A REVIEW ON PROTOCOLS FOR IN VITRO REGENERATION OF PLANTS

S.B SADAU*

Sani, I*

ABSTRACT

In the present review, an attempt has been made to set up protocols for *in vitro* regeneration of plant from young leaves, seedlings as explant source. For *in vitro* callus inductions auxins such as IAA, NAA and 2, 4-D in combination with cytokines like BAP and KN areused. Awell-developed greenish plant is obtained on MS medium when supplemented with BAP and NAA.The maximum number of shoot bud regeneration (14.0 shoots /explant) is observed on MS medium containing 5.0mg/l BAP, ofthe two cytokines used, BAP is found to be best for shoot bud regeneration. For root induction, elongated shoots were transferred to MS medium supplemented with various concentrations of (0.5-2.0 mg/l) IAA, IBA and NAA and in combination with 0.5 mg/l BAP and KIN on half strength MS medium. Among the different concentrations of auxin tested NAA was induced maximum percent of rooting (100%). The maximum number of roots (9.0 roots/shoot) was observed on MS medium supplemented with 0.5 mg/l NAA. Rooted plantlets were transferred into soil and sand in the ratio (2:1) and subsequently, survival plants were acclimatized in the field.

Keywords:*in vitro* culture, plant regeneration, callus induction, multiple shoots, indirect organogenesis,

* Department of Science and Technology Education, Department of Plant Biology, Bayero University, Kano, NIGERIA.

INTRODUCTION

Plant tissue culture has been identified as an excellent surrogate method to overcome the problems connected with utilization and conservation of plant (Bajaj *et al.*, 1998). Micro propagation is a substitute method to the conventional methods of vegetative propagation with the unbiased of improving the rate of multiplication (Kaur, 1998). For increase in production and productivity plant tissue culture has been noticed as an important technology for enhancing the competence of selected best high yield varieties.

Plant tissue culture is a collection of techniques used to maintain or grow plant cells, tissues or organs under sterile conditions on a nutrient culture medium of known composition. Plant tissue culture is widely used to produce clones of a plant in a method known as Micropropagation. Tissue cultures are started from pieces of whole plants. The small organs orpieces of tissue that are used are called explants. Explants can therefore be consisting of many different kinds. Plant grow in external environment are easily susceptible to many diseases. The correct choice of explant material can have an important effect on the success of tissue culture.When cultured *in vitro*, all the needs of the plant cells, both chemical and physical, have to meet by the culture vessel, the growth medium, and the external environment (light, temperature, etc.).

A callus culture system offers many advantages as a model system for several biological investigations. Callus cultures have been used widely in various physiological and related studies in plants (Weinstein *et al*., 1962) and in the citrus(Altman *et al.*, 1982).Even callus has proved better for synthesis of alkaloids in several cases (Bhat, 1995).

The growth medium has to supply all the essential mineral ions required for growth and development. In many cases (as the biosynthetic capability of cells cultured *in vitro* may notreplicate that of the parent Plant), it must also supplyadditional organic supplements such as amino acids and vitamins. Many plant cell cultures, as they are not photosynthetic, also require the addition of a fixed carbon source in the form of a sugar (most often sucrose). One other vital component that must also be supplied is water, the principal biological solvent. Physical factors, such as temperature, pH, the gaseous environment, light (quality and duration), and osmotic pressure, also have to be maintained within acceptable limits

Types of *In vitro* Culture

- Culture of intact plants (seed and seedling culture)
- Embryo culture (immature embryo culture)

- Organ culture
  - Shoot tip culture
  - Root culture
  - Leaf culture
  - Anther culture.
- Callus culture.
- Cell suspension culture.
- Protoplast culture**.**

Three Fundamental Abilities ofPlants

- **Totipotency:** the potential or inherent capacity of a plant cell to develop into anentire plant if suitably stimulated.It implies that all the information necessary for growth andreproduction of the organism is contained in the cell
- **Dedifferentiation:** Capacity of mature cells to return to meristematic condition anddevelopment of a new growing point, follow by redifferentiation whichis the ability to reorganize into new organ
- **Competency:** the endogenous potential of a given cells or tissue to develop in aparticular way.

