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***In-vitro* Plant Regeneration Studies of *Arachis hypogaea* L. (Ground nut) for its Heavy Metal Tolerance ($K_2Cr_2O_7$).**

P Krishna Chaitanya, BVM Santhi Priya, NVH Sailesh Babu, P Avinash, S Asha, and R Bharath Kumar.

School of Biotechnology, Vignana's University, Vadlamudi, Guntur-522213 Andhra Pradesh, India.

ABSTRACT

Groundnut (*Arachis hypogaea* L.) is an annual legume, which is also commonly known as peanut, earthnut, monkey nut and goobers. It has been placed by 13th most important food crop and the 4th most important oilseed crops of the world. Groundnut seeds (kernels) contain 40-50% fat, 20-50 % protein and 10-20 % carbohydrate. Groundnut seeds are a nutritional source of vitamin E, niacin, folic acid, calcium, phosphorus, magnesium, zinc, iron, riboflavin, thiamine and potassium etc.[1]. Due to its wide industrial use, chromium is considered a serious environmental pollutant. Toxicity of Cr to plants depends on its valence state: Cr (VI) is highly toxic and mobile whereas Cr (III) is less toxic. Since plants lack a specific transport system for Cr, it is taken up by carriers of essential ions such as sulfate or iron. Toxic effects of Cr on growth of ground nut (*Arachis hypogaea* L) and development include alterations in the germination process as well as in the growth of roots, stems and leaves, which may affect total dry matter production and yield[9]. The absorption capacity of the ground nut (*Arachis hypogaea* L) depends on the pH of the chromium solution[2]. The absorption capacity increases with increase in rate of dilution and minimum at the control conditions.

Keywords: *In-vitro* Plant Regeneration, *Arachis hypogaea* L. Heavy metal tolerance, $K_2Cr_2O_7$

***Corresponding author**



INTRODUCTION

Groundnut

Groundnut (*Arachis hypogaea* L.) is an annual legume which is also known as peanut, earthnut, monkey nut and goobers. It is the 13th most important food crop and 4th most important oilseed crop of the world. Groundnut seeds (kernels) contain 40-50% fat, 20-50 % protein and 10-20 % carbohydrate. Groundnut seeds are a nutritional source of vitamin E, niacin, folic acid, calcium, phosphorus, magnesium, zinc, iron, riboflavin, thiamine and potassium [1,8]. Groundnut kernels are consumed directly as raw, roasted or boiled kernels or oil extracted from the kernel is used as culinary oil. It is also used as animal feed (oil pressings, seeds, green material and straw) and industrial raw material (oil cakes and fertilizer). These multiple uses of groundnut plant make it an excellent cash crop for domestic markets as well as for foreign trade in several developing and developed countries.

Groundnut is one of the few plant species in which a fertilized ovary of aerial flower must be buried in the soil for the fruit (pod) to grow further and mature [9]. After flowering and fertilization above ground, further embryo development and fruit expansion are suspended while an intercalary meristem at the base of the ovary produces a stem-like gynophore [27]. The gynophore, carrying the ovary at its tip, bends and elongates downwards to penetrate the soil. Once the ovary is sufficiently buried (5-10 cm deep), embryo development is resumed and fruit expansion occurs.

Chromium

Chromium (Cr) was first discovered in the Siberian red lead ore (crocoite) in 1798 by the French chemist Vauquelin. It is a transition element located in the group VI-B of the periodic table with a ground-state electronic configuration of $Ar\ 3d^5 4s^1$. The stable forms of Cr are the trivalent Cr (III) and the hexavalent Cr (VI) species, although there are various other valence states which are unstable and short-lived in biological systems. Cr (VI) is considered the most toxic form of Cr, which usually occurs associated with oxygen as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) oxyanions. Cr (III) is less mobile, less toxic and is mainly found bound to organic matter in soil and aquatic environments [9,15]. Contamination of soil and ground water due to the use of Cr in various anthropomorphic activities has become a serious source of concern to plant and animal scientists over the past decade. Cr, in contrast to other toxic trace metals like cadmium, lead, mercury and aluminum, has received little attention from plant scientists. Its complex electronic chemistry has been a major hurdle in unraveling its toxicity mechanism in plants [4,11].

Diseases of Groundnut and their control measures in *in-vivo* and *in-vitro* conditions

Virus Diseases

The major virus diseases are bud necrosis, clump, rosette, peanut stripe, and peanut mottle. Peanut clump virus is transmitted by the fungus *Polymyxa graminis* which may cause damage to crop yield [32].

Nematode Diseases

Several nematodes are parasitic to Groundnut and Chick pea. These are root-knot nematodes (*Meloidogyne arenaria*, *Meloidogyne hapla*, *Meloidogyne javanica*), root lesion nematode (*Pratylenchus brachyurus*), ring nematode (*Macroposthonia ornata*), sting nematode (*Belonolaimus longicaudatus*), and testa nematode (*Aphelenchoides arachidis*) [32].

