RESEARCH ARTICLE

AN ISOLATION STUDY OF MICROORGANISM FROM DIFFERENT STAGE OF *IN VITRO* PROPAGATION

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Abstract: Fungi and bacteria contaminants were found associated with the cultured plant materials. The bacterial isolates include *staphylococcus aureus, Gram positive, staphylococcus epidermis, staphylococcus aureus, Gram positive*. And fungal contaminants include *Fusarium sp., Aspergillus niger, chaetomium sp., Acremonium sp., Aspergillus sp.* Microbes are living, biological contaminants that can be transmitted by infected people, animals and indoor air, and they can also travel through the air and get inside homes and buildings. It was discovered that the microbial population is higher in the preparatory room than the incubating rooms. This might be unconnected with the fact that more people frequent the preparatory room. Flaningan and Morey (1996), reported that presence of bacteria in a room indicate the presence of people and their levels may get high when the building is heavily populated. Consequently, adequately training of operators and high standards laboratory cleanliness is a vital pre-requisite to successful plant tissue culture. Most of these bacteria contaminants have been reported to increase culture mortality and the presence of latent infections can result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting (Kane, 2003) The fungi were identified on the bases of morphological characteristic such as colony form and color, type of mycelium, fruiting bodies and spores (Commonwealth Mycological Institute Descriptions of Pathogenic Fungi and Bacteria. The present research study aims to a) examine the possible bacterial contamination of plant tissue cultures initiated from medicinal plants of Sinai, b) identify the bacterial species contaminating those plant cultures, c) determine the antibiotic sensitivity of those covert bacteria and d) suggest the best strategy to minimize loss of plant materials during short- and long-term cultures.

Keyword: Contamination, Culture, Fungi, Bacteria

INTRODUCTION

Plant tissue culture is the *in vitro* technique of growing "sterile" plant cells, tissue or organs separate from the mother plant on artificial/synthetic medium. It (micro-propagation) is a tool, which allows the rapid production of many genetically identical plants using relatively small amounts of space, supplies and time. Among various uses an important technique for rapid multiplication of plant materials from tissue and cells of desirable plants. Basically the technique consists of taking a piece of a plant (such as a stem tip, node, meristem, embryo, or even a seed) and placing it in a sterile (usually gel-based) nutrient medium where it multiplies. The formulation of the growth medium depends upon whether it is intended to produce undifferentiated callus tissue, multiply the number of plantlets, grow roots, or multiply embryos for "artificial" seed. Micropropagation, an in vitro vegetative propagation method using pathogen-free propagules, has been considered significant in agriculture and forestry for producing pathogen-free stock plants or genetically superior clones that cannot be propagated by seeds or whose propagation efficiency is low in conventional vegetative propagation.

However, the widespread use of micropropagated transplants is still limite by high production costs, mostly attributed to a low growth rate, a significant loss of plants in vitro due to microbial contamination, poor rooting, low percent survival at the *ex vitro* acclimatization stage and high labor costs. Recent research, however, has revealed that most chlorophyllous explants/plants *in vitro* have the ability to grow photoautotrophically (without sugar in the culture medium), and that the low CO2 concentration in the air-tight culture vessel during the photoperiod is the main cause of the low net photosynthetic and growth rates of plants in vitro.

Although aseptic conditions are usually employed but many plant cultures do not stay aseptic *in vitro* as they get contaminated. Contamination with micro-organism is considered to be the single most important reason for losses during *in vitro* culture of plants, such micro-organisms include viruses, bacteria, yeast, fungi, mites and trips have been shown to be harmless to the plant though they introduce fungi, yeast and bacteria into sterile plant culture which are considered harmful to the plant cultures. The nutrient media in which the plant tissue is cultivated is a good source of nutrient for microbial growth.

These microbes compete adversely with plant tissue culture for nutrient. The presence of these microbes in these plant cultures usually results in increased culture mortality, the presence of latent infections can also result in variable growth, tissue necrosis,

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reduced shoot proliferation and reduced rooting (Kane, 2003).

