate, phosphoric acid and phosphatic fertilizer ready to use are imported in India. It accounts for a lot of foreign exchange burden to the Indian economy. Further, the phosphorus use efficiency of the crop plants is hardly 10-20 per cent. It has been estimated that India has a low grade Rock phosphate (17-25% P_2O_5) reserves to the tune of 240 m.t (Gaur, 1990). However, it is not suited for fertilizer production (Anon, 1994). It is estimated that if this low grade rock phosphate is used directly it can save Rs 3000/ton of foreign exchange on phosphorus fertilizer imported.

Several phosphorus solubilizing bacteria and microorganisms have been developed and they are being used as biofertilizers to improve the phosphorus use efficiency. Since the phosphorus use efficiency is very low by the crop due to fixation of added P fertilizers into chemical from viz., iron phosphate, calcium phosphate, aluminum phosphate etc. (Larsen, 1967; Stevenson, 1986) which are insoluble and thereby not available to the crop plant, though it is present in the rhizosphere of crop plants. It has been reported that the production of organic acids like citric acid, fumaric acid, gluconic acid, lactic acid, α-ketogluatric acid by the bacteria (Duff *et al.*, 1963 ; Pareek and Gaur, 1973 ; Rao *et al.*, 1982 ; Banik and Dey, 1981a, 1982 ; Illmer and Schinner, 1992 Babukhana *et al.*, 1995) or the root exudations of the plant (Dong *et al.*, 1987) are known to solubilize the insoluble mineral phosphorus complexes and made available to the plants.

The imported high grade rock phosphate is used for production of superphosphate etc, which requires sulphuric acid. For production of sulphuric acid, sulphur is required, which is also imported since we do not have sulphur deposit in the country. These fertilizer factories are known to produce lots of pollutants in and around these production units.

In view of the above to over come the above problems constant attempt is made to improve the phosphorus use efficiency by developing efficient biofertilizer and use the crop variety which is able to grow in the P deficient soils.

In year 1989, Lynda *et al.* isolated a gene *Citrate synthase* from the soil bacterium *Psedomonas aeruginosa* which was responsible for the production of citric acid and it was introduced to the tobacco plants by De la Fuente *et al.* (1997) and Jose *et al.* (2000). They were able to show that these transgenic tobacco plants were able to grow in the presence of toxic amount of aluminum and other heavy metal. It has been found that the citric acid in the root zones of the plant by expression of the transgenic plant were able to chelate the toxic heavy metal and there by able to protect plant from toxic metal at the same time it was also observed that in such soils the availability of P is increased for the plant uptake.

Groundnut regeneration through somatic embryogenesis was already standardized by many workers (Venkatachalam *et al.*, 2000 ; Ramanjini Gowda *et al.*, 2001). Based on this available information regeneration protocol through somatic embryogenesis for groundnut cultivar JL- 24 was further standardised.

We have found that the dark incubation plays an important role in the callus formation. Zygotic embryo kept on the medium supplied with 15 mg/l 2,4-D and kept for 12 days dark incubation showed the best result in terms of size of calli obtained (Plate 2).

Twelve day old calli were cultured on the MS medium containing 15 mg/l 2,4-D with different concentration of TDZ to study the effect of TDZ on callus growth and somatic embryogenesis. It was found that the calli which were kept on the medium containing 15 mg/l 2,4-D with 0.75 mg/l TDZ showed better callus growth and somatic embryo formation than those calli which were allowed to grow only on 2,4-D medium (Table 3 & Plate 4). In addition to callus growth we also found better results interms of number of somatic embryos formed and the number of days taken for somatic embryo formation from the treatments containing TDZ along with 15mg/l 2,4-D. Calli kept on the medium containing 15 mg/l 2,4-D and 0.75 mg/l TDZ showed early embryo development and more number of somatic embryos formation compared to the plant supplied only with the15 mg/l 2,4-D. Here the concentration of TDZ at even very low concentration 0.2 mg/l with 15 mg/l 2,4-D was able to improve the somatic embryogenesis in groundnut.

Though the gene construct in the present study used by us had both *npt-II* and *hpt-I* as selection marker genes, we used hygromycin for the selection of transformed plants, because it has been reported to be more toxic to plants at very low concentration. Only 3 mg/l of hygromycin was sufficient enough to kill tomato explant (Jayalakshmi, 2001). Hence, the experiment was carried out to find out the concentration of hygromycin at which untransformed calli would be killed. From the studies carried out on this account, the groundnut cv. JL- 24 used in this investigation was highly sensitive to the hygromycin concentration of 25 mg/l. So, this concentration was chosen for the selection of transformed plants.

