THE UNIVERSITY OF ZAMBIA
SCHOOL OF AGRICULTURAL SCIENCES
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MULTIPLICATION OF CASTOR LINES USING TISSUE CULTURE TECHNIQUES

BY

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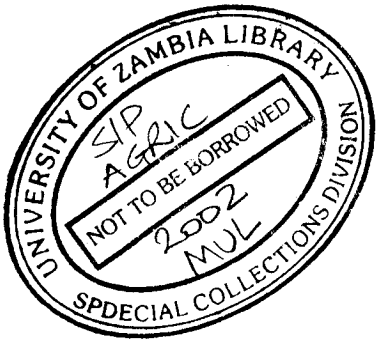

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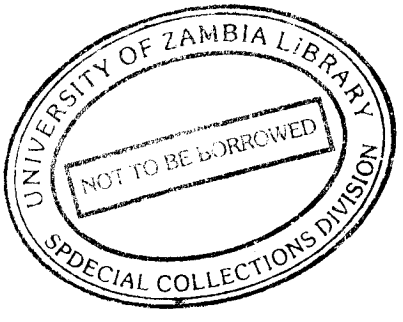
DEDICATION

To Pappa, Mum and my brothers and sisters for their Faith and Patience.

Also for my Late Uncle Sikota May His Soul Rest in Eternal Peace.



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Last but not least many thanks without number to the Mighty God for always loving me.

Psalms 16:1 " keep me safe, O Lord, for in you I take refuge."

Abstract

A study was conducted between February and September by a series of experiments at the school of Agricultural Sciences tissue culture Laboratory. The aim of the investigation was to develop a tissue culture propagation method for various castor plant varieties i.e. to investigate the effects of different Media components on both shooting and root formation.

Castor plantlets were produced via tissue culture using shoot buds from recently germinated or young castor plant as explants. Explants were transferred and maintained on Shoot and Root induction Media for 8 weeks and 4 weeks respectively. The optimum tissue culture conditions were; MS Medium containing 1.5 ppm 6 Benzlaminopurine (BAP) and 1.0 ppm Naphthaleneacetic acid (NAA).

Micropagation success was genotype-dependent. Varieties 2 (L162# 2) and 4(L46#6) exhibited the best performance overall with regards to shooting and rooting. While all varieties proved themselves amenable to shoot culture, only 2 and 4 were responsive to this concentration of rooting Media. In addition, average multiplication rates varied among experiments from 1-4 shoots per explant.

Rooting was however the most difficult phase in the propagation process. Most of the plantlets had small, very thick and highly branched growth habit when growing in vitro.

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CHAPTER ONE.

1.0 Introduction.

One of the most serious problems confronting castor plant (Ricinus communis L.) breeding is the long generation interval (Wagih, 1990).

Castor plant propagation by seed has sometimes proved unsatisfactory due to poor germination of seeds of castor plant, failure of seed formation or perhaps seed is formed but quickly lose their viability (Dean, 1982).

Young vegetative and inflorescence can be severely injured by cool temperatures that occur irregularly in many subtropical regions. Low temperatures also adversely affect fruit set. Enhanced cold tolerance would therefore extend the geographical range of castor plant production and would improve the regularity of bearing by eliminating a major source of fruit and flower drop due to cool weather.

Castor seeds appear to lose viability within two-three months after storage at ambient room temperature and normal moisture content. It has been suggested that castor seeds can remain viable for longer period if moisture content (percentage fresh weight) of the seeds is reduced (Maretzki, 1987)

Both, seed moisture content and storage temperature are found to affect seed viability. Ellis and Robert (1980). It is also known that pollen stored at low temperature remained viable for long period of time (Wang and HU, 1980)

In addition, castor plant propagation by seed can be as equally unsatisfactory due to heterozygous progenies obtained as it is a cross-pollinated crop.

With the above reasons, the need still exists for methods that would shorten generation time as well as create true to mother type of castor plants, and consequently, greatly benefiting castor plant breeding.

Furthermore, in such cases where conventional approaches have not been sufficient further improvements of castor plant can be achieved by plant tissue culture/ micropropagation. Although castor breeding is based mainly on classical breeding methods, these biotechnolologial research results can be incorporated into it as a complementary tool.

1.1 Micropropagtion.

Micropropagtion involves the production of plants from very small plant parts, tissues or cells grown aseptically in a test tube or other container where the environment and nutrition can be rigidly controlled. The growing of plant tissue such as callus, cell suspensions and various plant organs (stems, flowers, roots and embryos) in artificial medium is known as tissue culture. Sometimes, tissue culture is synonymous for micropropagation. The main advantage of this technique is rapid mass propagation of clones and can produce disease free seedlings.

In addition, plant tissue culture helps regenerate a plant in large numbers under disease-free (especially virus-free) conditions and thus providing opportunity for genetic improvements. For the pathogenic plant viruses, no effective cure exists for already infected plants in the field. They cause considerable economic losses and are therefore of major concern to worldwide phytosanitary agencies. In order to tackle the problem, both detection and elimination procedures need to be improved in order to provide virus free planting material to the growers. This combination of disease elimination and disease- indexing on in vitro plants would considerably reduce the efforts and contribute to savings of time, money and labour.