Factors Affecting Plant TissueCulture

Growth Media:

- Minerals, Growth factors, Carbon source, Hormones

Environmental Factors:

- Light, Temperature, Photoperiod, Sterility, Media

Explant Source:

- Usually, the younger, less differentiated explant, the better fortissue culture
- Different species show differences in amenability to tissue culture
- In many cases, different genotypes within a species will havevariable responses to tissue culture; response to somaticembryogenesis has been transferred between melon cultivarsthrough sexual hybridization

STEPS INVOLVED IN *IN VITRO* REGENERATION OF PLANTS.

Plant material:

The explant source can be from leaves, root, and shoot

Explants preparation:

Plant material must first be surface sterilized to remove any bacteria or fungal spores that are present. This is aimed to kill all microorganisms, but at the same time not cause any adverse damage to the plant material.

- The explant should be cut into small sections of florets about 1 cm across. If using a rose or other cuttings, cut the shoots into about 5 to 7 cm lengths. Whole African violet leaves can also be used.
- Wash the prepared plant material in a detergent-water mixture for about 20 minutes. If trying hairy plant material scrub with a soft brush (toothbrush). This will help remove fungi etc., and the detergent will help wet the material and remove air bubbles that may be trapped between tiny hairs on a plant.
- Transfer the washed plant material to the sterilizing chlorox solution. Shake the mixture for 1 minute and then leave to soak for 10-20 minutes. Carefully pour off the bleach solution using the lid to keep the plant tissue from coming out and then carefully cap the container.

Culture medium:

All media used for the present study were based on Murashige and Skoog (MS) [16] medium.

Murashige Minimal Organic Medium recipe (MMOM)

Inorganic salts	**mg/L**
NH_4NO_3	1,650.00
KNO_3	1,900.00
$CaCl_2$ (anhydrous)	332.20
$MgSO_4$ (anhydrous)	180.70
KH_2PO_4	170.00
Na_2EDTA	37.25
$FeSO_4.7H_2O$	27.80

H_3BO_3	6.20
MnSO4.H2O	16.90
$ZnSO_4.H_2O$	5.37
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
CuSO4 (anhydrous)	0.016
CoCl2 (anhydrous)	0.014
Sucrose	30,000.00
i-Inositol	100.00

Thiamine. Hcl 0.40

The pH is adjusted to 5.7 using 0.1 M Hcl or NaOH.

Basis for Plant Tissue Culture:

- Two Hormones Affect Plant Differentiation:
- Auxin: Stimulates Root Development.
- Cytokinin: Stimulates Shoot Development.
- Generally, the ratio of these two hormones can determine plant development:
- High auxin conc. and low cytokinin conc. =Root development.
- High cytokinin conc. And low auxin conc. =Shoot development.

Multiple shoots initiation:

Explants from 7-day- old seedlings ,young leaves were cultured on MS medium fortified with different concentration of KIN and BAP (0.5-5.0 mg/l) and/or in combination with 0.5 mg/l NAA. The cotyledonary parts of the explants elongated visibly after 4 day ofculture on MS mediumsupplemented with BAP and KIN. The frequency of shoot regeneration in KIN was relatively low and there were fewer shoots. Multiple shoots observed in all concentration of BAP used, but better regenerationresponse with maximum number of shoots 14.0 shoots/explants were produced at 5.0 mg/l BAP .The important role of BAP for shoot bud differentiation in legumes has been reported previously by Gulati and Jaiwal. Among the BAP concentration with NAA tested, the maximum number of multiple shoot was noticed on MS medium containing 5.0 mg/l BAP + 0.5 mg/l NAA (Fig. 1).The result of this study indicates that the addition of NAA

increased the frequency of shoot bud regeneration and root formations are consistent with results of Venkatachalamet.al., (1994). The percentage of shoot bud differentiation and the mean number of shoots per culture increased at higher concentration of cytokines in combination with auxin (Palanivel. *Et al.*, 2002).The cytokinin BAP has been commonly used for shoot bud initiation in legumes. The involvement of cytokinins especially in shoot bud formation has been reported in many plant species. The combinations of auxins and cytokinins at definite proportions are very critical and found to be essential for the induction of shoot root in many species (Venkatachalam.*et al.*, 1998).