The main prerequisites for plant cell and tissue culture initiation are appropriate explants). Therefore, plant cell and tissue cultures are usually considered to be free of microorganisms. However, there have been cases where covert bacterial contamination has been present in tissue cultures without affecting growth or being macroscopically visible . Contamination could occur at any point in the tissue culture operation. The focus should be to keep the incidences to the absolute minimum. A diverse range of bacteria may contaminate plant tissue cultures including: plant pathogens, epiphytes, endophytes and accidental contamination from air or from human during handling (Stead et al. 1998; Nowak et al. 1998). Plant endophytes and pathogens may often cause more obvious symptoms than saprophytic and other contaminant not normally considered pathogens of plants (Fellner *et al.* 1996). A range of other organisms may also contaminate plant tissue cultures (Williamson *et al*. 1998). A substantial list of bacteria, fungi and insects that contaminate cell cultures was reported (Leifert & Woodward 1998). Many of these are regularly associated with plant tissues and are difficult to detect because they need the knowledge of appropriate culture media and culturing conditions.

In general, some plant/ microbe relationships are physiologically meaningful to the plant. For example, the symbiotic interaction of Soybean/ Bradyrhizobium japonicum in nitrogen fixing roots nodules. Also, the genetic transformation of plant tissues by Agrobacterium spp. is beneficial to the plant. On the contrary, tissue cultures may experience unappreciated plant/ microbe relationships (Wildholm 1996). Covert bacterial contamination of plant tissue cultures may represent the unappreciated kind of interactions between plants and bacteria that take place routinely in lab benches and in culture vessels (Yang 1989).

MATERIALS AND METHODS

Preparation of MS Media

MS medium were categorized as stock with their solubility and stability such as A,B,C,D,E and F.nStock A was prepared from major components 82.5 gm NH_4NO_3 dissolved in 10 to 50 ml distilled water and shaked well with magnetic stirrer and make the final volume up to 1000 ml using distilled water. Stock B was prepared from major components 95.0 gm KNO_3 dissolved in 10 to 50 ml distilled water and the make the final volume up to 1000ml using distilled water. Stock C was prepared from minor components (I) 34.0 gm KH_2PO_4 dissolved in 10ml distilled water and added 30ml distilled water, (II) 1.24 gm H_3BO_3 dissolved in 10 ml distilled water the added 20 ml distilled water, (III) 0.05 gm $Na_2Mo_4.2H_2O$ dissolved in 5ml distilled water mixed properly and added 15ml distilled water, (IV) 0.166 gm KI dissolved in 5ml distilled water and added 15ml distilled water properly, (V) 0.005gm $CoCl_2.6H_20$ dissolved in 2 ml distilled water and the solution I,II,III,IV & V mixed and make the final volume 1000ml by using distilled water. Stock D was prepared from 88.0gm $CaCl_2.2H_2O$ dissolved in 10ml distilled water and make the final volume 1000ml by using distilled water and mixed properly. Stock E was prepared from minor components (I) 74.0 gm $MgSO_4.7H_2O$ dissolved in 10ml distilled water and made 120 ml by using distilled water, (II) 3.38gm $MnSO_4.4H_2O$ dissolved in 10ml distilled water and made 120ml by using distilled water, (III) 1.72gm $ZnSO_4.7H_2O$ dissolved in 10ml distilled water and made 120ml by using distilled water, (IV) 0.005gm $CuSO_4.5H_2O$ dissolved in 2ml distilled water and made 50ml by using distilled water. And solution I, II, III and IV mixed and made final volume 1000ml by using distilled water. Stock F was prepared by dissolving two component, component I, 7.46 gm Na_2-EDTA with 10ml distilled water and made 400ml by using distilled water. Component II, 5.50 gm $FeSO_4.7H2O$ in separate container with 10ml distilled water and made 400ml by using distilled water. Solution of $FeSO_4.7H_2O$ was slowly poured into stirring solution of Na_2-EDTA using magnetic stirrer and made volume 1000ml. Stirring was continued for 3-4 h at 60˚C temperature till pale yellow colour appeared of the stock solution.