During the transformation study through somatic embryogenesis as a regeneration system, we have standardized different parameters *viz.*, time for which calli where dipped in the bacterial culture, cocultivation period and acetosyringone concentration.

We found that calli when immersed in the bacterial suspension that read 1 OD at 660nm wave length, for 8 minutes followed by three days cocultivation and kept on the MS medium with cefotaxime (350 mg/l) were healthy even after seven days of cocultivation (Plate 7 & Table-4).

During transformation study we found that *Agrobacterium* does not infect the groundnut without acetosyringone. To improve the transformation event 150 µM of acetosyringone was added (Table-6). At this concentration, we observed that out of a total cocultivated calli, which were kept on selection medium (25 mg/l hygromycin), 44 per cent were found to survive at the seventh day after cocultivation. Similar results were also reported by Cheng *et al.* (1996).

So, for the cocultivation, calli were immersed in the bacterial suspension of 1 OD at 660nm wave length with 150 µM of acetosyringone for 8 minutes and then cocultivated for three days.

Following somatic embryogenesis regeneration technique, we found that large number of cocultivated calli was surviving on the selection medium (Plate 8) containing 25 mg/l hygromycin. However, most of these did not regenerate or showed abnormal growth . We were able to produce only one plant (Plate 9), which was healthy and well rooted out of several such surviving calli. Later this plant was hardened. This results shows that groundnut is highly recalcitrant to *Agrobacterium* mediated transformation through somatic embryogenesis. Lacrote *et al.* (1991) and Franklin *et al.* (1993) reported earlier similar results.

Because somatic embryogenesis did not respond well through *Agrobacterium* mediated transformation technique we used another method namely (*In planta* transformation) half seed method for the transformation of the

groundnut with *Citrate synthase* gene. In this method no tissue culture step was involved (Rohini and Sankara Rao, 2001; Mc Kently *et al.*, 1993).

This method is easy because regeneration of the plant does not involve the tissue culture step. Another advantage of this method is that problem associated with somoclonal variation in tissue culture can be also overcome. Large number of plants were produced (Plate 12) and analyzed for presence of *Citrate synthase* gene using *npt-II* primers. PCR result shows the presence of the gene in most of these plants (more than 50%), confirming they are transgenic (Fig. 2).

PCR analysis of plant obtained through somatic embryogenesis confirmed it as transgenic. This is only fourth report where, transgenic plants were produced through somatic embrygenesis. In all the previous studies reported (Venkatachalam *et al.*, 2000; Krishnamurthy, 2000; Sowmya, 2001), they used kanamycin as a selection marker. We are the first to develop transgenic groundnut plant through somatic embryogenesis using hygromycin (*hpt-I* gene) as a selection marker. This is the first attempt to develop transgenic groundnut plant with *Citrate synthase* gene.

The half seed method although found highly efficient in producing the transformed plants, further studies are necessary to evaluate whether the integrated gene is inherited to next generation normally and stably, since it is likely to form chimera unlike the plants derived from single transformed cell as somatic embryo.

The attempt made in this investigation is for the first time with *Citrate synthase* gene. The usefulness of the technique and the improvement if any in the transformed plant in solubilization of insoluble **P** and **P** use efficiency needs to be studied further.

Further, improvement in the regeneration protocol through somatic embryogenesis of the transformed calli is necessary with different medium combination to obtain more somatic embryo with efficient generation into complete plant.

SUMMARY

VI SUMMARY

Making food supply to growing population is a challenge for this century. One of the major constraints in crop production is low uptake of phosphorous by plants because it is highly reactive and forms complexes, which are not available to plant. There is large foreign exchange drain on account of import of **P** sources to the country.

Groundnut (*Arachis hypogaea* L.) is an important oilseed crop grown in arid and semiarid regions where the phosphorous fixation is a major problem. If by any means phosphorous availability is increased production of this crop can also be increased.

As a solution to one of the existing problems of phosphorous deficiency, *Citrate synthase* gene from the *Pseudomonas aeruginosa* was used for the development of the transgenic groundnut plants. Over expression of this gene in plant will enhance the efflux of citric acid in the rhizosphere, which helps in the solubilization of insoluble **P** and thereby its uptake by plants.

Keeping the above thing in mind present investigation was carried out with the objective of developing transgenic groundnut plant and to confirm the presence of the gene using PCR technique.

The regeneration protocol for groundnut cv. JL-24 via somatic embryogenesis was further improved over previously reported protocols. Dark incubation of the zygotic embryo explant for 12 days gave good response for callus production. When these calli were shifted to another medium containing 15 mg/l 2,4-D and varying concentration of TDZ (0.2, 0.5, 0.75 and 1.0 mg/l), we found that the presence of TDZ in the medium even as low as 0.2 mg/l was able to enhance the callus production and somatic embryogenesis.