Indirect shoot organogenesis from nodal derived callus:

Induction of callus was essential for the vegetative plant proliferation.Organogenic callus was observed on nodal explants only. Callus initiation was attained 10 days after incubation on MS basal medium with any one of the auxins like NAA, IAA and in combination with either cytokines (BAP or Kn).

Plant propagation through callus required the induction or organogenic callus and it is the prerequisite for adventitious shoot formation and also other in vitro genetic improvement including induction of somaclonal variations and embryoids.The presence of cytokines along with auxin is necessary for indirect shot induction was noted(Skoog and Miller, 1957).

***In vitro* flowering and fruiting:**

*Invitro*flowering was induced on SIM9, SIM10, SIM13 and SIM 14 after six weeks of culture followed by *in vitro* rooting. Whereas floral buds formation was observed within four weeks of culture in Tim (300mg/L) + SIM 14 medium (Fig 2C). However on SIM14 with Tim an early flowering was induced from regenerated shoots within one week after the change of cultures from SIM14 to SIM14+Tim 300mg/l medium. This shows the positive effect of Tim on early flowering. More number of floral buds / shoot and maximum frequency of plants showed flowering on 300 mg/L Tim + SIM 14. The flowers were self-fertilized and formed mature fruits (Fig 2D). Furthermore, less pollen formation and low fertility status are other serious hurdles in this endeavor. *Invitro* fertilization is a feasible proposition to overcome this incompatibility barrier. It also enables the production of precious hybrid seeds in off-season too (Sheeja and

Mandal, 2003). Hence *in vitro* flowering and fruiting may significantly contribute to the genetic improvement of tomato Micro-MsK.

Root induction:

For rooting, elongated shoots were cultured on MS medium supplemented with various concentration of (0.5-2.0 mg/l) IBA, IAA and NAA among the different concentration of auxin tested NAA was found to be most effective in inducing roots. The highest frequency of root initiation 100% was noticed at 0.5 mg/l with maximum root number (9.0 roots/explants). Higher concentration of NAA produced callusing at the cut end of shoots kept for rooting. In contrast, Venkatachalam *et al.,* (1998) had used a medium plus NAA or IBA to induce rooting. Asyllin-ozudogru *et al.,* (2005) reported that Virginia-type peanut shoots have a natural capacity to form adventitious roots in medium without plantgrowth regulators.

Hardening and Acclimatization:

Subsequently well-developed rooted plantlets were gently removed from the culture tubes, washed initially to remove adhered agar and traces of the medium to avoid contamination. Then they were transferred to plastic cupscontaining a mixture of red soil and sand in the ratio 1:2. Polyethylene bags were covered over the plants to ensure high humidity during first few days and watered regularly. Then they were transferred to the green house and finally acclimatized to the field.

a

b

c **d**

Figure 1.Plant regeneration from peanut.**a**. Multiple shootinduction **b**. Shoot elongation, **c.** Rooting and **d**. Plantlet is growingin plastic cup.