Stock solutions of vitamins, Thiamine-HCl, Nicotinic acid and Pyridoxine-HCl were prepared as 1000 ppm (1mg/ml) concentration in double distilled water. Glycine stock solution was prepared as1000ppm (1mg/ml) concentration in double distilled water. In order to prepare stock solution of Auxines (IAA, IBA, NAA and 2,4-D), ethanol or freshly prepared 1N NaOH were used as a solvent and for cytokinins (Kn and BAP),1N HCl was used as a solvent. Auxins and Cytokinins stocks were prepared as 1000 ppm (1mg/ml).All the media stock solutions (A to F), Vitamine, amino acid and growth hormones were stored at 10˚C temperature in refrigerator. Other media were prepared by direct weighing of individual chemicals without preparation of stock solutions. Required quantity of myo-inositol, sucrose, additive and agar-agar were added directly in the medium. For the preparation of medium, measured volume of distilled water was boiled in conical flask using microwave oven. MS medium, stocks were added in the boiled distilled water, followed by sucrose and myo-inositol. Agar-agar was added slowly with continoues and gentle shaking of the flask to avoid clumping of agar. Growth hormones were used as per the requirement of experiment. Media pH was adjusted to 5.8 without additives and 6.2 with additives using freshly prepared NaOH (1N) and HCl (1N). Sterilization of media was carried out in autoclave at 15 psi, 121˚C temperature for 15 minute using vertical autoclave.

Composition

Potato dextrose agar

Ingredients		gm /liter
Potato infusion from	-	200.0
Dextrose	-	20.0
Agar	-	15.0
Potato dextrose Agar	-	39.0
Distilled Water	-	1000

pH of the media is adjusted to 5.6 using 1N NaOH and 1N HCl. Then medium was autoclaved at 121˚C for 15minute.

Nutrient agar media

Ingredients		gm/ liter
Peptone	-	5.0
Sodium extract	-	5.0
Beef extract	-	1.5
Yeast extract	-	1.5
Agar	-	15.0
Nutrient agar	-	28
Distilled Water	-	1000ml

pH of the media is adjusted to 7.4 using 1N NaOH and 1N HCl. Then medium was autoclaved at 121˚C for 15minute.

PDA media preparation

100 ml PDA media was prepared by adding PDA 3.9 gram and agar 1.5 gram dissolved in 10 ml distilled water and made 90 ml by distilled water. Then adjusted pH 5.2 with help of 1N HCl or 1N NaOH and autoclaved at 15 psi for 121˚C temperature.

NAM media preparation

100 ml NAM Media was prepared (NAM 2.8 gm) as per the composition then maintain pH 7.4 with help of 1N HCl or 1N NaOH and autoclaved the media at 121ºC for 15 min hold steam under15 lbs pressure.

Stain

Gram staining :- Gram's Iodine , Crystal Violet and Saffranin.

Fungal staining :- Cotton Blue

Cotton blue solution :- 1gm of cotton blue mixed with 100 ml of distilled water .

Antibiotic

Antibacterial-Tetracycline, Chloramphenicol,

Antifungal-Ketocanazole

Stock Solution of Antibiotics

Tetracycline - 5 mg tetracycline dissolved in 1 ml ethanol. Filtered and Sterilized.

Cefotaxime- 200 mg Cefotaxime dissolved in 1 ml ethanol .Filtered and Sterilized.

Cholorompheni**col:-** 34 mg Choloropenical dissolved in 1ml ethanol. Filtered and Sterilized.

Ketocanazole**:-**These antifungal are used to reduce fungal contamination .2 mg Ketocanazole dissolved in 1 ml distilled water. Filtered and Sterilized.

Methods

Explant collection, Sterilization and Inoculation

Explants were collected from fresh plants and transferred in polythene covers, kept in ice box and brought to Tissue culture laboratory. The collected shoots were cut into nodal segments and washed with tap water, followed by treatment with 1% Bavistine for 4-5 minutes to reduce fungal contamination, followed by washing 3-4 times by distilled water. Followed by treatment with Tween20 for 10minute to remove dust from explants surface and washed with distilled water for 4-5 times. Explants were surface sterilized under aseptic condition in laminar air flow. Surface sterilization were carried out by using 0.2% Mercuric Chloride for 4-5minute followed by distilled water for 3-4 times .This is followed by washing with 70% ethanol for 15-20Sec and finally 3-4times by distilled water. Surface sterilized explants were inoculated vertically in the medium for shoot induction and leaf horizontally on the surface of the medium for callus induction. Culture was kept at $25_{+\_}$ 2C in culture room at 2500lux light intensity for 12h photoperiod.