Bacterial Diseases

Seed rots and seedling diseases are the main bacterial diseases. Many of them are soil habituated fungi, which infects the seed and germinating seedlings of groundnut. They may be identified by fungal spores that give characteristic colorations to the seed, e.g., gray spores indicate *Rhizopus arrhizus* [33].

Foliar diseases

The major foliar diseases caused by fungi are rust (*Puccinia arachidia* Speg.) late leaf spot (*Cercosporidium personatum* recently renamed *Phaeoisariopsis peraanata* Berk & Curt), and early leaf spot (*Cercospora arachidicola* Hori). Rust and late leaf spot are important diseases in India and most of the semi-arid tropic (SAT) regions [31,33].

Selected disease Control Measures for ground nut

- Follow the crop rotation practices, i.e., cereal-cereal-groundnut.
- So we get good quality and disease-free seed.
- Avoid damage to the seed testa and deep placement of seed at sowing.
- Treat the seed with thiram 3 g kg-1 seeds or with carbendazim 2g kg-1 seeds.
- Use cereal-cereal-groundnut crop rotation and seed treatment with thiram.
- Harvest at proper maturity and discard the wilted and dead plants as such plants are likely to have seeds infected by *Aspergillus flavus*.
- Sprays of Bordeaux mixture and dithiocarbamate have been found effective to control rust and late leaf spots. Chlorothalonil 0.2% spray has been found effective against rust and late leaf spot, when sprayed 30 days after germination till 15 days before harvesting at regular 10-15 day intervals. However, this schedule could be modified using a suitable disease forecast system based on temperature, humidity, cloudy weather, and rainfall pattern to save the fungicide and reduce the spray cost. Calixin is effective against rust but not against leaf spots, whereas benomyl is effective against leaf spots but not against rust [12,29].

MATERIALS AND METHODS

Plant Profile

The peanut, or groundnut (*Arachis hypogaea*), is a species in the legume "bean" family (Fabaceae) native to Mexico, South America and Central America. It is an annual herbaceous plant growing 30 to 50 cm (0.98 to 1.6 ft) tall. The leaves are opposite, pinnate with four leaflets. After pollination, the fruit develops into a legume 3 to 7 cm (1.2 to 2.8 in) long, containing 1 to 4 seeds, which forces its way underground to mature. *Hypogaea* means "under the earth" [27]. Peanuts are known by many local names, including earthnuts, ground nuts, goober peas, monkey nuts, pygmy nuts and pig nuts. The term "Monkey nut" is often used to mean the entire pod. (The terms *earthenut*, *groundnut* and *pignut* can also refer to *Conopodium majus* or to tubers of the Bunium family.)