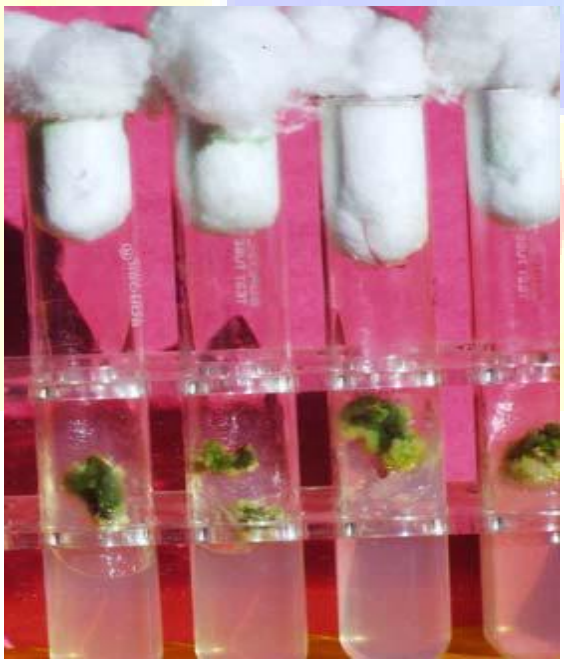


Fig.2

Fig.3

Fig.2. Callus induction in *R. sepentina*from leaf explants in MS media containing BAP(1.0) and IAA(0.5)mg/l. Shoot regeneration from callus on BAP (2.5) + IAA (0.4) mg/l MS media.

Fig.3. Rooting regeneration on BAP (2.5) + IAA (0.5) + NAA (0.5) mg/l MS media.

APPLICATIONS OF PLANT *IN VITRO* REGENERATION

Developing Sterile Cultivars: One focus of our research is to develop improved, non-invasive, seedless nursery crops. One of the most effective means for developing seedless plants is to create triploids (plants with three sets of chromosomes)(Ranney, 2004). While triploids grow normally, they are unable to divide equallyduring meiosis and typically fail to produce viable gametes. Triploids have been developedfor many food crops including watermelon, bananas and grapes. Triploidscan be bred by hybridizing tetraploids with diploids.

In North Carolina, miscanthus has escaped cultivation and naturalized in manyareas. We have recently utilized tissue culture to develop a series of triploid miscanthus.Somatic embryogenesis was induced from callus derived from seed orshoot apices. Callus was maintained in the dark and subcultured to fresh mediaevery 4 weeks. To induce polyploidy, callus was treated with the

mitotic inhibitororyzalin that resulted in approximately 60% of the regenerated plants being tetraploids.Tetraploids were transferred to the glass house and hybridized with diploidsto create triploids.A common problem associated with the successful development of sterile triploidsis a phenomenon referred to as "triploid block," generally thought to result fromimbalances in the ploidy levels between the embryo and endosperm. Triploid blocksare common in interploid crosses and typically results in embryo abortion. For miscanthus,embryo rescue techniques were required to overcome this phenomenon.Immature embryos were rescued from aborting seed using a microscope and germinatedin vitro. Recovery rates remained low with approximately 600 plantlets beingobtained from over 10,000 seed to date.

Wide Crosses: Wide crosses refer to hybridizations between species of the sameor different genera. Wide crosses are useful in crop improvement as they allowfor recombination of diverse genes and traits. However, post fertilization barriersresulting from genome incompatibility and ploidy levels often make hybridizationsdifficult. Similar to interploid crosses, seed from wide crosses often fail to developand abort. Embryo rescue techniques (i.e., rescuing an embryo from an abortingseed) and ovule culture can be used to recover progeny from wide crosses. Wehave used this technique in developing wide crosses between *Derivilla*and *Weigela*(Touchell et al., 2006).Progeny from wide crosses may also be sterile due to uneven chromosome paringleading to meiotic failure. Chromosome doubling can help in restoring fertilityto wide hybrids by providing an exact homologous duplicate of each chromosomethat can pair together during mitosis. Tissue culture procedures, particularly somatic embryogenesis, provide a mechanism to induce and recover polyploids. Forexample, *Rhododendron* 'Fragrantissimum Improved' is a sterile wide hybrid. Wehave developed a protocol to induce embryogenesis from leaves using a combinationof the cytokininthidiazuron (TDZ) and an auxin indole acetic acid (IAA). Embryogeniccultures were treated with oryzalin at different concentration and durationsand will be assessed for ploidy.

