IN VITRO REGENERATION OF *THEVETIA PERUVIANA* PERS.K.SCHUM, FAMILY APOCYNACEAE

P. O. Odhiambo[1], M. Makobe[2], H. Boag, A. Muigai[2] and H. Kiesecker[3]
[1]Masinde Muliro University of Science and Technology, Kakamega, Kenya
[2]Jomo Kenyatta University of Agriculture and Technology
[3]DSMZ
E mail: pissoonyango2@yahoo.com

Abstract
The objective of this study was to develop and optimize *in vitro* protocol for regeneration of soma clonal variants of *T.peruviana* aimed at obtaining glycoside free or low glycoside plantlets with high oil and proteins content through tissue culture technique in order to develop new varieties which has novel traits compared to the existing landraces. *T.peruviana* seedlings germinated from mature fruits of orange flowering variety of *T. peruviana* trees were used. Young but enlarged leaves next to the shoot tip were excised as source of explant. Callus initiation media consisted of MS salts, vitamins plus 8 g / l agar agar, 30 g / l sucrose, 2.0 mg / l dichlorophenoxyacetic acid (2,4-D) supplemented with 0.1 mg / l of kinetin at pH of 5.75 at 22^{o}C to 24^{o}C and 12 hour darkness during the night and 12hrs lighting from fluorescent tubes during the day. Shoot regeneration medium consisted of MS media salts, vitamins, supplemented with 3 mg / l of 2 ip, 8 g / l agar agar, 30 g / l of sucrose, at pH of 5.75 were incubated at 25^{0}C-27^{0}C, with continuous lighting from florescence tubes in the growth chamber. Rooting was initiated by incubating single shoots into each media bottle containing 30 ml of MS media salts, vitamins, 1 g of glycine, 0.2 g of biotin, 3 mg/l of Indole-3- butyric acid (IBA), 8 g of agar agar and 30 g/l sucrose. It was noted that plantlets of *T.peruviana* regenerated after 12-24 months.

Key words: *Thevetia peruviana*, *in vitro* regeneration, Murashige and Skoog, callus

1.0 Introduction

Thevetia peruviana Pers.K.Schum synonym *T.nerifolia* A.Juss belongs to family Apocynaceae. The family is a major centre of interest to botanists, phyto-chemists, pharmacologists and agriculturalists since the members are well known for drugs, arrow poisons, alkaloids, rubber and as ornamentals (Van Beek *et al.*, 1984). *T. peruviana* has great potential for vegetable oil, pharmaceuticals and proteins production (Supinya *et al.,*2002,Usman *et al.,* 2009, Quilichini and Bertucat, 1956, Arora and Battacharya, 1967, Jain and Yadav, 1991).

In vitro culture of higher plants is the culture on nutrient media under sterile conditions of plants, embryos, organs, explants, tissues, cells and protoplasts of higher plants, (Pierik, 1992). Murashige and Skoog's culture medium is the most used tissue culture medium, (Murashige and Skoog, 1962). The establishment of cell cultures, the cell growth, and accumulation of secondary metabolites can be affected by the illumination conditions, the hormonal composition of the culture medium, and the organic and in organic composition of the medium itself, (Duchefa, 1998-9). Plant growth regulators in the tissue culture medium can be manipulated to induce callus formation and subsequently changed to induce embryos from the callus. Furthermore subculture of responsive embryos or embryogenic callus produces shoots or somatic embryos depending on the medium and plant growth regulators used and vary with plant type. *In vitro* culture technology has been proven to be effective in some cases for the production of secondary plant metabolites such as taxol. However little attention has so far been put on the development of *in vitro* cultures of *T.peruviana.* Most of the studies on *T.peruviana* have focussed on somatic embryogenesis rather than secondary metabolites, (Abha Sharma and Anjani Kumar, 1994 and Anjani Kumar, 1992).

In this study the main objective was to develop and optimize an *in vitro* regeneration protocol for soma clonal variants of *T.peruviana*, aimed at obtaining glycoside free or low glycoside plantlets with high oil and proteins content through tissue culture as a step towards development of new varieties with novel traits compared to the existing landraces in Kenya.