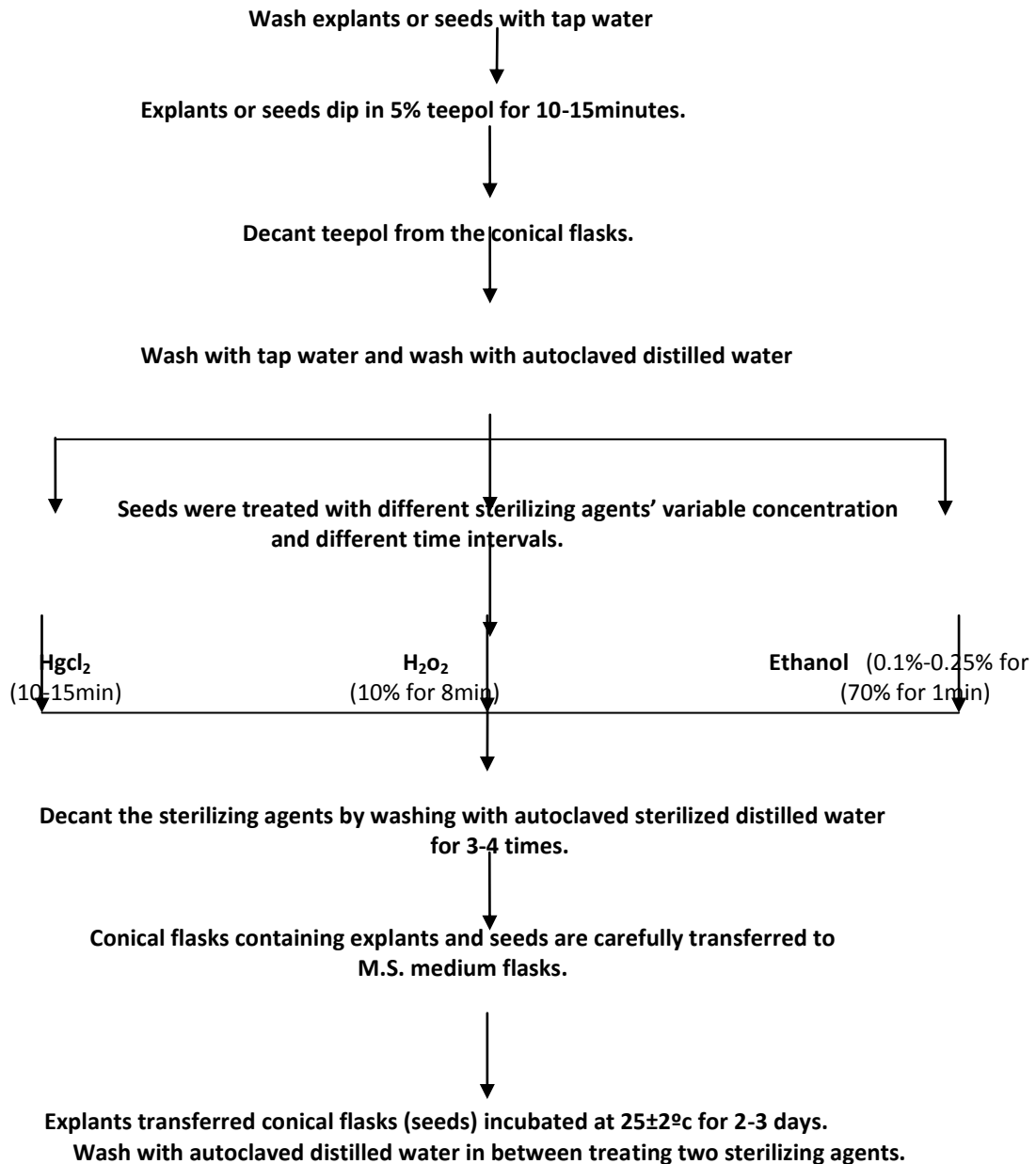
Scientific classification

Binomial name	<i>Arachis hypogaea</i> Linn.
Family:	Fabaceae
Ln/En:	Groundnut, Peanut, Earthnut

The cultivated *Arachis hypogaea* is an allotetraploid 2n=40 with the basic chromosome number of the genes *Arachis* as 10 and most wild species being diploid with 2n=20. In the evolutionary history of peanut species the rate of partitioning to fruit was related to survival in different environments [2,13].

Collections of Explants/seeds:

Fresh and clean seeds or explants of ground nut and chick pea are taken and subjected to surface sterilizations .The surface sterilizations technique is shown in the below flow chart[25].



***In-vitro* Culture Techniques**

Sterilization of Equipments and Glass ware and other necessary materials:

All operations for *in vitro* culture were carried out inside a laminar air flow cabinet under aseptic conditions using sterilized plant materials, equipments, glass materials and chemicals. A horizontal laminar flow cabinet with HEPAfilter was used. The hood surface was wiped clean with paper towel soaked in 70 % ethanol and sterilized by germicidal ultraviolet light for at least 10 min prior to use. All surgical instruments, glassware and other accessories were sterilized in autoclave at 121 °C with15 psi for 30 min and then dried in oven. Surgical instruments like scalpel, forceps, and scissors were sterilized by dipping in 100 % ethyl alcohol and flaming prior to use[20,25].

Culture Room

The explants were incubated in a culture room where the temperature was maintained at 25-26 °C, humidity at 85 % and either under continuous dark or under a photoperiod of 16 h light(2000 flux) and 8 h dark.

Preparation of Culture Media

Media-making can be time-consuming. Nowadays the plant tissue culture media most commonly used are available in the market as dry powders. The simplest methods of preparation media is to dissolve these powders contain inorganic and organic nutrients in some quantity of distilled water. After the content have been thoroughly mixed in water, sugar and agar other organic supplements are added. Finally, the volume is made up to one lt. The pH is adjusted and the medium autoclaved[19,22].

Powdered media are useful for propagation of plant species requiring nutrients according to the recipe of standard media. In experiments in which changes in the quantity and quality of media constituents become necessary, it is desirable to weight and dissolve each ingredient separately before mixing them together. Another convenient procedure is to prepare stock solutions which, when mixed together in appropriate quantities, constitute a basal medium. Four stock solutions are prepared, consisting of

- Major salts
- Minor salts
- Iron
- Organic nutrients except sucrose

MS (Murashige and Skoog 1962) inorganic salts, organic supplements and vitamins, they were used as basal media for seed germination, callus induction, callus multiplication, shoot and root induction. The formulation and composition of MS medium is given in below[25,26].

Preparation of Stock Solution/s

Stock solutions of the major components, such as macronutrients, micronutrients, vitamins, and plant growth regulators of the media were prepared and stored in refrigerator

Stock solution-1

MgSO ₄ .7 H ₂ O	-	370mg/lt
CaCl ₂ .2 H ₂ O	-	440mg/lt
KNO ₃	-	1900mg/lt
NH ₄ NO ₃	-	1650mg/lt
KH ₂ PO ₄	-	170mg/lt

Stock solution-2

MnSO ₄ .4H ₂ O	-	22.3mg/lt
ZnSO ₄	-	8.6mg/lt
CuSO ₄ .5 H ₂ O	-	25µg/lt (0.025mg/lt)
H ₃ BO ₃	-	6.2mg/lt
Na ₂ MoO ₄ .2 H ₂ O	-	0.25mg/lt

Stock solution-3

Add -83mg/lt (or) 0.83g/lt -KI into 100ml Dist.H₂O.