Polyploid Induction: The development of new polyploid, through chromosomedoubling, may increase ornamental characteristics, expand breeding opportunities,and restore fertility in sterile hybrids — ultimately leading to the development ofimproved cultivars (Contreras, et al., 2007; Olsen et al., 2006a,b; Ranney, 2006).Polyploid induction has been employed to improve ornamental characteristics andfacilitate breeding programs for a wide range of plant taxa (Allum et al., 2007;Dunn and Lindstrom, 2007).*Rudbeckia*is one genus in which we are exploring the

effect of polyploidy on ornamentalcharacteristics. Embryogenic systems were used to establish clonal linesin tissue culture. In-vitro treatments, ranging from 15 to 60 µM oryzalin over 3 to 5days, were effective at inducing polyploidy, depending on taxa. New tetraploids of*R. maxima, R. subtomentosa,* and a novel interspecific hybrid were successfully developedand will be evaluated for ornamental characteristics (Palmer et al., 2008).

Stabilizing Chimeras: Chimera's occur when a plant or part of a plant is composedof genetically different layers. Chimeras often result in leaf variegations. Assomatic embryogenesis offers a unique capability of regenerating a plant from asingle cell, it is possible to regenerate plantlets with different genetic compositionsfrom different layers of a single leaf or flower.*Rhododendron* 'Little John' has a leaf variegation resulting from an apparentmutation causing the L1 layer (outer layer) to produce red pigments. Embryogenicprocedures have allowed for the development of both red and green plants to beinitiated from the same leaf.

Mutation Breeding: Induced mutations using irradiation or chemical mutagensis another advance in biotechnology that may have potential benefits for theproduction of sterile plants and novel forms (dwarfs) and foliage types (variegation).Mutation breeding is also beneficial to increase variability in species withlow genetic diversity such as *Hypericumfrondosum*. Gamma irradiation has beenused for several decades for whole plants and seed; however, more recently theprocedures have been used to induce mutations in tissue cultures (Ahloowalia andMaluszynski, 2001; Charbaji and Nabulsi, 1999). It is particularly desirable to treatcallus cultures and to regenerate plants from single cell lines to eliminate chimera tissue. In our laboratory we have developed embryogenic systems for *Hypericum*and investigated the effects of gamma irradiation and chemical mutagens.

LIMITATION OF IN VITRO REGENERATION

While plant tissue culture provides powerful mechanisms to assist inbreeding programs, there are several problems to overcome. Regeneration systemshave proven difficult for many woody plants and only a small percentage of ornamentalspecies have successfully been introduced into tissue culture. Comprehensive researchneeds to be conducted on a species-by-species manner to optimize regenerationprotocols that can be applied to a breeding program. Further, some species are recalcitrantto tissue culture and may be better suited to conventional propagation protocols.

CONCLUSIONS

In the present study on protocols for in vitro regeneration of plants from plant parts such as leaves, shoots, as explant source, we can achieve a simple, clear, and reliable protocol for large scale production.This protocol could be useful for production of high frequency of transformants in plants using recombinant DNA technology in the near future.