Dedifferentiation and loss of thevetin in *T. peruviana* callus was first reported in 1981. The cotyledonary callus tissue of Yellow Oleander was found to contain thevetin, a cardioactive glycoside in the early callus tissue which gradually disappeared after six months of regular sub-culturing. Modification of environmental conditions or nutrition could not induce thevetin synthesis in the old callus tissue. Slight variation at genotypic level has also been reported. Cardenolide content of different genotypes of *T. neriifolia* and *Nerium odorum* were investigated. In spite of distinct morphological differences between varieties of *T.neriifolia* and *Nerium odorum* all varieties of *T.neriifolia* were similar in their cardenolide profiles. However there was slight variation in thevetin content at varietal level in *T. neriifolia,* (S.Gopa and Datta P.C, 1981).

Development of anthoxanthins and carotenoids in *T.nerifolia* anther and ovarian tissues in vitro had been reported. The ovaries and wall layers of anthers of *T.neriifolia* proliferate into snowy callus showing rapid growth in about a fortnight. The callus tissues showed development of anthoxanthins and carotenoids. Anther tissues contained kaemferol, quercetin, carotenes, and cyaniding-3-glycosides abundantly whereas these were absent in the ovarian tissues. Lutein and anthocyanin precursor flava 3:4 diols were present in both the tissues but only ovaries developed polyoxy pigments, (Marthur *et al.,* 1976).

Biotransformation of cardenolides by cell suspension cultures in *Digitalis lanata* and *T.peruviana* had also been reported. In cell suspension cultures of *T.peruviana*, cymarin was glycosilated to k-strophanthin, peruvoside was glucosylated to a diglycoside and also reduced at the C-10 formyl group to give neriifolin, thevetin A was deglucosylated to the corresponding diglycoside and then to peruvoside, thevetin B was deglucosylated to its diglycoside and then to neriifolin, lanatoside A was deglucosylated and deacetylated to digitoxin. Similar biotransformations were reported on *D.lanata*. However neither of the two plant cultures was able to remove the digitose moiety *in vitro,* (Doeller, P.C *et al.,* 1977).

Biotransformation of cholesterol to thevetin in early callus of *T.peruviana* had also been reported. The study found out that thevetin synthesis by cotyledonary callus of *T.peruviana* on Murashige and Skoog's culture medium supplemented with cholesterol increased with increase in cholesterol concentrations up to 300mg/100mL;

however thevetin yield gradually decreased at higher concentrations. Callus growth however declined in response to cholesterol, (Gosh Gopa *et al.,* 1983).

Elicitation with methyl-jusmate has been reported to stimulate peruvoside production in cell suspension cultures of *T.peruviana*. The elicitor effect of Methyl –jusmate was reportedly studied in cell suspension cultures established in Schenk and Hildebrandt (SH) medium. The best results were obtained at a concentration of 100mg l^{-1} of Methyl jusmate applied at the beginning of the culture which induced extra cellular peruvoside production of 8.93 mg l^{-1}, (Mario Arias Zabala *et al.,* 2009).

Sharma and Kumar, 1994 regenerated plantlets from leaf derived callus of a mature *T. peruviana* plant. The callus were regenerated in MS media containing 2mg/L-2,4D plus 0.1mg/L Kinetin. The calli were sub-cultured in a lower concentration of 0.1mg/L, 2,4D supplemented with 2mg/L 2ip for embryogenesis. The developing embryos were then transferred to MS media lacking growth regulators for embryo maturation. The mature embryos were then sub-cultured into modified half strength MS media resulting into 80% regeneration of plantlets *in vitro*.

2.0 Materials and Methods

The tissue culture experiments were carried out at the Institute for Biotechnology Research (IBR) at JKUAT and DSMZ Tissue culture Laboratories in Germany.

2.1 Source of Explants

T.peruviana seeds were harvested from mature fruits of orange flowering variety of *T. peruviana* trees cultivated in an experimental plot at Jomo Kenyatta University of Agriculture and Technology (JKUAT). The seeds were planted in polythene pots measuring 5 cm wide and 10 cm high. The pots were filled with loamy soil watered once a day in the morning at 8.00 am. The pots were kept on the laboratory side bench, next to the windows and in green house conditions until germination occurred. At DSMZ yellow oleander seeds were germinated under laboratory condition set with tropical temperatures ranging from 20-30^{0}C and watering regimes similar with JKUAT.