Stock solution-4

Dissolve - 373mg Na₂ EDTA/279mg of Fe.SO₄.7 H₂O - in 50ml of dist.H₂O.

Stock solution-5

Vitamins		Actual Conc.(mg/lit)	100X(mg)
Gltcine	-	2mg	200
Tymine	-	0.1mg	10
Pyridoxine HCL	-	0.5mg	50
Nicotinic acid	-	0.5mg	50

Stock solution-6

Dissolve 1000mg in 50ml distilled water
Use 5ml to prepare 1000ml of culture medium.

Media

- Sucrose -20to30gm/lit
- P^H -5.6 to 5.8
- Agar –Agar -4to8 (as per the requirement)

To prepare 1lt of media has stocks to be taken as –stock1-50ml, stock2-1ml, stock3-1ml, stock4-5ml, stock5-1ml, stock6-2ml.

Growth Regulators

Auxins and cytokinins were the two major phytohormones are taken in different concentrations and combinations in various media for induction and growth of callus, root and shoot[20,23].

Auxins

Powders of auxin were dissolved in 1N NaOH and made up the volume with sterilized distilled water and then used or stored in freezer as stock for further use. One auxin/s is used in the present study were 2, 4-Dichlorophenoxyacetic Acid (2, 4-D) and Two different concentrations (2 and 4µM) of 2, 4-D were tested in MS medium[18,20].

Cytokinins

Two different cytokinins 6-Benzyl adenine (6-BA) and Kinetin (Kn) are taken and they were dissolved in 1N NaOH and make in to them for the required volume solution. They were kept in refrigeration and stored for further utilization[20,23].

Two concentrations of 6 BAP (2 and 4 µM) and two concentrations of Kn (5µM and 10µM) were added to the basal medium of MS salts[9,25].

Seed Germination

The sterilized seeds were given a cut at the non-embryo side and placed in petri dishes, test tubes embryo side up in a hormone-free MS medium solidified with agar 0.8 % (w/v). For shoot multiplication one accession was used.

Inoculation of Explants & Seed Material

The seeds of Ground nut (*Arachis hypogaea* L) are inoculated in the M.S medium of about 30 test tubes and incubated at 25±2°C. The seeds were germinated in a conical flask containing MS basal medium in dark hypocotyls, epicotyls, axillary bud, immature leaves and cotyledonary nodes of 10 days old seedlings were used as explants. Immature embryos were dissected out from sterilized pods 35-45 days after pollination[11,14].

Seedlings of explants were cultured on MS salts supplemented with B5 vitamins and various growth hormones. The nutrient media consists of major and minor salts, according to Murashige and Skoog (1962), B5 vitamins (Gamborg et al. 1968), 3.0% (W/V) Sucrose and 0.7% (W/V) Agar. It was variously supplemented with 6-Benzyl adenine (6-BA) and Kinetin (Kn) [20,25.]

Shoot Multiplication and Maintenance

The explants were sub cultured onto fresh media every 15 days. When the explants started to multiply, well grown axillary shoots were separated with the help of a sterile scalpel under the hood and put in the same media for further multiplication. The shootlets derived from each seed were tracked individually to determine the total number of plants produced from single seed and their subsequent genetic identity [6,21]. The explants were inoculated in different concentrations of growth hormones of MS basal medium combinations are given here:

1. MS medium+6-BA
2. MS-medium+2, 4-D
3. MS-medium+ (Kn)
4. MS-medium+6-BA+2,4-D
5. MS-medium+2,4-D+ (Kn)
6. MS-medium+6-BA+ (Kn)
7. MS-medium+6-BA+2, 4-D+ (Kn)
 - 2, 4-D- 2, 4 Dichloro pheenoxyacetic acid, 6-BA-6 benzyl adenine, (Kn)- Kinetin

Uptake and Estimation of chromium in shoots

The germinated shoots are sub cultured in different dilutions of Chromium (Chromium is prepared at 1 molar with dilutions of 10^{-1} to 10^{-9}) in above mentioned combinations (A,B,C,D,E & F).

The sub cultured samples are subjected to A.A.S to determine the toxic effect on the selected plant species (ground nut) [24,26].