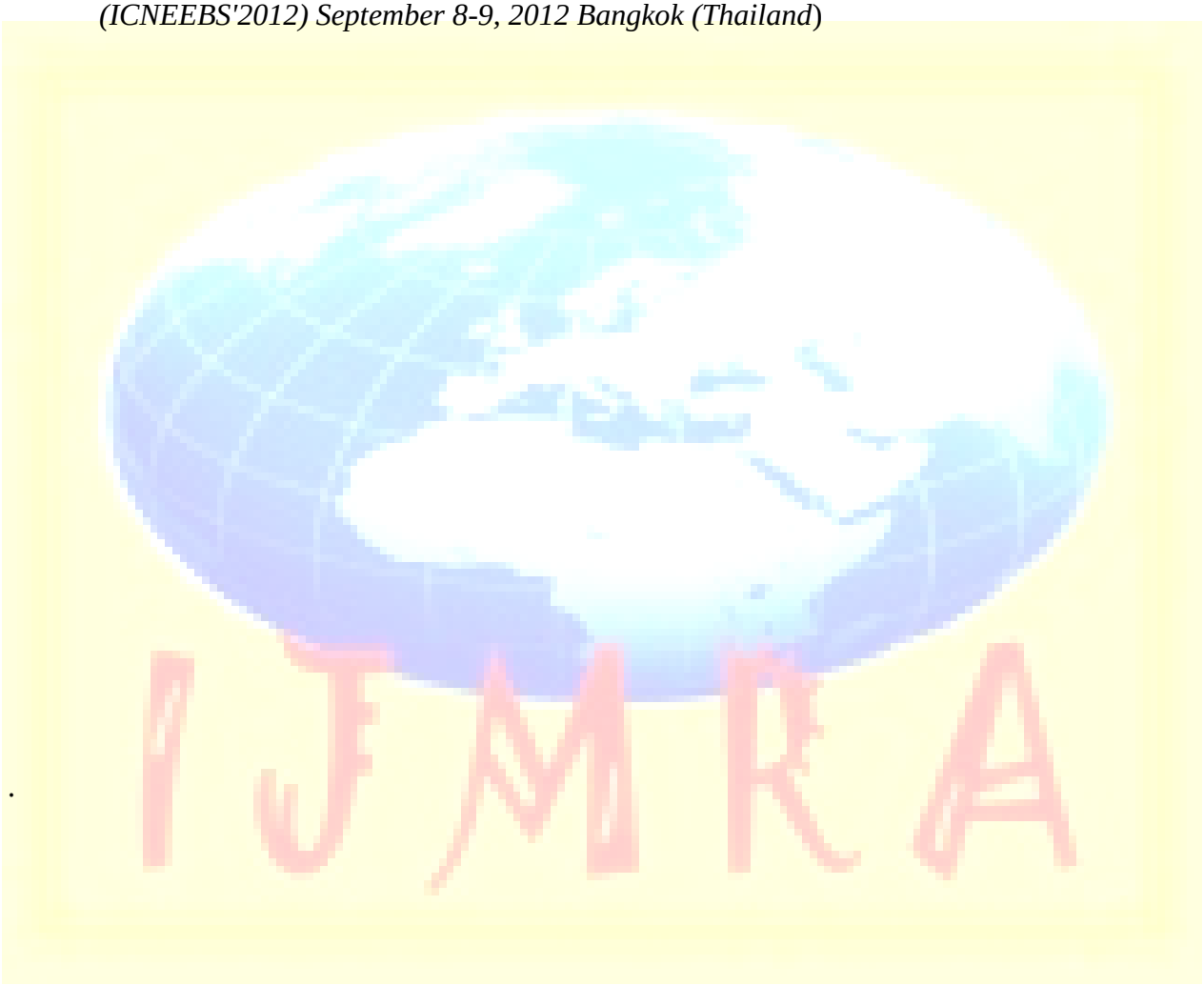
REFERENCES

1. Agastya, P.., Lincy Williams, Ignacimuthu,S. 2006 .*In vitro propagation of Justicia gendarussa.*
2. Ahloowalia, B.S., and M. Maluszynski. 2001. Induced mutations — A new paradigm inPlant breeding. Euphytica 118:167–173.
3. Asyllin-Ozudogru, E., Ozden-Tokatli, Y. and Akcin, A. 2005. Effect of silver nitrate on multiple shoot formation of Virginia-type peanut through shoot tip culture. *In vitro*Cell Dev. Biol. Plant. 41: 151–156.
4. Burm F. –A medicinal plant. *Indian journal of biotechnology*,**5**: 246-248.
5. Bhatia P, Ashwath N, Senaratna T, David M (2004) Tissue culture studies of tomato (*Lycopersiconesculentum*) Plant Cell, Tissue and Organ Culture78: 1–21, 2004.
6. Charriere, F., Sotta, B., Miginiac, E. and Hahne, G. (1999). Induction of adventitious shoots or somatic embryos on *invitro*culture. *Plant Physiol.Biochem*. **37(**10):752-757.
7. Chaudhury, A. and Qu, R. (2000). Somatic embryogenesis and plant regeneration of truf-type Bermuda grass:effectof 6-Benzaldehyde in callus induction medium. *Plant cell tiss. Org.Cult*ure**60**:113-120.
8. Charbaji, T., and I. Nabulsi. 1999. Effect of low doses of gamma irradiation on in vitroGrowth of grapevine. Plant Cell, Tissue and Organ Cult. 57:129–132.
9. Contreras, R.N., T.G. Ranney, and S.P. Tallury. 2007. Reproductive behavior of diploid Andallotetraploid*Rhododendron* L. 'Frangrant Affinity'. Hort. Science 42(1):31–34.
10. Cucco, M., Rossi, A.2000 Protocol for regeneration *in vitro* of *Arachishypogaea*L. EJB Electron J. Biotechnol. 3(2): 154–160.
11. Deore AC & Johnson TS, Plant Biotechnol. Rep, **2** (2008)