Young but enlarged leaves next to the shoot tip were excised from the young(5-10 days old) germinated seedlings and cut into leaf discs measuring 5 mm in diameter using heat sterilized scalpel. The seedlings aged 10-14 days were used as explants source, Figure 1 below:

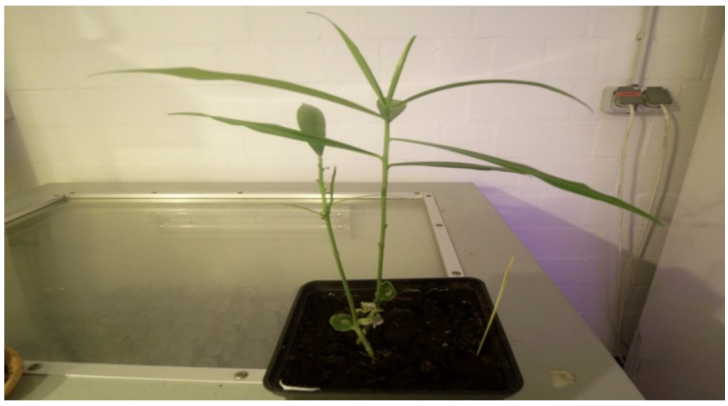

Figure 1: Young T.peruviana seedlings used source of as explants germinated at DSMZ

2.2 Preparation of the Explants for Callus Initiation and Growth

The excised leaf discs were surface sterilized by immersing in 50 ml of 70 % ethanol for 1 minute and then transferred into 50 ml of 2 % sodium hypochlorite solution for 15 minutes. The sodium hypochlorite solutions were prepared from commercial “jik” containing 3.5 % sodium hypochlorite by diluting 57 ml of the “jik” to 100 ml with distilled sterilized water.

In a lamina flow the sterilized explants were then transferred into sterilized petri dishes and rinsed thrice with distilled sterilized water. Using heat-sterilized forceps, the leaf discs were inoculated one each into each sterilized culture glass bottles measuring (15 x 5 cm) and disposable petri dishes containing 30 ml of Murashige and Skoog (MS) culture medium and the screw caps and top covers tightly closed.

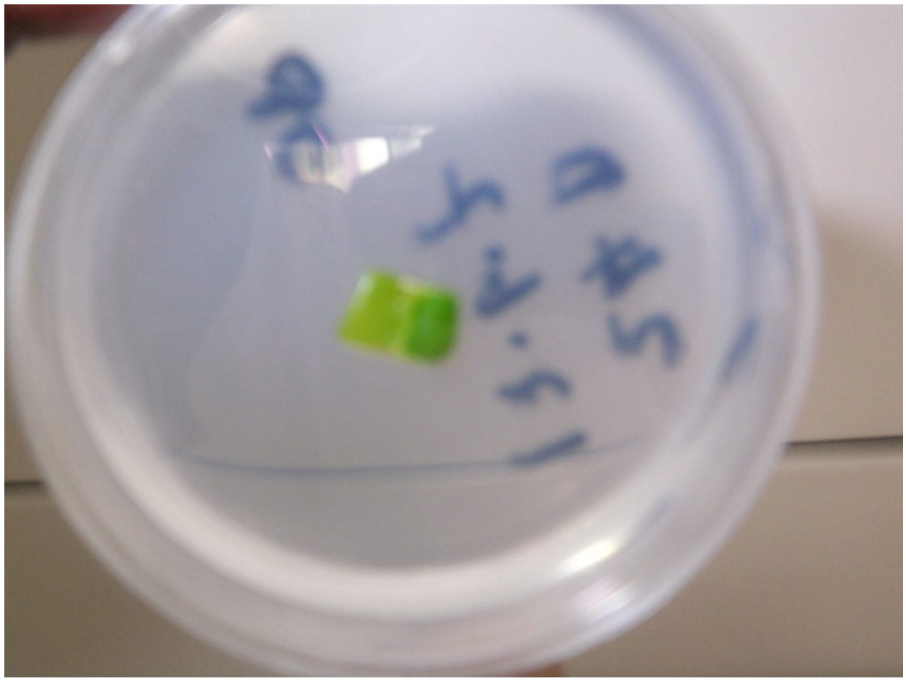

Figure 2: Leaf disc in MS media enclosed in a petri dish for callus initiation

2.3 Callus Initiation Medium

The callus initiation media consisted of MS salts and vitamins plus 8 g / l agar agar, 30 g / l sucrose, 2.0 mg / l dichlorophenoxyacetic acid (2,4-D) supplemented with 0.1 mg / l of kinetin.