1.2 Objectives

The aim of the investigation presented here is to develop a tissue culture propagation method for castor plant. Specific objectives include:

Evaluate the effect of growth regulator on both Shooting and rooting of each of the castor varieties

Establish the best root inducing media of castor mirocuttings (microshoots).

CHAPTER TWO

2.0 Literature review

2.1 The plant: Biology and Economic Importance of Ricinus communis.

Ricinus communis, or Castor Bean, is not a true bean, but rather a member of the spurge family or Euphorbiaceae (Zomlefer, 1994). The Euphorbiaceae is a family of primarily tropical distribution, however 47 genera including Ricinus occur in the U.S. and Canada (Zomlefer, 1994). Ricinus is native to tropical South-east Africa. The Castor Bean is usually planted in the U.S. as an ornamental, however it is known to naturalize in the wild worldwide. According to Jones, one naturalized population of Ricinus communis, or the Castor Bean, has been officially recorded in Georgia (1988). The plant is becoming a common weed in the Southwest U.S. Ricinus communis is characterised as a 4-12 cm shrub-like annual herb with alternate, palmate lobed green to reddish-purple leaves. The lobes of the leaf are 5-11 cm long with a serrate margin. The highly toxic seeds are characterised by their elliptical shape and glossy, mottled black or brown and white coloration (Hardin 1974). The seeds also possess a small projection termed a caruncle that serves as a sight for water absorption during germination. The seeds are three carpellate, with one seed per carpel and the carpels split apart at maturity. The plants are monecious and the flowers are found in dense, terminal clusters with the pistillate flowers above and the staminent flowers below. Both types of flowers are apetalous and the female flowers have red, feathery, multi-lobed stigmas. Wind is the primary mechanism for pollination. Ricinus communis is commonly naturalized along stream beds, bottomlands, and river beds as well as other locations with warm, well-drained, high plant nutrient habitats.

2.2 Derivation of the name castor.

It is interesting to trace the origin of the name "castor." Castor is the generic name of the North American beaver (Castor canadensis) and one of the brightest double stars in the constellation Gemini. In Greek and Roman legend, Castor was one of the twin sons of

Jupiter and Leda. According to E. A. Weiss (1983), writing in Castor, Sesame and Safflower (1971), the name "castor" has nothing to do with beavers, luminous stars, or offspring of Greek and Roman Gods. Castor was apparently coined by English traders who confused it with the oil of another shrub, Vitex agnus-castus, which the Spanish and Portuguese in Jamaica called "agno-casto." Although it is commonly known as the castor bean plant, the seed is not a true bean and it is not related to the Bean or Legume Family (Fabaceae). There are many other examples of "beans" that are technically not beans, such as Mexican Jumping "beans" and coffee "beans."

The scientific name for the castor plant, Ricinus communis, has a much more logical derivation. Communis means common in Latin, and castor plants were already commonly naturalized in many parts of the world when the eighteenth century Swedish naturalist Carolus Linnaeus (Karl von Linné, 1982) was giving scientific first and last names to plants and animals over 200 years ago. Ricinus is the Latin word for tick and is the specific epithet for the Mediterranean sheep tick (Ixodes ricinus). Apparently, Linnaeus thought the seeds looked like ticks, particularly large ticks engorged with blood

In addition, the derivation of the name of the castor bean plant (common and Latin names) is an interesting note. Apparently, the first English traders to encounter the plant mistook it for another species, Vitex agnus-castus, which was known in Jamaica as agno-casto. The Latin name has a more creative origin. Ricinus communis was first described and named by Carl Von Linne (1983).

The whole plant is very poisonous and even one seed has been known to be lethal to children. The seedcoat contains an extremely lethal poison that was once used by the KGB to dispose off their enemies. The leaves are only mildly poisonous. The toxic principle is water-soluble so is not found in the oil.

2.3 Physical characteristics.

An evergreen shrub with a spacing growing to 1.5 m by 1 m at a fast rate. It is frost susceptible and in leaf all year, in flower from July to September, and the seeds ripen from September to November. The flowers are monoecious (individual flowers are either male or female, but both sexes can be found on the same plant). It is rated 2 out of 5 for usefulness.

The plant prefers light (sandy), medium (loamy) and heavy (clay) soils, requires well-drained soil and can grow in heavy clay soil. The plant prefers acid, neutral and basic (alkaline) soils. It cannot grow in the shade. It requires moist soil.

2.4 Oil

The seed contains 35 - 55% of edible oil, used in cooking. The seed is a rich source of phosphorus, 90% of which is in the phytic form.

2.5 Medicinal uses

The oil from the seed is a very well known laxative that has been widely used for over 2,000 years. It is considered to be fast, safe and gentle, prompting a bowel movement in 3 - 5 hours, and is recommended for both the very young and the aged (Grieve M., 1931). It is so effective that it is regularly used to clear the digestive tract in cases of poisoning. It should not be used in cases of chronic constipation, where it might deal with the symptoms but does not treat the cause. The flavor is somewhat unpleasant, however, and it can cause nausea in some people (Carter. A, 1997). The oil has a remarkable antidandruff effect and is well tolerated by the skin and so is sometimes used as a vehicle for medicinal and cosmetic preparations (Mazzola. M, 1997).