12. Dunn, B.L., and J.T. Lindstrom. 2007. Oryzalin-induced chromosome doubling in *Buddleja*to facilitate interspecific hybridization. Hort. Science: 42(6):1326–1328.
13. Gulati, A. and Jaiwal, P. K. 1994.culture conditions affecting plant regeneration fromcotyledons of *Vignaradiate*(L.) Wilczek. Plant Cell Tiss. Org. Cul. 23: 1-7.
14. Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant 15:473-497.
15. Mahadev D, Chandra Panathula, Naidu C. V. 2014. *Efficient protocol for in vitro induction and direct plant regeneration of Solaniumviarum (Dunal) _.int J.Med. Arom .Plants.4(2) 1-2*
16. Olsen, R.T., T.G. Ranney, and Z. Viloria. 2006a. Reproductive behavior of induced allotetraploid $X*Chitalpa*and in vitro embryo culture of polyploid progeny. J. Amer. Soc. Hort. Sci. 131(6):716–724.
17. Olsen, R.T., T.G. Ranney, and D.J. Werner. 2006b. Fertility and inheritance of variegated
18. and purple foliage across polyploid series in *Hypericumandrosaemum*L. J. Amer. Soc. Hort. Sci. 131(6):725–730.
19. Palanivel, S., Parvathi, S. and Jayabalan, N. 2002. Callus Induction and Plantlet Regeneration from Mature Cotyledonary Segments of Groundnut *(Arachishypogeal*.). J. Plant Bio. 45(1): 22-27.
20. Palmer, I.E., D.H. Touchell, and T.G. Ranney. 2008. In-vitro polyploid induction of *Rudbeckia*spp. Proc. SNA Research Conference. In pres.
21. Ranney, T.G. 2004. Population control: Developing non-invasive nursery crops. Comb.
22. Proc. Intl. Plant Prop. Soc. 54:604–607.
23. Ranney, T.G. 2006. Polyploidy: From evolution to new plant development. Comb. Proc.
24. Intl. Plant Prop. Soc. 56:137–14.
25. Sheeja TE, Mandal AB (2003) In vitro flowering and fruiting in tomato (*Lycopersiconesculentum)*Mill.Asia Pacific Journal of Molecular Biology and Biotechnology 11 (1): 37-42.
26. Sujatha M &Mukta N, Plant Cell Tiss Org Cult, 44 (1996) 135
27. Sujatha M, Makkar HPS & Becker **K**, Plant Growth Reg, **47** (2005) 83.
28. Tallury, S. P., Hilu, K. W., Milla, S. R., Friend, S. A., Alsaghir, M., Stalker, H. T. and Quandt, D. 2005. Genomic affinities in *Arachis*section *Arachis*(Fabaceae): molecular and cytogenetic evidence. Theor. Appl. Genet. 111: 1229–1237.

29. Venkatachalam, P., Geetha, N. and Jayabalan, N. 1998. Influence of growth regulators on plant regeneration from epicotyl and hypocotyl cultures of two groundnut (*Arachishypogaea*L.) cultivars. J. Plant Biol. 41(1): 1-8.

30. Venkatachalam P.., Kavipriya V..,(2012) *Efficient Method for In Vitro Plant Regeneration from Cotyledonary Node Explants of Peanut (ArachishypogaeaL.) International Conference on Nuclear Energy, Environmental and Biological Sciences (ICNEEBS'2012) September 8-9, 2012 Bangkok (Thailand*)



.