Isolation of microbial contaminants

From the contaminated plant tissue culture bottles, emerging microbes were isolated by inoculating them on respective media. Sterilized NAM was poured in petriplates and kept for 24 h. Then petriplates containing solidified PDA was inoculated with culture of Albizia procera, Nepenthes khasiana, Rauwolfia serpentine, Orchid and Bacopa Monnieri. Then all petri plates were kept for incubation for 2 day and observed for bacterial growth. Sterilized PDA media was poured in petriplates and kept for 24 h. Then petriplates containing solidified PDA was inoculated with culture of Albizia procera, Nepenthes khasiana, Rauwolfia serpentine, Orchid and Bacopa Monnieri. Then all petri plates were kept for incubation for 7 day and observed for fungus. Pure isolates obtained from repeated sub-culturing of the isolates which were placed in an agar slant and stored at 40C in a refrigerator.

Identification of Microbes

Bacterial isolates were identified using microscopic and morphological characteristics. Microscopic identification using gram`s staining. All bacteria was stained with gram`s staining method after bacterial staining observe under microscope purple colour shows the gram positive bacteria Or pink colour shows the gram negative bacteria. Bacterial smear are prepared on a glass slide and thereafter heat fixed. A drop or two of crystal violet is put on the smear. After 1-2 minutes the slide is washed with tap water to remove excess of stains. The washed slide is subjected to few drops of grams iodine and waited for 1- 2 minutes and thereafter washed with tap water for decolorization. The slide is put in 95% ethanol. Safranin is added to the slide after 1-2minutes and washed with tap water. The slide was observed under the microscope for the colour of bacterial colony.

The fungal isolates were identified using cultural characters, morphology, microscopic and by comparison with standards (Barnet and Hunter, 1972). Fungal colony were taken in clean glass slide then added 1-2 drop of cotton blue and fungal colony then observed under the microscope.

Antibiotic Sensitivity Test

Antibiotic test was performed on bacterial and fungal isolates.

Antibacterial test preparation

800 ml NAM Media was prepared as per the composition then maintain the media pH 6.2 (NAM) with help of 1N HCL solution or 1N NaOH and divided media in eight parts of 100ml and then autoclaved the media at 121ºc for 15 min hold steam under pressure. After autoclaving cooled the media at 45ºc and added 50µl Streptomycin, Penicillin, tetracycline and chloramphenicol in each 100ml media and 100µl Streptomycin, Penicillin tetracycline and chloramphenicol in each 100ml media. Then poured the media in petriplates and after solidifying, streaking the staphylococcus gram positive bacteria and incubated in 28c for 2 days and observed for the growth.

Antifungal test preparation

400 ml PDA Media was prepared as per the composition then maintain the media pH 6.2 (PDA) with help of 1N HCL solution or 1N NaOH and divided media in four parts 100ml and then autoclaved the media 15 lb at 121ºc for 15 min hold steam under pressure. After autoclaving cooled the media at 45ºc and added 50µl, 100µl, 200µl,400µl Ketoconazole, Gliseofuluin and Fluconazole in respective media respectivelly. Then poured the media in petriplates and after solidification, inoculated the fungus and incubated in 37c for 7days and observed for the growth.

RESULTS AND DISCUSSION

The inoculated explants showed good growth on MS media and along with that some fungal and bacterial contaminant were found associated with cultured plant materials. The rate of fungal isolates was higher than that of bacterial isolates. Ten microbial contaminants (consisting of five bacteria and five fungi) were found associated with the tissue culture plants and the laboratory environments (Table 1 and 2) The bacterial contaminants includes, *Staphylococcus aureus*, *Staphylococcus epidermis* and some Gram's positive bacteria While Fungi isolates were *Fusarium sp., Chaetomium sp., Acrimonium sp., Aspergillus niger and Aspergillus species*.(Fig.1-9)