Castor oil congeals to a gel-mass when the alcoholic solution is distilled in the presence of sodium salts of higher fatty acids. This gel is useful in the treatment of dermatosis and is a good protective in cases of occupational eczemas and dermatitis (Ruth Hazzard et al., 1999).

2.6 Castor oil as a cure-all elixir.

Castor oil has been used medicinally in the United States since the days of the pioneers. As Americans moved west after the Civil War, settlers were very attracted to Indian medicines and popular "cure-all" remedies. The stronger smelling and the more vile tasting the concoction, the better, and some medical historians have described the latter part of the 1800's as the "age of heroic cures." Travelling medicine men peddled their elixirs throughout towns of the west, and often their products contained up to 40 percent (80-proof) alcohol. Castor oil was one of the old-fashioned remedies for everything from constipation to heartburn. It is indeed a very effective cathartic or purgative (laxative) and is still used to this day; however, there are milder, less drastic methods of inducing regularity in bowel movement. Castor oil is also used as lubricant: It is sometimes applied externally as a soothing emollient for dry skin, dermatitis, other skin diseases, sunburn, open sores, and it is the primary ingredient of several brand name medications. Several additional little-known uses for castor oil include hair tonics, ointments, cosmetics, and contraception creams and jellies. One remarkable old remedy mentions administering castor oil to induce labour during pregnancy.

2.7 Other uses:

2.7.1 Insecticide.

The seed contains 35 - 55% of a drying oil. It is an ingredient of soaps, polishes, flypapers, paints and varnishes (Dow. L, 1994). It is also used as a lubricant and for lighting and as an ingredient in fuels for precision engines.

A fibre for making ropes is obtained from the stems (Mazola, 1997). The growing plant is said to repel flies and mosquitoes. When grown in the garden it is said to rid it of moles and nibbling insects. The leaves have insecticidal properties Cellulose from the stems is used for making cardboard, paper etc (Grieve. M, 1931).

2.7.2 Castor oil in paints

The castor plant has many uses, particularly the thick, yellowish or almost colourless oil obtained from the seeds. There are an astonishing number of industrial applications for castor oil and its derivatives, and new ones are continually being discovered. When dehydrated, castor oil is converted into quick-drying oil used extensively in paints and varnishes. In fact, one of the largest single markets for castor oil in the United States is in the paint and varnish industry. Some experts say that dehydrated castor oil has qualities superior to linseed oil and tung oil, two of the most important drying oils. Castor oil is one of the world's most versatile natural products. Its water-resistant qualities make it ideal for coating fabrics and for protective coverings

2.7.3 Castor oil in nylon.

Castor oil is the primary raw material for the production of sebacic acid, which is the basic ingredient in the production of nylon and other synthetic resins and fibers. Approximately three tons of castor oil is necessary to produce one ton of nylon. Sebacic acid is a 10-carbon dicarboxylic acid with a carboxylic group (C-OOH) at each end of the molecule. It is reacted with 1,6-hexanediamine, a 6-carbon molecule with an amino group ($C\text{-}NH_2$) at each end. The free carboxylic and amino ends of these molecules begin bonding together in a chain reaction called condensation polymerization, in which a water molecule is produced at each link. The resulting nylon polymer is called Nylon 6,10 to denote the 6-carbon diamine and 10-carbon sebacic acid.

2.7.4 Castor bean motor oils.

The superior "oiliness" of castor oil and its ability to "cling" to very hot moving parts make it outstanding racing oil for high performance engines. In fact, it is the basic ingredient of Castrol-R racing motor oil for high-speed automobile and motorcycle engines. Castor oil is a popular fuel additive for two cycle engines, and imparts a distinctive aroma to the exhaust of these engines. Castor wax, a hard wax produced by the hydrogenation (chemical combination with hydrogen) of pure castor oil, is used in polishes, electrical condensers, carbon paper, and as a solid lubricant.

Considering the existing food scarcity in developing countries, utilisation of castor plant oil as cooking fuel does not compete with production or use as food (GTZ, 1995, Franke, 1981, Rehm and Espig, 1996). Utilisation of castor plant oils as cooking fuel provides employment and income opportunities for rural people. Independent energy supply in both rural and urban regions reduces dependence upon imported fossil fuels. Therefore use of plant oils as fuel secures a long –term supply of cooking energy, which guarantees proper preparation of meals and provides heat that is necessary for basic hygienic needs such as boiling water.

2.7.5 Fruit flavors from castor oil.

Although castor oil is rather malodorous and distasteful, it is the source of several synthetic flower scents and fruit flavours (esters), such as jasmine, apricot, peach, plum, rose, banana, and lemon. The chemicals (esters) responsible for these flavours and aromas are obtained from ricinoleic acid, one of the important ingredients of natural castor oil. In fact, ricinoleic acid comprises about 90% of the total triglyceride fatty acids of castor oil. Castor oil is also used in making soap, inks, and plastics; for preserving leather; as an illuminant; in Turkey red oil for dyeing and finishing textiles; and in brake fluids and certain insecticidal oils. Even after the oil has been removed, the poisonous crushed seeds or oil cake (pomace) makes an excellent fertilizer.

