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Standardization of *in vitro* sterilization procedures and molecular identification of endogenous contamination in cultures of black ginger (*Kaempferia parviflora* wall ex. Baker)

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Abstract
The successful initiation of culture depends on the technique of surface sterilization of the explants. In the present study, an attempt was made to remove endogenous fungal contaminants from the surface of the plant material, thereby obtaining axenic cultures with the highest survival rates. Black ginger is one of the most important medicinal plants in the world. However, in micro propagation, surface sterilization is the most important step in explants preparation. The effect of different fungicides, antibiotics and disinfectants: Mercury (II) chloride ($HgCl_2$), sodium hypochlorite (NaOCl), hydrogen peroxide (H_2O_2) and ethanol have been evaluated to disinfect *Kaempferia parviflora* rhizome shoots by varying their concentrations and exposure times. Fungal contaminants formed during culture initiation were identified by DNA sequencing. In the second experiment, different nanoparticles were evaluated for their ability to disinfect rhizomes. The rate of bacterial contamination, fungal contamination, total contamination and survival rates of the culture were observed. The results showed that among all the sterilization methods, 100% AgNP for 60 min was the most effective treatment method. The highest percentage of uncontaminated cultures was achieved at 90.00% with this treatment.

Keyword: *Kaempferia parviflora*, contamination, surface disinfection, rhizome shoots

1. Introduction
Kaempferia is a medium-sized genus in the family Zingiberaceae. *Kaempferia parviflora*, also known as Thai black ginger, Thai ginseng, or Krachaidum, is a herbaceous plant. *Kaempferia parviflora* is a diploid (2n = 22) species (Nopporncharoenkul *et al.* 2017) [13]. It is commonly found in northeastern India, especially in the regions of Arunachal Pradesh, Manipur, Meghalaya, Nagaland and the Himalayas. *Kaempferia parviflora* has been the subject of increasing scientific interest in recent centuries. *Kaempferia parviflora*'s health benefits and potential therapeutic functions increase its market value as an herbal product.

However, *in vitro* culture of plants in the field is susceptible to microbial contamination. Contamination of plant culture is still an ongoing problem that can lead to the loss of plant culture, not only some number, but in the whole batch of cultures. Sterilization is necessary because bacteria and fungi can contaminate culture and pose a constant threat during the growing stages. The production of sterile and viable plantlets *in vitro* is necessary to increase the efficiency of clonal propagation in plant tissue culture. It may be necessary to use the correct sterilization method in tissue culture techniques to save time and effort (Mahmoud *et al.* 2016) [11].

In vitro fungal and bacterial contaminations are also among the most important limiting factors, especially in rhizomes. To overcome fungal and bacterial infections during the sterilization phase, various surface disinfectants, including sodium hypochlorite, hydrogen peroxide, antibiotics, ethanol, and mercury chloride have been used in laboratories in all over the world. The effectiveness of sterilization methods depends on various parameters such as physiological state of the mother plant, size, age and type of explant, disinfectant concentration and exposure time. (Teixeira da Silva *et al.* 2015) [18]. Therefore, it is difficult to come up with a standard sterilization procedure for all plants and explants.

Therefore, sterilization procedures must be optimized for any species to obtain the maximum number of sterilized viable explants. Tissue culture technology is very powerful and has opened up vast areas of research for biodiversity conservation. *In vitro* plant regeneration is a biotechnological tool that offers a significant potential solution for the propagation of endangered and superior genotypes of medicinal plants that can be released into natural habitats or grown on a large scale for the pharmaceutical product of interest. Tissue culture procedures have been developed for a variety of medicinal plants, including endangered, rare and threatened plants.

2. Materials and Methods

2.1 Explants preparation and sterilization: The experiment was conducted at the Centre for Advanced Research in Plant Tissue Culture, Department of Agricultural Biotechnology, Anand Agricultural University, Anand, and Gujarat with the aim of evaluating the effectiveness of different disinfectants on explants of *Kaempferia parviflora* (black ginger) under *in vitro* conditions. Healthy rhizomes are kept in a greenhouse for germination. Explants were prepared as shown in Figure 1 for surface sterilization and initiation. The rhizome is cut into small pieces and the dead skin is removed. Care has been taken to ensure that each piece has a bud and is intact. The size of the explant was reduced to 2±0.5 cm. Explants were surface disinfected with various antifungal and antibacterial agents (i.e. 50% carbendazim, cefotaxime, kanamycin, streptocycline and $HgCl_2$) prior to inoculation.