The pH of the media was set at 5.75 using 0.1 M hydrochloric acid and 0.1 M sodium hydroxide solutions. The media was autoclaved for 15 minutes at 1.5 kg cm^{-1} pressure and temperature 121^{o}C. For initiation of callus, the culture bottles were incubated in the growth chamber maintained at temperatures between 22^{o}C to 24^{o}C and 12 hour darkness during the night and 12hrs lighting from fluorescent tubes during the day.

The hormone concentrations were arrived at after performing trial experiments involving (0.5, 1.0, 1.5, 2.0, 3.0 mg / l of 2, 4-D and 0, 0.05, 0.1, 0.15, 0.2, 0.3mg / l of kinetin. Each concentration for trial experiment had 20 replicates. After the trial experiments 2 mg / l of 2, 4-D and 0.1 mg / l of kinetin were determined as optimum for callus induction and used in subsequent experiments.

Other auxins, indoleacetic acid (IAA) and napthaleneacetic acid (NAA) at concentrations as above were also used in an attempt to induce callus without success.

2.4 Callus Proliferation Medium

For further growth and multiplication, the callus was maintained by culturing in MS media salts, vitamin solutions, 8 g agar agar, 30 g / l of sucrose supplemented with 3 mg / l of BAP plus 0.1 mg/l 2, 4-D. The pH was 5.75 for two months with regular sub-culture into fresh medium after every two weeks. Other concentrations at (1, 2, 3 and 4 mg / l of BAP and 2 ip and 0, 0.05, 0.1, 0.15 0.2 mg / l of kinetin and 2,4-D were used to arrive to an optimum of 3 mg / l of BAP and 0.1 mg / l 2,4-D for callus proliferation.

2.5 Callus Maturation andEmbryogenesis

Callus maturation was attained by sub culturing 14 weeks old calli into hormone free MS media supplemented with B5vitamins. The pH was maintained at 5.75 and inoculants kept at 22-24^{0}C with regular subculture into fresh maturation media.

2.6 Cell Suspension Culture

Cell suspension cultures were established using liquid MS medium and subcultured into fresh liquid MS media after every 2 weeks. The liquid MS media without agar was supplemented with 3 mg / l of BAP plus 0.1 mg/l 2,4-D. The pH was 5.75. In a lamina flow about 1cm^3 of callus was suspended in 15ml of the media in 50ml sterilized conical flasks covered with sterilized aluminium foil. The inoculated conical flasks were then placed on a rotary at 50 revolutions per minute at between 22^{o}C to 24^{o}C and 12 hour darkness during the night and 12hrs lighting from fluorescent tubes during the day. The large clumps of embryogenic calli in the liquid media were then plated on the solid regeneration MS media supplemented with.

2.7 Regeneration and Rooting

For plant regeneration embryogenic callus, which was greenish in colour was separated from whitish non-embryogenic callus using sterilized scalpel. About 1-2 g of excised embryogenic callus was inoculated into 30ml shoot regeneration media.

The shoot regeneration medium consisted of MS media salts, vitamins, supplemented with 3 mg / l of 2 ip, 8 g / l agar agar, 30 g / l of sucrose, at pH of 5.75 and incubated at 25^{0}C-27^{0}C, with continuous lighting from florescence tubes in the growth chamber at IBR and at DSMZ. This concentration of 2 ip was arrived at after several trial experiments involving use of (1, 2, 3, and 04 mg / l of 2 ip). The regenerated shoots measuring 2-5 cm was transferred to rooting media after 13 weeks of regular sub-culture into fresh shooting media after every two weeks.