2.7.6 Ricin: a deadly protein.

The active poison in castor seeds is ricin, a very deadly protein called a lectin. Ricin is found in the meal or cake after the oil has been extracted. That who occasionally takes castor oil may be assured that ricin does not occur in the pure oil (Hussey 1976). When a gram of ricin is compared with equivalent weights of other toxic substances, it turns out to be one of our deadliest natural poisons. It has been estimated that, gram for gram, ricin is 6,000 times more poisonous than cyanide and 12,000 times more poisonous than rattlesnake venom. Ricin mixed with food and used, as bait is highly toxic to certain pest animals, such as some rodents and insects. E. A. Weiss (1971) states that a dose of 0.035-milligram (approximately one millionth of an ounce) may kill a man, and even small particles in open sores and in the

eyes may prove fatal. According to the Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals (1997), a dose of ricin weighing only 70 micrograms or two millionths of an ounce (roughly equivalent to the weight of a single grain of table salt from a salt shaker) is enough to kill a 160 kilogram person. As few as four ingested seeds can cause death in an adult human and lesser amount may result in symptoms of poisoning, such as vomiting, severe abdominal pain, diarrhoea, and convulsions. Of course the degree of poisoning depends upon the amount ingested and the age and general health of the individual. There are numerous documented cases of ricin poisoning and death when horses, livestock, and poultry accidentally ate castor seeds or meal.

With the exception of certain pathogenic bacteria, lectins include some of the most insidious plant toxins affecting people. In addition to ricin from castor bean seeds, another very poisonous lectin called abrin occurs in the seeds of rosary bean (Abrus precatorius), a common tropical vine in the Legume Family (Fabaceae). According to the Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals, 1983), one thoroughly masticated seed of rosary bean can cause fatal poisoning. Brightly coloured rosary beans are commonly strung for seed jewelry in Mexico and Central America. Sometimes the seeds are boiled in order to facilitate the piercing of their hard seed coats, and this heating would undoubtedly denature the toxic proteinaceous lectins inside. Of course, the undisputed record for the deadliest natural toxin goes to the anaerobic bacterium of spoiled food (Clostridium botulinum). A fascinating article on botulism appeared in Scientific American, April 1968. So deadly is the toxin (even deadlier than strychnine, arsenic and snake venoms), that an amount equal to the weight of ink in a printed period in a textbook is enough to kill 30 adult humans. One ounce could theoretically kill 30 million tons of living matter and one pound could kill the entire human population

The poisoning mechanism of ricin is very complicated, but it apparently causes clumping (agglutination) and breakdown (hemolysis) of red blood cells, haemorrhaging in the digestive tract, and irreparable damage to vital organs such as the liver and kidneys. It is most toxic when taken intravenously or inhaled as fine particles. In fact, the possibilities of

ricin dust in chemical warfare are horrendous. There are even documented cases of ricin poisoning in murders by paid assassins.

2.7.7 Ricin in cancer research.

Although it is a very potent poison, ricin has been shown to possess antitumor qualities and has been used in cancer research and chemotherapy during recent years. One of the most promising uses of ricin is in the production of immunotoxins, where the protein ricin is joined to monoclonal antibodies. The antibodies are produced in a test tube (in vitro), and have protein receptor sites that recognise the specific target cells of a tumour. The resulting ricin-antibody conjugate is called an immunotoxin. By arming these antibodies with ricin, the deadly toxin can be carried directly to the site of the tumour in a cancer patient. Thus, ricin can destroy the tumour cells, without damaging other cells in the patient.

To produce monoclonal antibodies, B-lymphocytes (plasma cells) from mice are exposed to a specific tumour antigen so that they can produce antibodies against the tumour.

2.8 Tissue culture media.

Tissue culture media are nutritive solutions presented to the explant in a liquid or semi-solid state. Explants were positioned above the medium using gelled agar, which support the explant and keep it from submerging in the medium, yet allow diffusion of the medium ingredients into the plant tissues.

The employed media was the medium developed by Toshio Murashige and Folke Skoog (Murashige, T. and F. Skoog. 1962). The Murashige and Skoog medium is so well known to the scientific community that it is often abbreviated simply as the MS medium.

Basically this nutritive medium contains macronutrients (minerals required in rather large concentrations such as N, P, K, Mg, S, Ca), micronutrients (also called trace elements because they are required in low concentrations such as Fe, Zn, B, I, Mn, Mo, Co, Cu), vitamins (thiamine, nicotinic acid, riboflavin most commonly), sugars (sucrose and glucose are common) and sugar alcohols (myo-inositol in particular). A medium may also include

amino acids, nucleic acid bases, or other organic molecules. If the medium has no phytohormones or plant growth regulators, it is referred to as a basal medium.

Phytohormones or plant growth regulators may be added to the basal medium to stimulate the growth and development of the explant in a particular fashion. There are five classes of phytohormones: auxins, cytokinins, gibberellins, abscisic acid and ethylene. Auxins and cytokinins are most commonly employed in tissue culture media.

In general, auxins are promotive of root initiation, although they may inhibit root elongation and subsequent development. Cytokinins on the other hand tend to promote the development of new vegetative buds or the opening and growth of existing vegetative buds. There are many exceptions to those generalizations, as is often the case with generalizations.