Microbes are living, biological contaminants that can be transmitted by infected people, animals and indoor air, and they can also travel through the air and get inside homes and buildings. It was discovered that the microbial population is higher in the preparatory room than the incubating rooms. This might be unconnected with the fact that more people frequent the preparatory room. Flaningan and Morey (1996), reported that presence of bacteria in a room indicate the presence of people and their levels may get high when the building is heavily populated. Consequently, adequately training of operators and high standards laboratory cleanliness is a vital pre-requisite to successful plant tissue culture. Most of these bacteria contaminants have been reported to increase culture mortality and the presence of latent infections can result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting (Kane, 2003) The fungi were identified on the bases of morphological characteristic such as colony form and color, type of mycelium, fruiting bodies and spores (Commonwealth Mycological Institute Descriptions of Pathogenic Fungi and Bacteria, 1996). In present investivagation fungal and bacterial contaminants were found associated with the six different culture plant materials.

The fungi identified in this study were observed to utilize the nutrients of the plant tissue culture medium for their metabolism and usually outgrown the culture materials. Blake (1994) has reported that thorough disinfection and strict hygiene in the laboratory have achieved effective control of microbial contaminants. Other sources of contamination include non-adequate decontamination of explants, which allows the carryover of associated organisms from the field, or from the compost manure.

Plants materials must be subjected to pesticidal treatments right from the field, and clean materials from these plants should also be planted in screen houses and be subjected again to pesticidal treatments. The mother plant materials will then have to be decontaminated again. Surface sterilization of dormant buds with mercuric chloride and sodium hypochlorite solutions has been reported to give high rates of freedom from bacterial infections, as did the use of meristems from growing shoots (Kunneman and Faaij- Groenen, 1998).

Antibiotics are used on against plant culture to inhibit the growth of microbes . It is not surprising that, since the antibiotic sensitive has emerged amongst the plant pathogens approache. Inclusion of the antibiotics, Tetracyline, cholorophenical, ketocanozole, flucocanozole at 100µl ml in the micropropagation media proved effective in suppressing growth of pure culture of the bacterial contaminants. (Table.3) Tetracyline at the same contaminants was effective against the use of antibiotics in the control of bacterial contaminants of plant tissue has been reported (Benjama and charkuol, 1997). Treatments that are often effective in removing contamination are also toxic to the plant tissue, contaminants (if present) will remain and will eventual grow and ruin the cultures. Antibiotics used for inhibiting the growth of microbial contaminant while it is not recommended for real plant tissue culture. (Table .4)

The following precautions have considerably helped in minimizing the contaminations and often obtaining contaminant free cultures: 1. Selection of clean, healthy part, which were free of soil. 2.

Adequate surface sterilization of explants for tissue culture:- i. 0.35% Sodium hypochlorite for 3 min for leaf explants; ii. Washing the roots under the tap to free them of any adhering soil; iii. Then immersing the root explants in 70% alcohol for 3 min followed by immersion in 0.1% mercuric chloride for 3 min. 3. All operations were performed in a sterile bench with other aseptic practices. With the above protocol, contamination of tissue culture materials has been reduced to manageable level**.** (Table.5,6)

Table 1. Fungal contaminant were found associated with the tissue culture plants

S.NO.	**Plant name**	**Sub culture**	**Fungi isolate**
1.	*Albizia procera*	Callus	*Fusarium sp.*
2.	*Roulfia serfitiana*	Callus	*Aspergillus niger*
3.	*Nepenthus khasiana*	Shoot	*Chaetomium sp.*
4.	*Andographic peniculata*	Shoot	*Acrimonium sp.*
5.	*Bacopa monneri*	Shoot	*Aspergillus sp.*

Table 2. Fungal contaminant were found associated with the tissue culture plants

S.NO.	**Plant name**	**Sub culture**	**Bacteria isolate**
1.	*Andographic peniculata*	Callus	Staphylococcus aureus
2.	*Andographic peniculata*	Root	Gram positive
3.	*Roulfia serfitiana*	Shoot	Staphylococcus epidermis
4.	*TerminaliaChabula*	shoot	Staphylococcus aureus
5.	*Albizia procera*	Shoot	Gram positive