Rooting of the shoot regenerates was initiated by incubating single shoot into each media bottle containing 30 ml of MS media salts, vitamins, 1 g of glycine, 0.2 g of biotin, 3 mg/l of Indole-3- butyric acid (IBA), 8 g of agar agar and 30 g/l sucrose. The incubation temperatures remained at 25^0-27^{0}C with 12hr darkness and 12hr lighting from fluorescent tubes. This concentration of IBA was reached after trial experiments using 0.1, 0.2, 0.3, and 0.4 mg/l of IBA. Each trial had 20 replicates.

3.0 Results

Callus formation was successful and estimated at 80%, observed after 2 weeks in culture media consisting of MS salts, vitamins plus 8 g / l agar agar, 30 g / l sucrose, 2 mg / l 2, 4-D supplemented with 0.1mg/l of kinetin. Figure greenish embryogenic (M) callus and regenerated into shoots (R) in Figure 1. Trials using IAA and NAA failed to induce callus growth. 2-4, D was very effective in induction of callus even at as low values as 0.5 mg / l.

Callus proliferation was attained in culture media consisting of MS media containing vitamins plus 8 g / l agar agar, 30 g / l sucrose, supplemented with 3 mg / l BAP and 0.1mg 2, 4-D. Cell suspensions cultures developed successfully in the liquid media with the formation of large clumps of embyogenic callus which regenerated into plantlets on plating on MS solid regeneration media supplemented with 3 mg / l of 2 ip, 8 g / l agar agar, 30 g / l of sucrose, at pH of 5.75 and incubated at 25^{0}C-27^{0}C, with continuous lighting from florescence tubes in the growth chamber at DSMZ.

Callus growth and maturation was achieved by maintaining the callus in hormone free MS media with B5 vitamins, 8 g agar agar, 30 g / l of sucrose at pH of 5.75 for two months with regular sub-culture into fresh medium after every 2-3 weeks regularly. The greenish embryonic callus was formed from the whitish non embryogenic callus during this stage.

The embryogenic calli were sub-cultured into shooting media. Shoots measuring 1-3 cm tall, with 2-4 green leaflets measuring 1-2cm long were obtained after 12 weeks. The shoot regeneration media consisted of MS media salts, vitamins, supplemented with 3 mg / l B.A.P, 8 g / l agar agar, 30 g / l of sucrose. Trial experiments with kinetin failed to induce shoot formation.

Rooting rate was as low as 40 % obtained after 3-6 weeks of incubation of the shoots into media bottle each containing 30 ml of MS media salts, vitamins, 1 g of glycine, 0.2 g of biotin, 0.3 mg / l of Indole-3- butyric acid (IBA), 8 g of agar agar and 30 g/ l sucrose, see plate 3. The whitish roots that measured 2-4 cm long appeared like prop roots formed at the base of the shoot and grew into the media. Although IBA induced rooting in presence of increased concentration in levels of glycine and biotin, it failed to induce root formation in the absence of the two amino acids.

Figure 3: Regeneration of T. peruviana in vitro via callus

L. embryogenic (greenish) and non embryogenic (whitish) calli of *T.peruviana*
M. embryogenic callus of *T. peruviana in vitro* R. *in vitro* shooting of *T. peruviana* from the embryogenic calli.

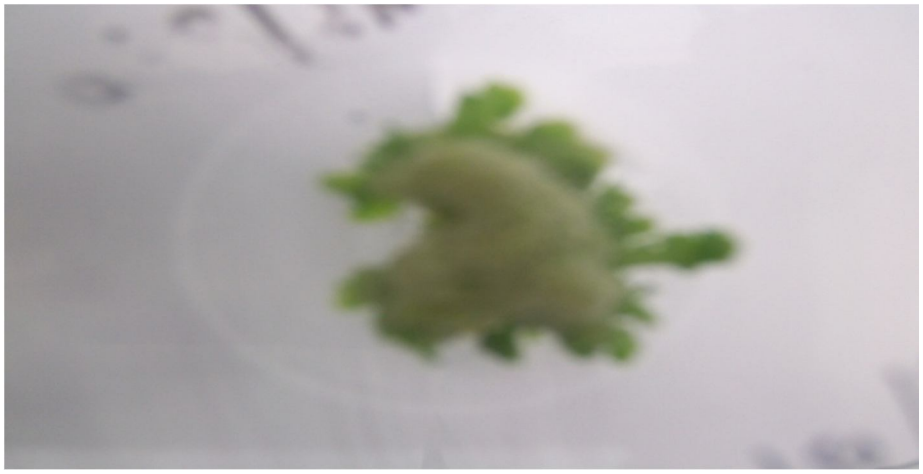

Figure 4: T.peruvaina callus undergoing somatic embryogenesis at DSMZ laboratory, Germany