RESULTS AND DISCUSSION

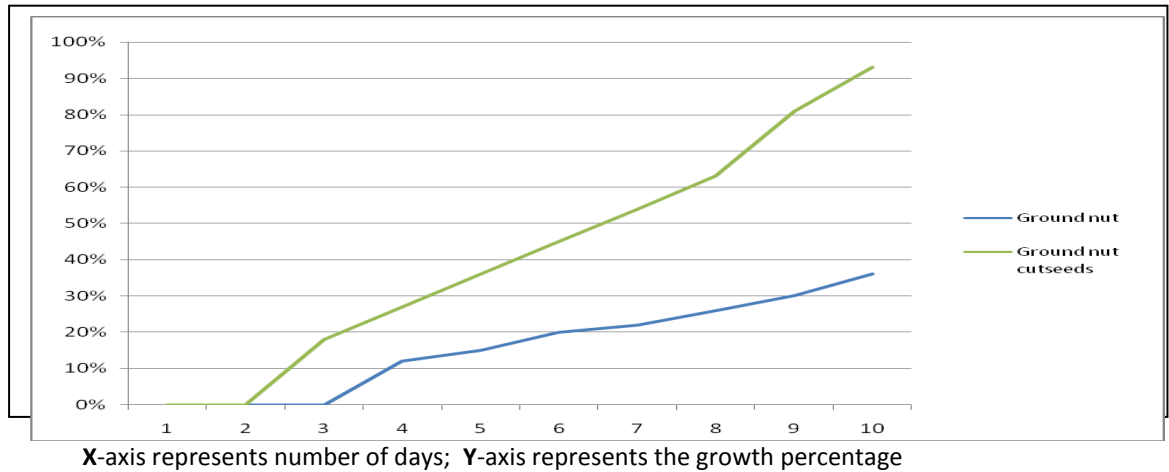
The present study was conducted to investigate optimal concentrations and combinations of plant growth regulators in the medium for efficient micro propagation in ground nut via nodal shoot culture, to determine the effect of Chromium in the selected plant species.

Seed Germination

Seeds of one accessions of ground nut and chickpea were used for germination test in the below table. The seeds were placed in MS medium without any plant growth regulator. When a cut was given to the seed at the opposite end to the embryo, there was remarkable increase in the percentage of germination. For example, 93 % of cut seeds of ground nut germinated after 10 days, whereas only 36 % germination was noticed for uncut seeds [11,22].

Growth percentage of Ground nut for 10 days

S.No.	Name of the plant species	Part of the plant	Day of inoculation	Percentage of Germination(days)										Contaminated		
				1 st	2 nd	3 rd	4 th	5 th	6 th	7 th	8 th	9 th	10 th	Petri dishes	Test Tubes	Conical flasks
1.	Ground nut	Seed	25.01.10	--	--	--	12%	15%	20%	22%	26%	30%	36%	3	---	---
3.	Ground nut	Cut Seed/s	08.02.10	--	--	18%	27%	36%	45%	54%	63%	81%	93%	2	4	2



Shoot Multiplication in Ground nut explants/seeds

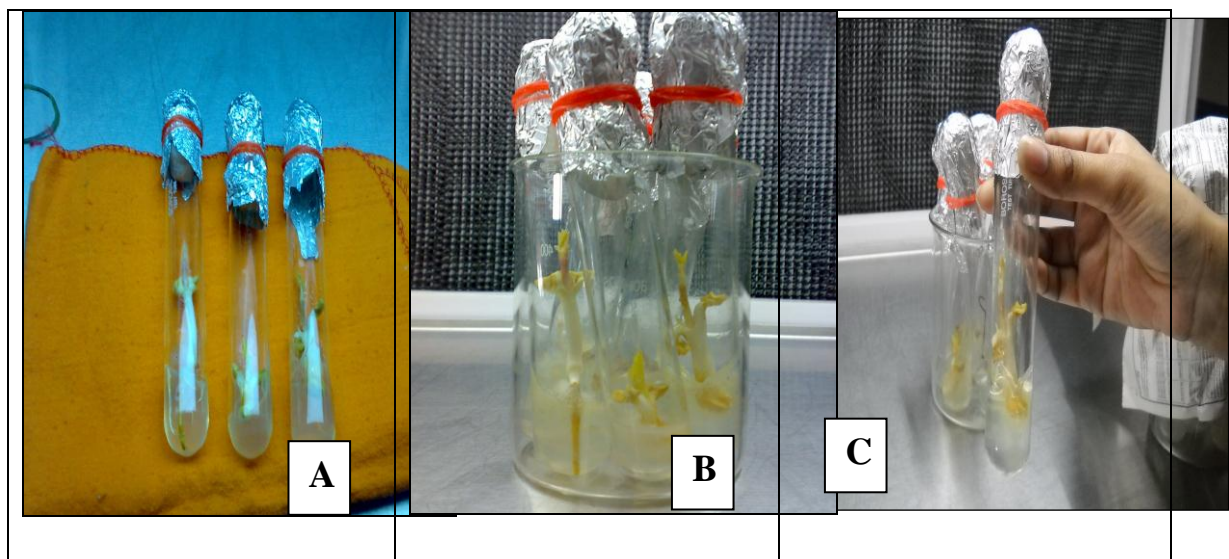
In a preliminary experiment of shoot multiplication from cotyledonary nodal explants, two accessions were tested with seven different M.S medium-combinations. There was no response in 2, 3, 5 (detailed in material and methods), where only vertical growth was observed. On the other hand, 1, 4, 6 and 7 media responded well to shoot multiplication. Eventually, two accessions were used for shoot multiplication in three media i.e., M.S+1, M.S+4, and M.S+6&7. The Responses of Shoot Multiplication in Ground nut are show in the table below[19,25].