Table 3. Effect of different concertration of Ketoconazole on different fungi

S.no.	**Name of fungi**	**Ketoconazole (in µl)**			
		50	100	200	400
1.	Fusarium spp.	S	S	R	R
2.	Aspergillus niger	R	R	R	R
3.	Chaetomium spp.	R	R	R	R
4.	Acremonium spp.	R	R	R	R
5.	Aspergillus spp.	S	S	S	S

S=Sensitive; R=Resistance

Table 4. Effect of different concertration of Gliseofuluin on different fungi

S.no.	**Name of fungi**	**Gliseofuluin (in µl)**			
		50	100	200	400
2.	Aspergillus niger	R	R	R	R
3.	Chaetomium spp.	R	R	R	R
4.	Acremonium spp.	R	R	R	R
5.	Aspergillus spp.	R	R	R	R

S=Sensitive; R=Resistance

Table 5. Effect of different concentration of fluconazole on different fungi

S.no.	**Name of fungi**	**Fluconazole (in µl)**			
		50	100	200	400
2.	Aspergillus niger		R	R	R
3.	Chaetomium spp.	R	R	R	R
4.	Acremonium spp.	R	R	R	R
5.	Aspergillus spp.	R	R	R	R

S=Sensitive; R=Resistance

Table 6. Effect of different concentration of Streptomycin, Penicillin, Tetracycline and Chloramphenicol on different Bacteria

S.No.	Name of bacteria	Streptomycin (in µl)		Penicillin (in µl)		Tetracycline (in µl)		Chloramphenicol (in µl)	
		50	100	50	100	50	100	50	100
1.	Staphylococcus aureus	R	R	R	R	S	S	S	S
2.	Gram positive	R	R	R	R	S	S	S	S
3.	Staphylococcus epidermis	R	R	R	R	S	S	S	S
4	Staphylococcus aureus	R	R	R	R	S	S	S	S
5.	Gram positive	R	R	R	R	S	S	S	S

S=Sensitive; R=Resistance

Fungal contamination in callus culture of *albizia procera*

Fig.1. a. Callus culture of *Albezia procera* , b.isolated fungus, c. identifed fusarium spp.

Fungal contamination in callus culture of *Rouwolfia serfentina*

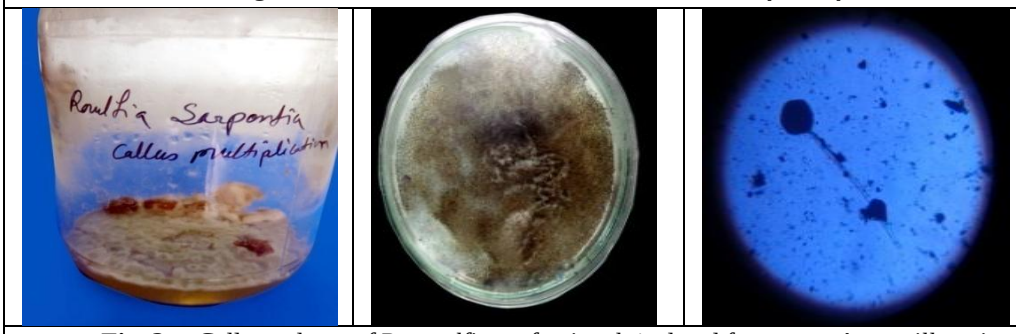

Fig. 2 a .Callus culture *of Rouwolfia serfentina*, b.isolated fungus, c. *Aspergillus niger*