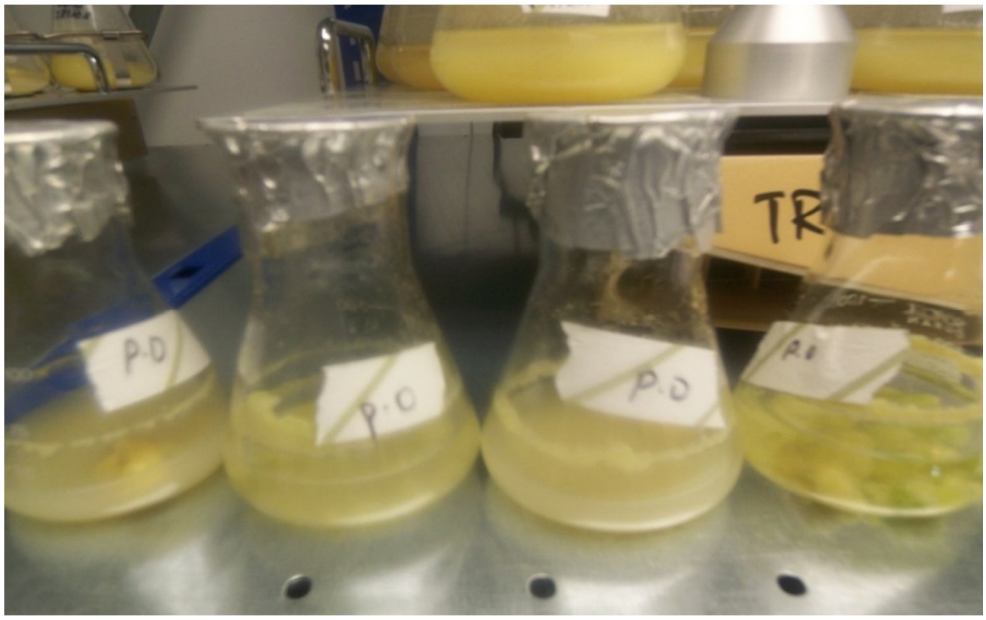

Figure 5: Cell suspension cultures of T.peruviana at DSMZ laboratory

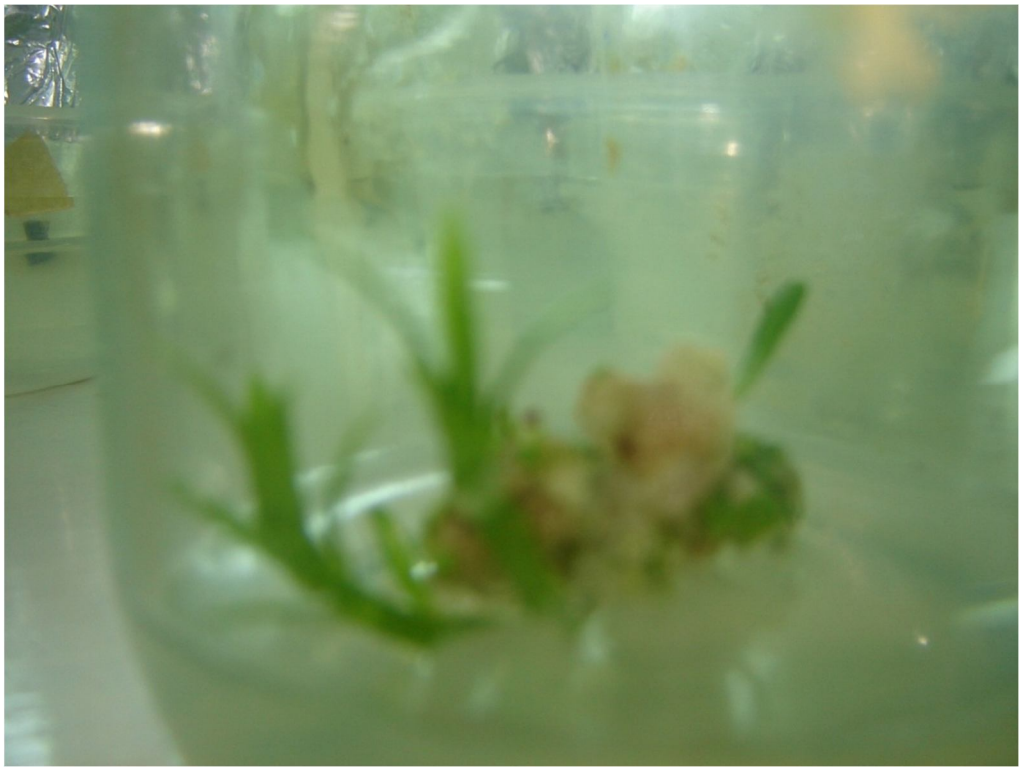

Figure 6: Regenerating T.peruviana plantlets at JKUAT, IBR laboratory

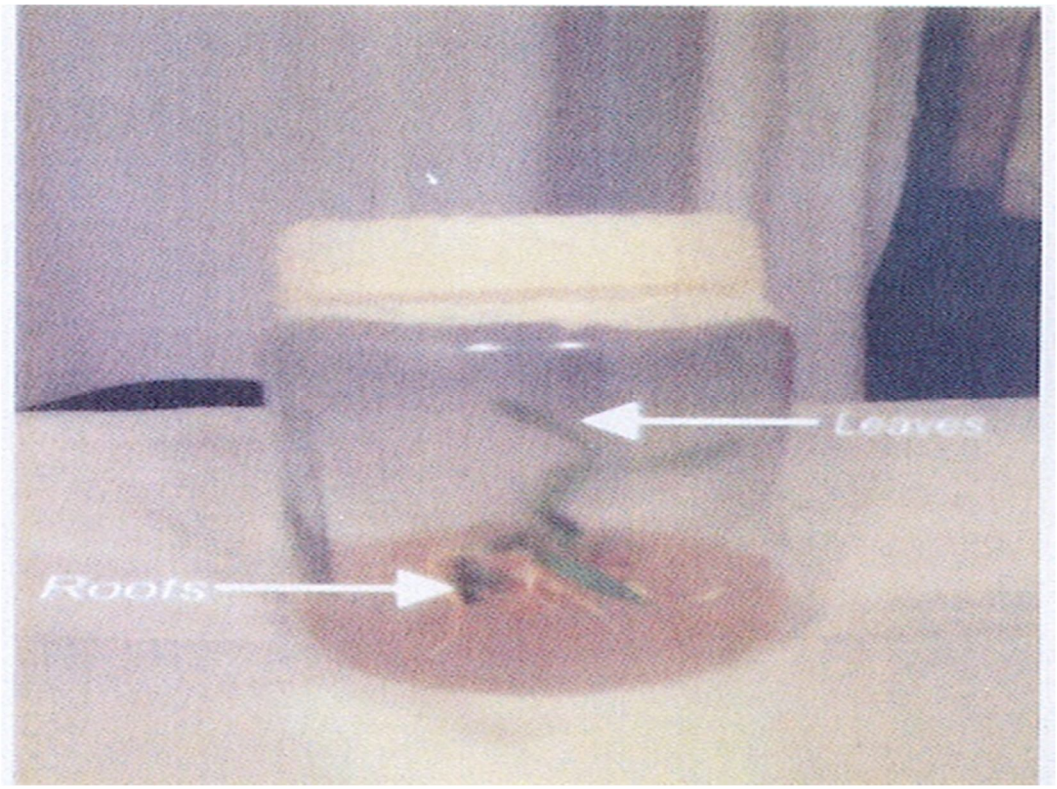

Plate 7: T.peruviana plantlet rooting in vitro at IBR, JKUAT

4.0 Discussion

The leaf discs from young *T. peruviana* seedlings exhibited good dedifferentiation characteristics into callus *in vitro* induced by plant growth regulator 2,4-D. This could be attributed to their young age and an early stage in their physiological development and due to the physiological effect of 2, 4-D on wounded plant parts like the leaf discs. 2,4D is largely used as a herbicide in plants due to its ability to retard plant growth and development.