The best result in terms of callus growth, number of days taken for somatic embryo formation and number of somatic embryos produced per explant was observed when the calli were cultured on 0.75 mg/l TDZ and 15 mg/l 2,4-D under light condition. This standardized protocol was used for transformation of *Citrate synthase* gene using *Agrobacterium* strain 105. Different parameters like cocultivation period and acetosyringone concentration were standardized for better transformation efficiency. Using somatic embryogenesis we could produce one transgenic plant, which was confirmed by PCR.

We also tried another method (*In planta*), which was tissue culture independent for transformation study. This method was found more efficient than somatic embryogenesis. PCR analysis of the putative transgenic plants confirmed that more than 50 percent plants were transgenic. These plants have to be analyzed for further level of confirmation for integration of gene and their evaluation for increase production of citric acid in the rhizosphere of the plant.

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*Originals not seen.

APPENDICES

VIII APPENDIX

REAGENTS AND BUFFERS

Phenol: chloroform: Isoamylalcohol, (25:24:1)

A mixture consisting of equal parts of equilibrated phenol and chloroform : Isoamylalcohol (25:24:1) was used to remove proteins and lipids from preparations of nucleic acids. Isoamylalcohol was used to reduce foaming during extraction.

Neither chloroform nor isoamylalcohol required treatment before use. The phenol : chloroform : isoamylalcohol mixture was stored in a brown bottle at 4^0C.

Preparation of commonly used stock solution:

1. 0.5 M EDTA (pH 8.0): 186.11gm of disodium ethylenediaminotetra acetate was added to 800ml of H_20. Stirred vigorously on magnetic stirrer. The pH was adjusted to 8.0 with NaOH (20gm of NaOH pellets) and dispense into aliquots and sterilized by autoclaving.

2. **Ethidium Bromide:** 1gm of eithidium bromide was added to 100ml of H_20. Stirred on a magnetic stirrer for several hours to ensure that the dye had dissolved. The container was wrapped with aluminium foil or the solution was stored in a brown bottle at room temperature.

3. **3M Sodium Acetate:** 408.1gms of sodium acetate $3H_20$ was dissolved in 800ml of H_20, the pH was adjusted to 5.2 with glacial acetic acid or the pH was adjusted to 7.0 with dilute acetic

acid. The volume was adjusted to 1 litre with water, dispensed into aliquots and sterilized by autoclaving.

4. 5M NaCl: 292.7gms of NaCl was dissolved in 800ml of water, adjusted the volume to 1litre with water, dispensed in aliquots and sterilised by autoclaving.

5. 10% SDS: 100gms of electrophoresis grade SDS was dissolved in 900ml of H_20, heated to 68^0C to assist dissolution. Adjusted pH to 7.2 by adding few drops of HCl (concentrated), adjusted the volume to 1 litre.

6. 1M Tris. 121.1gms of Tris-base was dissolved in 800ml of H20, adjusted the pH to the desired value by adding concentrated HCl. Allowed the solution to cool to room temperature before making final adjustment in pH, adjusted the volume to 1 litre, sterilized by autoclaving.

Composition of the Murashige and Skoog's (MS) media (1962)

Ingredients Inorganic salt	Salt concentration in stock solution (g L^{-1})	Group	Aliquot taken for one litre of medium (ml)	Final Concentration of Salt in 1 litre of Culture medium (mg L^{-1})
NH_4NO_3	66.00	I	25	1650.00
KNO_3	76.00			1900.00
$MgSO_4.7H_2O$	14.60			370.00
KH_2PO_4	6.80			170.00
$CaCl_2.2H_2O$	4.40	II	10	440.00
Na_2EDTA	7.45	III	5	37.20
$FeSO_4.7H_2O$	5.57			27.20
H_3BO_3	0.62	IV	10	6.20
$ZnSO_4.4H_2O$	0.66			8.60
$MnSO_4.4H_2O$	2.25			22.30
KI	0.083			0.83
$Na_2MoO_4.2H_2O$	0.025			0.25
$CuSO_4.5H_2O$	0.0025			0.025
$CoCl_2.6H_2O$	0.0025			0.025
Organic salt:				
Glycine	0.200	V	10	2.0
Myo-inositol	10.000			100.0
Thiamine HCl	0.010			0.10
Pyridoxine HCl	0.050	V	10	0.50
Nicotinic acid	0.050			0.50
Sucrose				30000 mg L^{-1}
Gelrite				2000 mg L^{-1}
PH				5.8