Folke Skoog and Carlos Miller, in a classic study, demonstrated the interaction of auxins and cytokinins when growing tobacco pith cells (Skoog, F. and C.O. Miller. 1957). They observed that when tobacco pith was exposed to a medium containing a high auxin concentration relative to the cytokinin concentration, the roots formed from the pith tissue. They also observed that when pith was exposed to a medium containing a high cytokinin concentration relative to the auxin concentration shoots developed from the pith tissue. When the concentrations of the growth regulators were relatively balanced, no organs were formed, but instead the pith cells proliferated in an unorganized fashion to form a tissue called callus.

An MS medium formulation was selected and the solution prepared, the pH of the medium was adjusted to the required pH range of 5.7 to 5.8. This pH range keeps most of the ions in a charged state where they are available for absorption by the plant cells.

A medium containing the proper concentration of nutrients, at the proper pH, and the agar dissolved in the medium was dispensed into test tubes. Test tubes were capped with a lid that vents to the atmosphere without letting microbes inside easily. The medium was then

sterilized heating under pressure in an autoclave (generally 15 minutes at 15 psi) Muyobo (2000) suggested that terminal buds were amenable to tissue culture.

2.9 World productivity of castor plant.

According to Meredith.et al., 1997, World annual production of castor oil is about 460,000 t (1.1 million tonnes of seeds); the main producers are India, Brazil, and China. The European Union uses about 90,000 t of castor oil and imports oil as well as about 30,000 t of seeds, which is mainly crushed in Germany. Due to new outlets for castor oil (such as anticorrosive products or odorant captivators), European demand should increase over the next ten years.

CHAPTER THREE

3.0 Materials and Methods

3.1 Explant preparation.

The apical (terminal) buds containing few leaves were removed from the plant and placed onto a sterile culture medium The first step of surface disinfection was a simple rinse with de-ionized water to remove any insects, loose spores and bacteria, and other debris that might be harbouring microbes.

Following the initial wash, explants were then transferred and soaked in 3% sodium hypochloride for 5 minutes. When the Explant was surface sterilized, it was removed from the sterilizing solution and rinsed several times in sterile, distilled water. This last step was performed inside a laminar flow hood to maintain the axenic condition of the explant and to prevent the re-introduction of contaminating microbes. The explant was then trimmed and placed onto a Basal Murashige-Skoog (MS) tissue culture medium.

3.2 Environmental conditions

The culture vessel containing the explant was placed in a controlled environment. The amount (quantity) of light, the spectral quality, the periodicity, the temperature (range and periodicity) all vary with the plant tissue involved and nature of the medium. However for this experiment explants were grown under 16:8-hour light-dark photoperiod at 26 °C.
Parameters measured include the number of roots per explant after 4 weeks and number of Shoots per explant after 8 weeks.

Three replicate plants were used per treatment. Data collected was then analysed using MSTAT by way of a 2-factor Completely Randomised Block. ANOVA detected significant interactions between Media and variety. The Duncan's Multiple Range was then used to test separate treatment means.

CHAPTER FOUR.

4.0 Results and Discussion

4.1 Effects of growth regulator concentration (BAP) on shoot formation.

Across all of the experiments, it was observed that there were significant interactions between Media and variety with regards to both Shoot induction and Rooting.

As regards the effect of media on the number of shoots per explant it was shown that varieties 1(Line 22), 2(Line 162#2) and 5(Line 44#4) had similar responses in both Media 1 and Media 2. However, with varieties 3(Line 109) and 4(Line 46#6) the number of shoots per explant, was higher in Media 2 than in Media 1 as shown in Fig.1 and 2.

Additionally, all Castor varieties except variety 1 were amenable to increasing Shoot inducing hormone. As the concentration of BAP was increased from the initial 0.5 ppm to 1.5 ppm, multiple Shoots were initiated in varieties 2,3,4 and 5. This is as Shown in Fig.3.