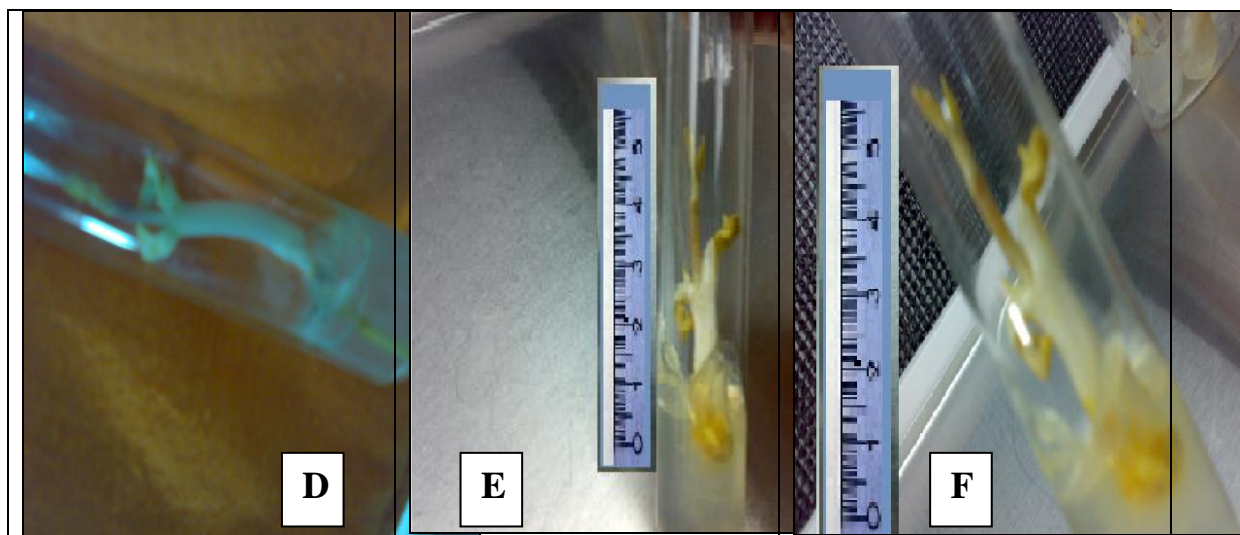
Growth response of Ground nut in different media Compositions

S.No.	Name of the plant species	Part of the plant	M.S+1	M.S +4	M.S+6	M.S+7	No of test tubes Inoculated	Response plant species of different combinations				Contami- nated
								M.S+1	M.S+4	M.S+6	M.S +7	
1.	Ground nut	Germinated seeds	2ml	1:1	1:2	1:2:4	10	93%	98%	90%	95%	2

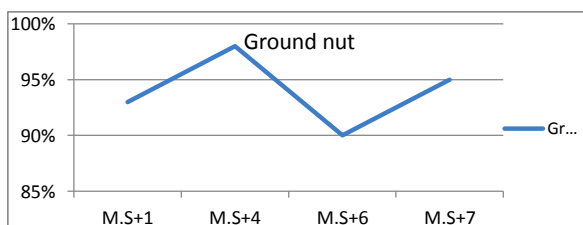
[Where as M.S+1= MS medium+6-BA, M.S+4= MS-medium+6-BA+2,4-D, M.S+6=MS-medium+6-BA+(Kn), M.S+7=MS-medium+6-BA+2,4-D+(Kn)].

In-vitro growth and development of Groundnut seeds (MS Salts + Supplements) on the medium (A,B,C,D,E &F)





Growth Curve of Ground nut



[X-axis represents the M.S medium with different combinations (M.S+1= MS medium+6-BA, M.S+4= MS- medium+6-BA+2,4-D, M.S+6=MS-medium+6-BA+(Kn), M.S+7=MS-medium+6-BA+2,4-D+(Kn))
Y-axis represents the growth percentage of ground nut]