The age and physiological state of the parent plant has been known to determine the success of the *in vitro* regeneration potential of the explant. Leaf explants from young pre-flowering, and healthy plants had been recommended (Evans *et al.,* 1981). The whitish callus developed into embryos, which were greenish in colour, which later regenerated into whole plantlets, when 2, 4-D was withdrawn and replaced with cytokinin (BAP) since 2, 4- D is known to inhibit embryo, shoot and roots development (Fujimura and Komamine, 1975). Somatic embryos are formed from plant cells that are not normally involved in the development of embryos. No seed coat or endosperm is formed a round somatic embryos. Application of this somatic embryogenesis include, clonal propagation of genetically uniform plant material, elimination of viruses, provision of source tissue for genetic transformation, generation of whole plants from single cells and development of synthetic seed technology, (Dodeman *et al.,*1997). In other reported cases, 2, 4-D has been used alone or with a low cytokinin concentration to initiate callus in over 57 % of successful embryogenic cultures in the world (Evans *et al.,* 1981). Generally a high concentration of auxin and a low concentration of cytokinin in the medium promote abundant cell proliferation with the formation of callus (John and Lorin, 1982).

Callus maturation and embryo development was realized in 2, 4 D free culture media. It was noted that BAP was very crucial during callus proliferation, embryo development and the subsequent shooting stage. Cytokinins are known to promote embryonic accumulation of proteins and biosynthesis of polyamines, required for rapid cell division. The concentration of 6, benzylaminopurine was very critical during embryo development and shoot regeneration *in vitro*. Cytokinins are very important for fostering embryo maturation and especially cotyledon development and change in auxin type or concentration is a prelude to embryo development (Fujimura and

Komamine, 1975). Amino acid glycine, vitamin solutions in the rooting media specifically biotin and the auxin (IBA) played a crucial synergistic role in the root development stage in the *in vitro* regeneration of *T. peruviana.*

Increased concentration and bioavailability of glycine promoted root development by acting as a source of organic nitrogen, which is essential for enzyme, and protein synthesis required for root development. The vitamins specifically biotin which were added in solution form to the media played a key role as well as the amino acid glycine during the root development stage by hastening the process. Vitamins are known to be physiologically significant as enzyme activators and indeed the biotin played a key role in the rooting stage by activating the enzymes required for protein synthesis. Earlier work shows that biotin is a water-soluble vitamin which is physiologically important as cofactor of four adenosine tri-phosphate (ATP) dependent carboxylases namely acetyl Co-A carboxylase, pyruvate carboxylase, propionyl-Co-A carboxylase, and β-methyl crotonyl-Co-A. It is used as ATP carrier in the carboxylation reactions (Tom, 1999). Vitamin D, its analogues, and other vitamins such as thiamine are anew class of plant growth substances known to promote, cell aggregation, growth and root development (Salisbury and Ross, 1986).

A short passage time of two weeks and sub-culture into fresh media was maintained since it is known that a shorter passage is recommended to maintain chromosome stability of cell cultures and replenish the nutrients in the growth media. Sub-cultures should be done frequently especially during late exponential growth phase and should never enter the stationery phase. Longer passage time before subculture is reported to be source of mutations in cultured plant cells (Kao *et al*., 1970).

Aseptic culture media was always maintained by the outlined sterilization procedures to provide the most favourable conditions containing essential macro and micro-nutrient salts, sugars, vitamins, amino acids and growth regulators to the cultured tissue as the same nutrients are in greater demand by the micro biota (John and Lorin, 1982). Cell suspension cultures produced large clumps of embryogenesis calli since there was uniform distribution of nutrients as well aeration due to the constant shaking.

5.0 Conclusion

This study revealed that plantlets can be regenerated by tissue culture via callus induction and can provide the basis for selection of plantlets with superior traits such as high oil yield, low or no glycoside content, rapid growth, and early maturity. The technique can also provide an opportunity for genetic engineering of the oil plant to incorporate other desirable genetic factors such as nitrogen fixation, which will be of great benefit to the farmers and vegetable oil industry. This study is useful since the species is currently growing in the wild without well documented information on its propagation and improvement. Tissue culture is one of the best methods so far for generation of superior planting materials without use of irradiation and chemical mutants in *T.peruviana*.

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