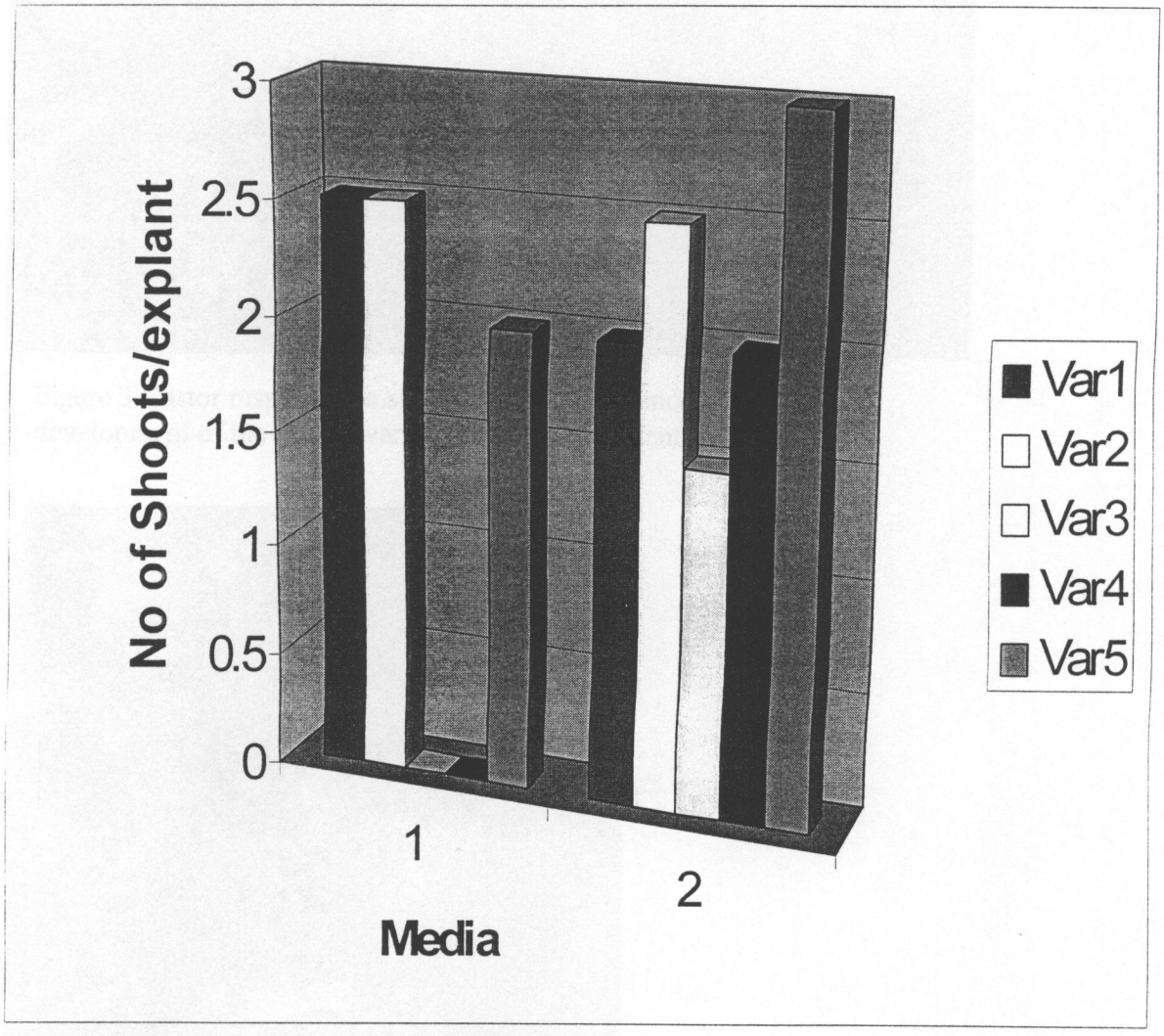

Fig. 1 Effect of different BAP concentration on shoot formation in different castor varieties after 8 weeks.

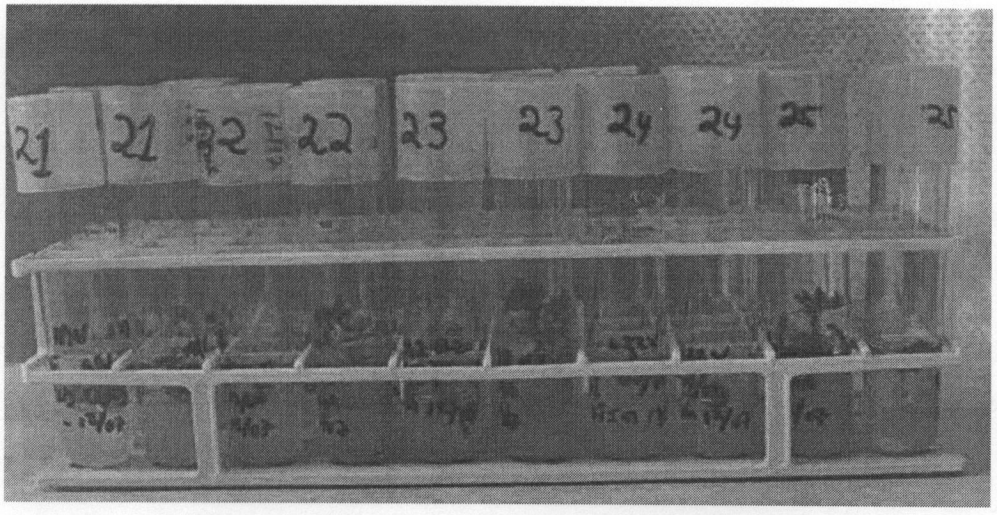

Figure 3 Castor responses to shoot culture- Differences in the development of the castor. varieties is clearly evident.