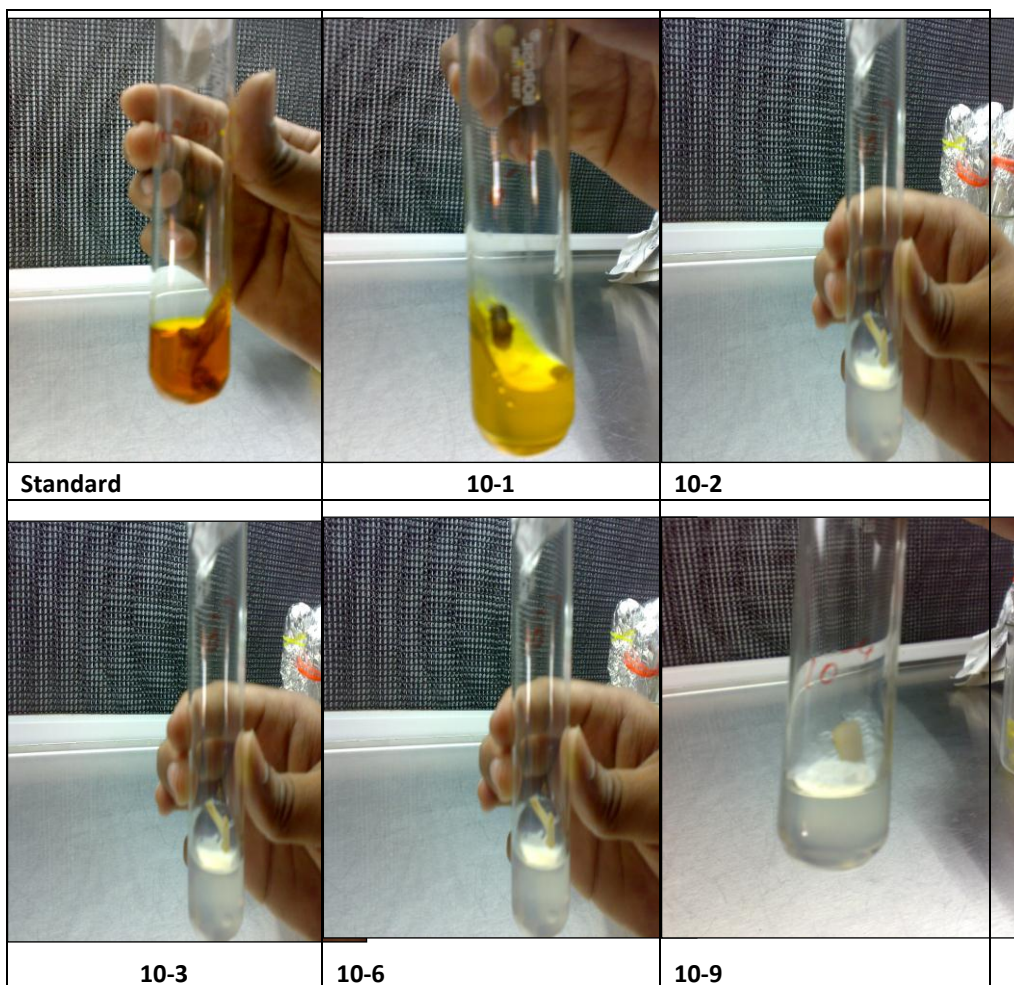
Estimation of Chromium

The Germinated plants species of Ground nut are surface sterilized and inoculated in to the media having the different dilutions of 10^{-1} to 10^{-9} . Selected media is taken i.e., M.S medium with 6BA and 2,4-D as mentioned in the above graph. Growth observed in the Chromium augmented media is shown in the table below. Chromium levels are estimated my submitting these Chromium Dilutions Samples to A.A.S (Atomic Absorption Spectrophotometer)[24,29].

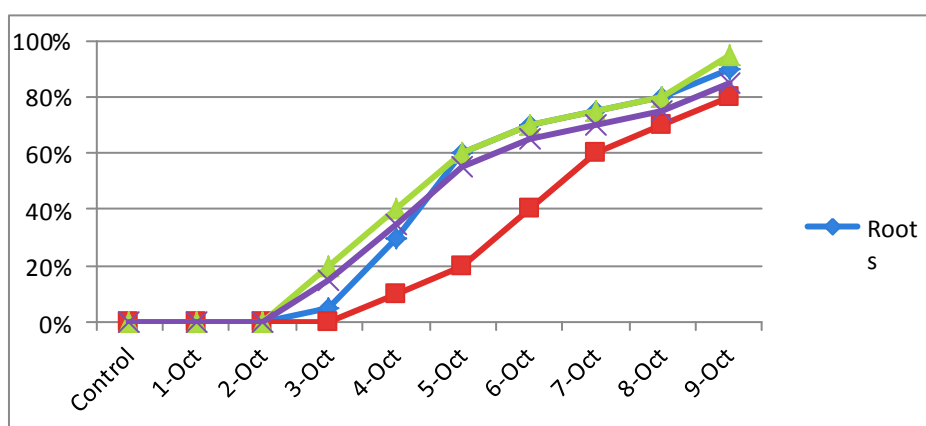
Growth of Ground nut in different dilutions of Cr