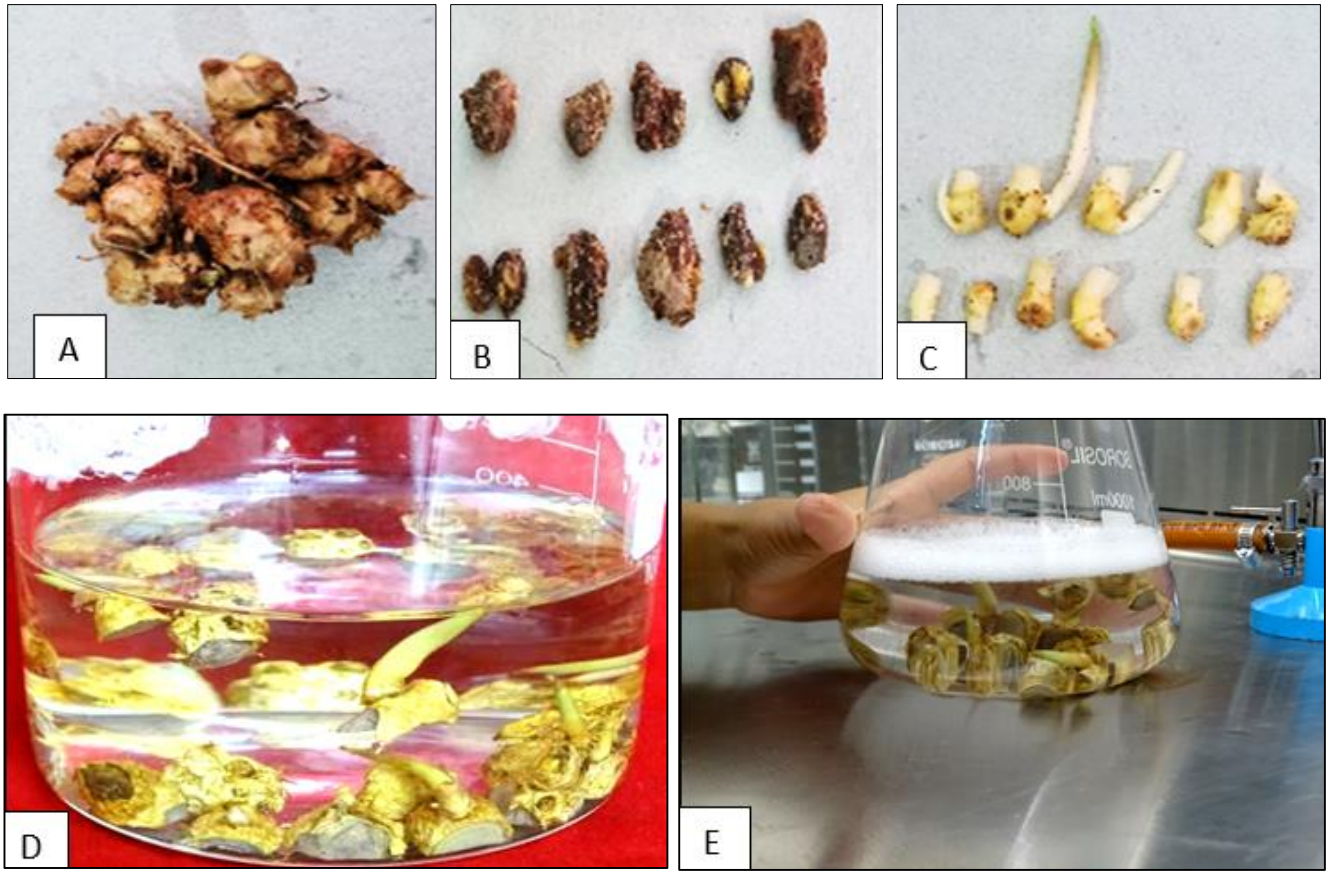

Fig 1: Preparation and surface sterilization of explants (A) Rhizome of black ginger (B) Rhizomes were excised to number of small pieces (C) sprouted rhizomes used as a explants (D) & (E) The explants were surface sterilized with various antifungal and antibacterial agents

2.2 Experiment 1: Effect of streptocycline, kanamycin, bavistin, Aureofungin, ethanol, sodium hypochlorite, hydrogen peroxide and $HgCl_2$ on the establishment of axenic cultures in black ginger

The germinated rhizome shoots were collected in a beaker or conical flask and kept under running water before being sterilized in the laminar airflow cabinet. For the experiment, different disinfectants, namely Bavistin (carbendazim 50%), streptocycline, kanamycin, Aureofungin, ethanol and $HgCl_2$ in the case of ST1 and ST2 treatments, respectively were tested for explant sterilization by varying their concentration and time of exposure (Table 1). In the last three treatments (ST3 - ST5), sodium hypochlorite (NaOCl), hydrogen peroxide (H_2O_2) and ethanol were used instead of antibiotics. Treated rhizome explants were thoroughly washed three times with sterilized distilled water in a laminar airflow cabinet. Surface sterilization of the rhizome samples in this study revealed no effective treatments for fungi, bacteria, overall contamination, and very little axenic culture was observed (Figure 2). Therefore, in order to find the appropriate surface disinfection treatment, further experiments are required to prevent contamination. The identification of endophytic fungi is necessary to establish *in vitro* cultures.