Fungal contamination in callus culture of *Nepenthes Khasiana*

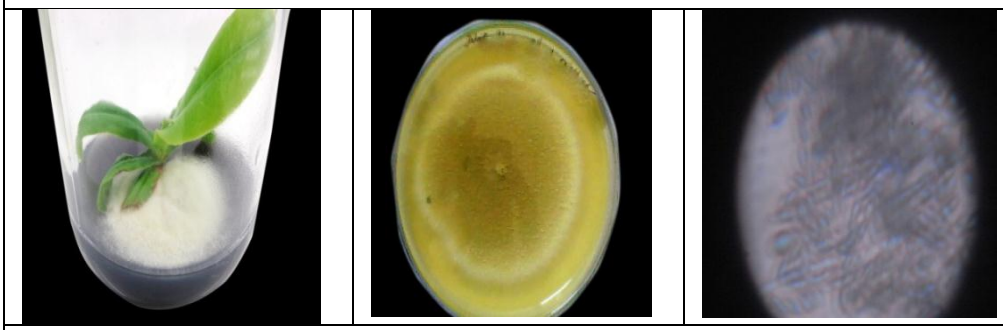

Fig. 3 a.Shoot culture *of Nepenthes Khasiana*, b.isolated fungus, c.Chaetomium Species.

Fungal contamination in shoot culture of *Andographis peniculata*

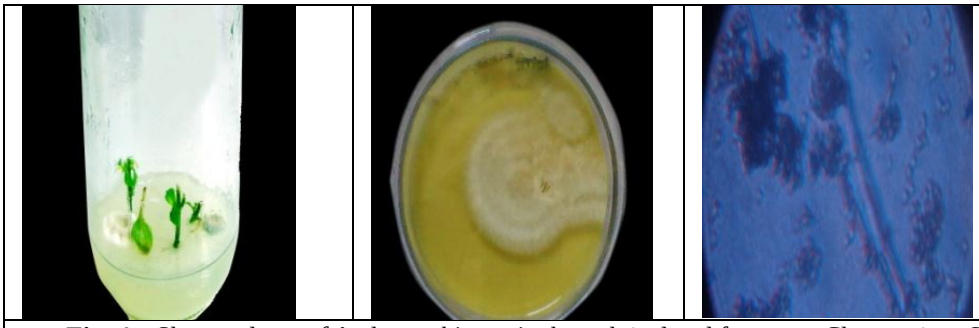

Fig. 4 a.Shoot culture *of Andographis peniculata* , b.isolated fungus, c.Chaetomium Species.

Fungal contamination in shoot culture of *Bacopa monneria*

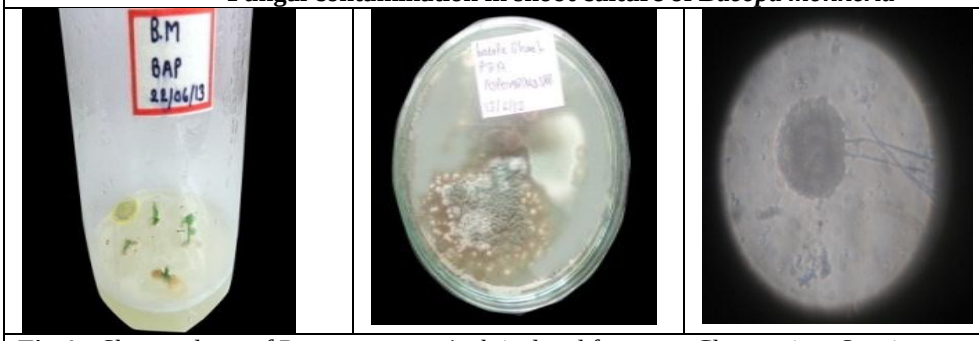

Fig.4 a.Shoot culture *of Bacopa monneria*, b.isolated fungus, c.Chaetomium Species.

Bacterial contamination in callus culture of *Andographis peniculata*

Fig. 5 a.callus culture *of, Andographis peniculata* b.isolated bacteria, c.Staphylococcus aureus