S.No.	Name of the plant species	Part of the Explants	M.S medium +6BA+2,4-D +Cr dilutions									
			Control	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
1.	Ground nut	Roots	----	----	----	5%	30%	60%	70%	75%	80%	90%
2.	Ground nut	Stem	----	----	----	----	10%	20%	40%	60%	70%	80%
3.	Ground nut	Seeds	----	----	---	20%	40%	60%	70%	75%	80%	95%
4.	Groundnut	Nodules	----	----	----	15%	35%	55%	65%	70%	75%	85%

Different phases of growth of Ground nut explants in different dilutions of Chromium is shown in the following figures



Growth curve of the Ground nut at different dilutions of Cr



[X-axis represents the Cr dilutions in the selected media (M.S+6BA+2,4-D) the dilutions ranging from 10^{-1} to 10^{-9} .
Y-axis represents percentage of ground nut growth at different dilutions of Cr.]

Uptake and accumulation of Cr on plant parts by using AAS:

Different dilutions in Chromium (Cr)	Root	Nodules	Stem	Seed
Standard	-----	-----	-----	-----
10-1	0.68	0.71	0.58	0.10
10-2	0.70	0.73	0.62	0.14
10-3	0.74	0.76	0.68	0.18
10-4	0.84	0.86	0.73	0.21
10-5	0.92	0.94	0.83	0.23
10-6	0.95	0.97	0.90	0.25
10-7	0.99	1.00	0.96	0.28
10-8	1.02	1.04	0.99	0.32
10-9	1.08	1.10	1.06	0.36

Uptake and Accumulation of Cr in

Roots

Chromium content of the root for different dilutions is recorded in the above table. Maximum chromium content of ground nut is observed in 10^{-9} (1.08) dilution and minimum is observed in control plants.

Nodules

Chromium content of ground nut for root is recorded in the above table. Maximum chromium content of ground nut is observed in 10^{-9} (1.10) dilution and minimum is observed in control plants.

Stem

Chromium content of ground nut for stem is recorded in the above table. Maximum chromium content of ground nut is observed in 10^{-9} (1.06) dilution and minimum is observed in control plants.

Seeds

Chromium content of ground nut for seeds is recorded in the above table. Maximum chromium content of ground nut is observed in 10^{-9} (0.36) dilution and minimum is observed in control plants.

CONCLUSIONS

Groundnut (*Arachis hypogaea* L.) is an annual legume which is also known as peanut, earthnut, monkey nut and goobers. It is the 13th most important food crop and 4th most important oilseed crop of the world. Groundnut seeds (kernels) contain 40-50% fat, 20-50 % protein and 10-20 % carbohydrate[1,2].

The present investigations were carried out on an important legume plants-Ground nut (*Arachis hypogaea* L). The main objectives of the present investigations were: To determine the heavy metal Tolerance (Chromium) of ground nut[5,30], to get disease-free plants that will lead to qualitative improvement of the crop and to check the toxic levels of chromium in *in-vitro* conditions at different concentrations[3,4].

All operations for *in vitro* culture were carried out inside a laminar air flow cabinet under aseptic conditions using sterilized plant materials, equipments, glass materials and chemicals described in standard literature source. The seeds of Ground nut (*Arachis hypogaea* L) are inoculated in the M.S medium of about 30 test tubes and incubated at $25 \pm 2^\circ\text{C}$. The seeds were germinated in a conical flask containing MS basal medium in dark hypocotyls, epicotyls, axillary bud, immature leaves and cotyledonary nodes of 10 days old seedlings were used as explants and they were maintained for under *in-vitro* conditions for further analysis of growth and germination percentage in regular intervals[6,7].

The present study was conducted to investigate optimal concentrations and combinations of plant growth regulators in the medium for efficient micro propagation in ground nut via nodal shoot culture. To determine the effect of Chromium in the selected plant species[5,9].

These explants were observed after incubation of 1 to 2 weeks of duration, their % germination results are given respectively in the table (table no. 1&2). The germination of groundnut explants in the combination of M.S+4 medium, were expressed considerable response (i.e. 98%), when compared with other combinations[10,20].

The heavy metal contamination is an alarming issue known a days due to the environmental pollution particularly the various industrial effluents and their residues will causing severe damage to the productivity of lands in at various levels which leads the severe damage to the plants in terms of yield, diseases etc to overcome these problems, phytoremediation is an emerging cleanup technology, can be implemented by using the selected microorganisms (*Chlorobium*, *Thiobacillus Rhizibium* sp.), grasses (aquatic grasses like *Typa*, *Cynodon* etc) or higher plants can be grown along with the crop plants in the fields, which can able reduce the effects heavy metals on food crops[12,13,14].

As per the modern aspects and developments in the field of biotechnological methods like Protoplast fusion, genetic engineering and in-vitro propagation technologies, can facilitates for the development of the transgenic strains of the species belongs to the promising food crops against to the heavy metal tolerance, salt tolerance and drought resistance etc. to meet our future needs of adequate food resources.

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