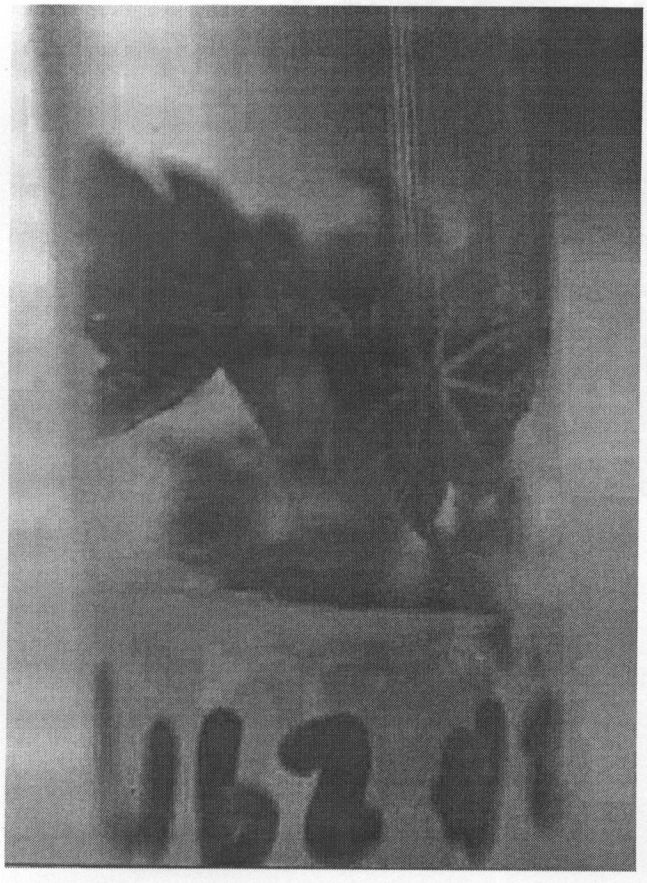

Figure 2. Multiple Shoot formation in the castor varieties.

4.2 Effect of NAA concentration on root formation.

With respect to effects of Media on number of roots per explant variety 1 only responded in Media 3 (Plain MS Medium) while variety 4 only responded in Media 1. Furthermore, variety 2 showed response in both Media 1 and Media 3. However, varieties 3 and 5 showed no response at all in Media 1,Media 2 or Media 3. The overall effect of Media on the number of roots per explant at 4 weeks is shown in fig.4.

With increasing NAA concentration from 1.0 – 2.0 ppm all varieties showed no response at all.

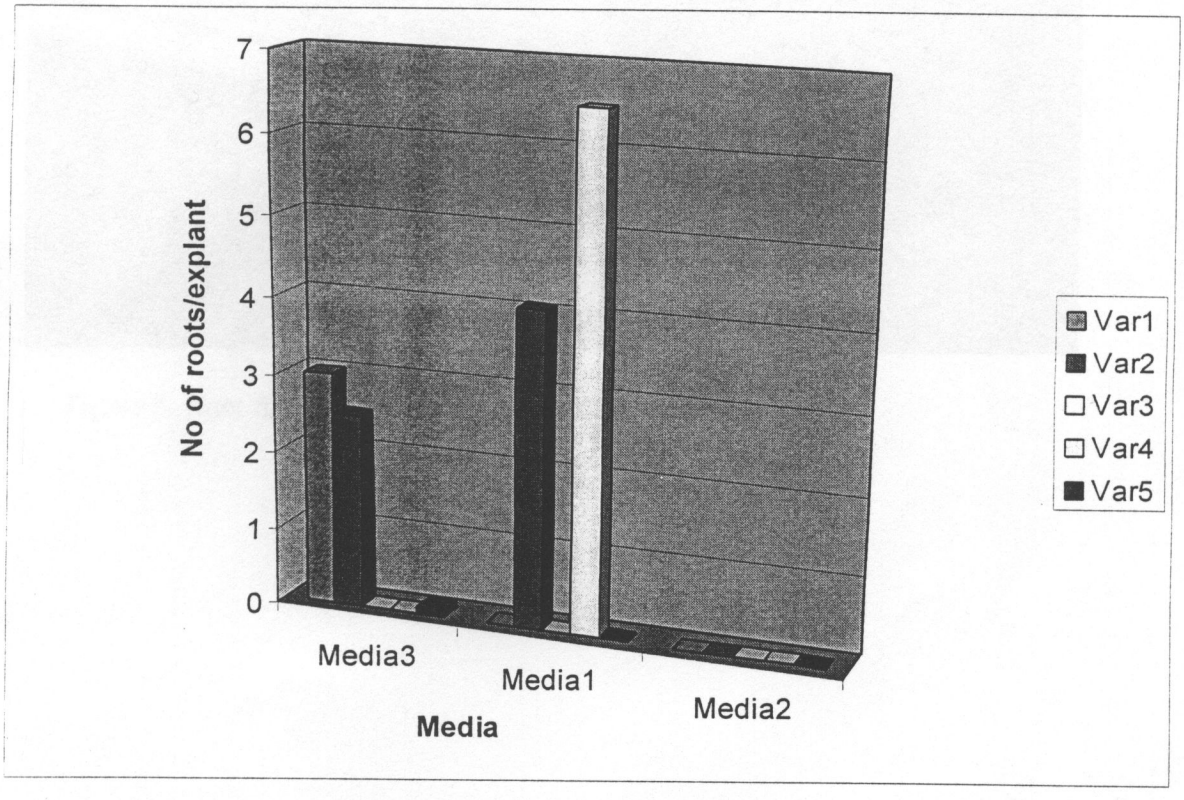

Fig.4: Effect of NAA concentration on number of root per explant of different castor varieties at 4 weeks