Bacterial contamination in root culture of *Andographis peniculata*

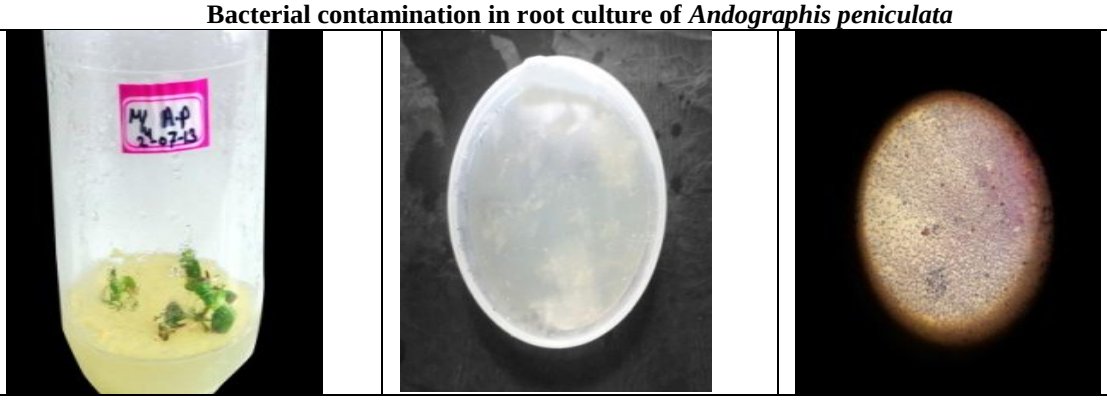

Fig. 6 a.Shoot culture *of Andographis peniculata* , b.isolated fungus, c. Gram positive

Bacterial contamination in shoot culture of *Roulfia serfentiana*

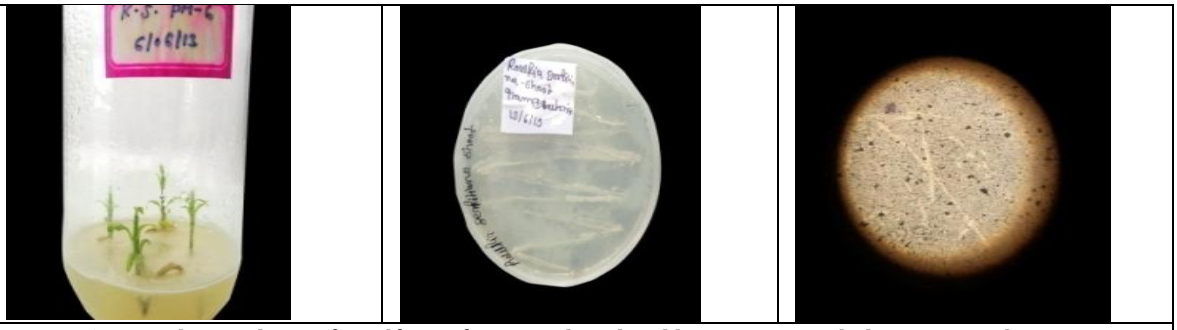

Fig. 7 a.Shoot culture *of Roulfia serfentiana*,, b.isolated bacteria, c.Staphylococcus epidermis .

Bacterial contamination in shoot culture of *Albezia procera*

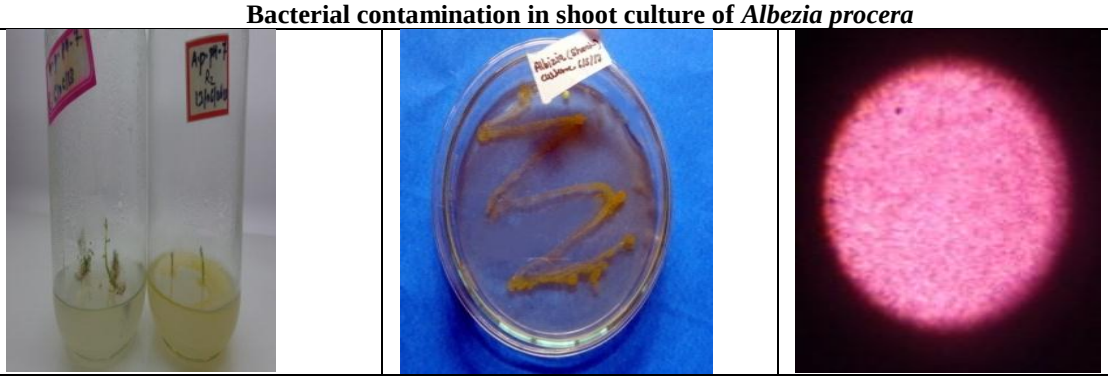

Fig. 8 a.Shoot culture *of Albezia procera*, b.isolated bacteria, c. Staphylococcus aureus .

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