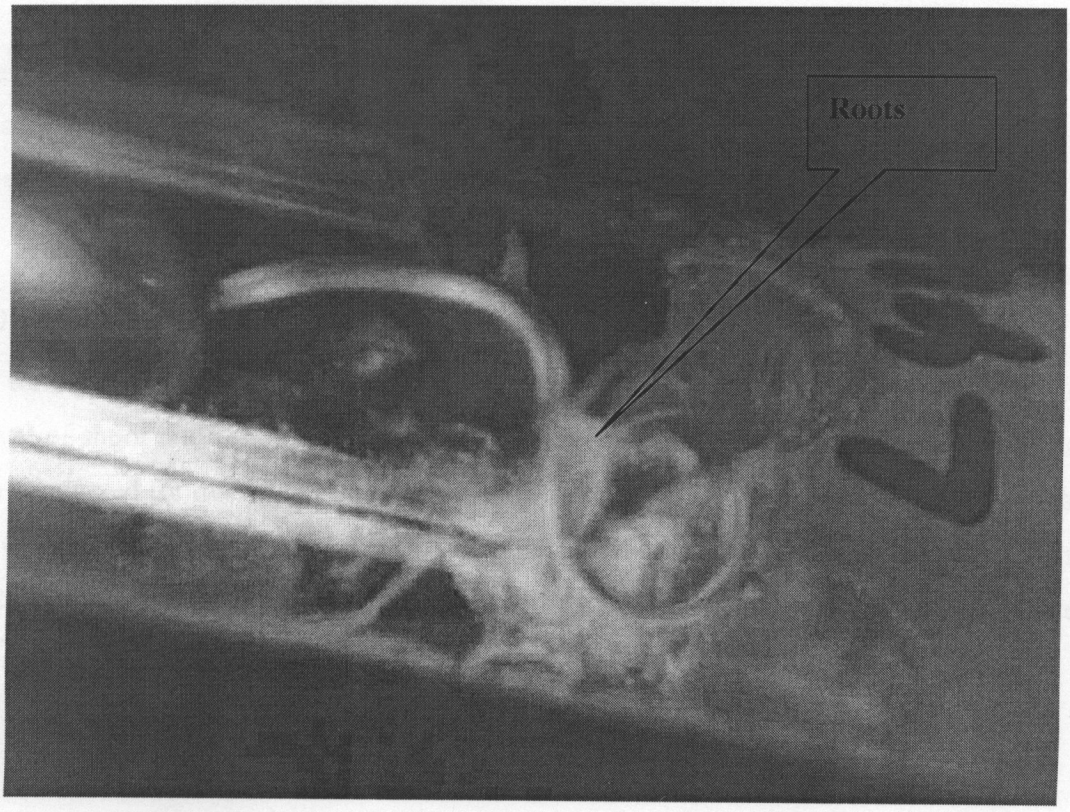

Figure 5. Root formation in castor Line 162 #2 and Line 46 #6

5.0 Conclusion

A protocol for micropropagating varieties 2 (Line 162#2) and 4 (Line 46#6) has been developed and could be adopted or adapted for Castor oil production in the country. However; rooting was the most difficult task especially in other varieties as they differ in their specificity of response to NAA.

6.0 Recommendations

It can therefore be recommended that a study be conducted that will establish up to what level of shoot and rooting hormone can castor respond to tissue culture. In addition, a study be conducted to investigate the impact of different NAA/BAP combination on both shooting and rooting of castor. Also, it can be recommended here that a study be carried out that will establish whether or not root production will be initiated at NAA concentrations lower than 1.0 ppm.

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APPENDICES

APPENDIX 1: ANOVA TABLES FOR PARAMETE STUDIED

ANOVA TABLE 1 FOR NUMBER OF SHOOTS PER EXPLANT.

Source	D.F	Sum of Square	Mean Square	F-value	Prob.
Factor A	1	3.200	3.200	16.000	0.0025
Factor B	4	11.700	2.925	14.625	0.0003
AB	4	4.300	1.075	5.375	0.0142
Error	10	2.000	0.200		

LSD Value = 0.9965 at alpha = 0.050

CV = 24.85 %

ANOVA TABLE 2 FOR NUMBER OF ROOTS PER EXPLANT.

Source	D.F	Sum of squares	Mean Square	F-value	Prob.
Factor A	2	22.351	11.175	164.156	0.000
Factor B	4	28.156	7.039	103.339	0.000
AB	8	62.813	7.852	115.334	0.000
Error	15	1.021	0.068		

LSD Value = 0.5558 at alpha = 0.050

CV = 24.24 %

APPENDIX 2:

TABLE 3 MEAN SEPARATION OF SHOOTING

MEAN	TREATMENT COMBINATION	NUMBER OF SHOOTS
1	M1V1	2.500 AB
2	M1V2	2.500 AB
3	M1V3	0.000 C
4	M1V4	0.000 C
5	M1V5	2.000 AB
6	M2V1	2.000 AB
7	M2V2	2.500 AB
8	M2V3	1.500 B
9	M2V4	2.000 AB
10	M2V5	3.000 A

CV = 24.24 %

Means with the same letter are not significantly different at 5 % level of probability.

TABLE 4 MEAN SEPARATION OF ROOTING.

MEAN	TREATMENT COMBINATION	NUMBER OF ROOTS
1	M1V1	0.000 D
2	M1V2	4.066 B
3	M1V3	0.000 D
4	M1V4	6.500 A
5	M1V5	0.000 D
6	M2V1	0.000 D
7	M2V2	0.000 D
8	M2V3	0.000 D
9	M2V4	0.000 D
10	M2V5	0.000 D
11	M3V1	3.000 C
12	M3V2	2.500 C
13	M3V3	0.000 D
14	M3V4	0.000 D
15	M3V5	0.0790 D

CV = 24.24 %

Means with the same letter are not significantly different at 5 % level of probability.