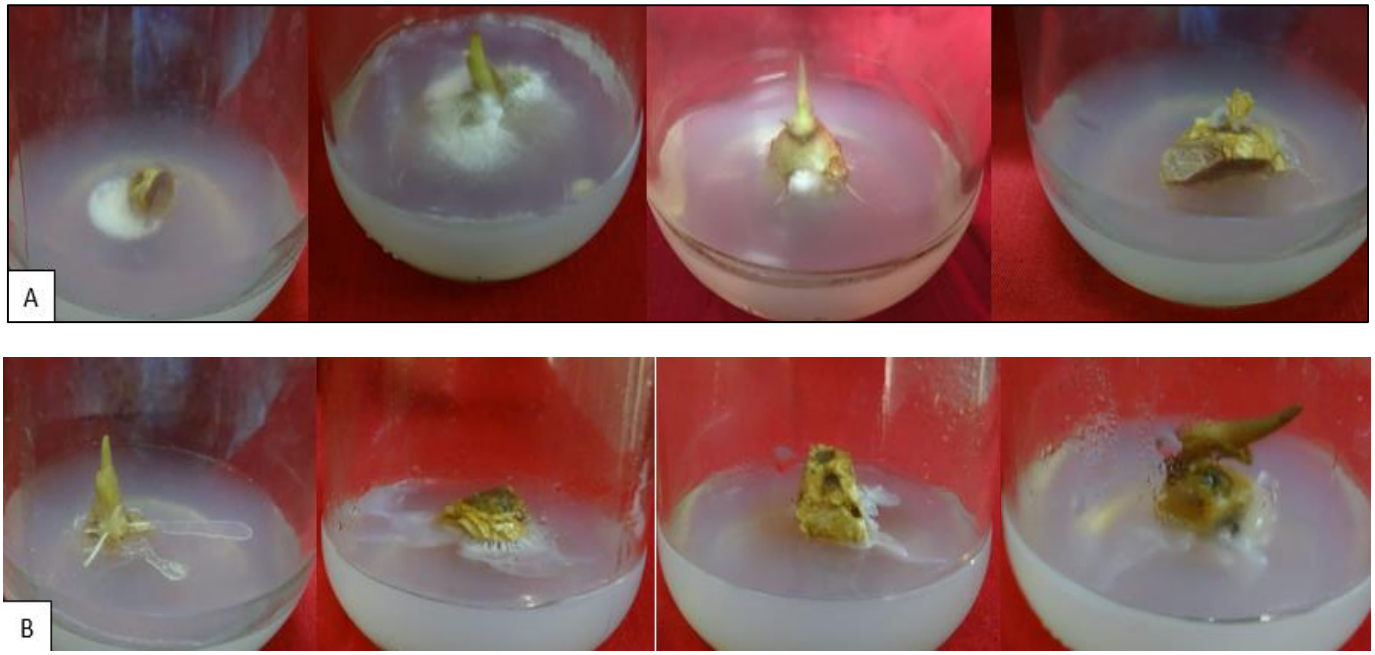

Fig 2: Overall contamination after sterilization process with different sterilizing agents (A) fungal contamination (B) bacterial contamination

Table 1: Different antibacterial, antifungal and surface sterilizing agents for sterilization of explant

Treatments	Surface sterilants	Concentration	Time (minute)	contamination			Axenic Cuture
				Bacterial	Fungal	Total	
ST_1	Ethanol	70%	1	30.00	66.67	96.67	3.33
	Kanamycin	2000 mgl^{-1}	15				
	Streptocycline	2000 mgl^{-1}	20				
	$HgCl_2$	0.1%	5				
	Bavistin (50% Carbendazim)	10000 mgl^{-1}	40				
ST_2	Ethanol	70%	30 s	43.33	50.00	93.33	6.67
	Aureofungin	1000 mgl^{-1}	7				
	Kanamycin	2000 mgl^{-1}	6				
	Cefotaxime	2000 mgl^{-1}	6				
	Streptocycline	2000 mgl^{-1}	7				
	$HgCl_2$	0.1%	1				
	Bavistin (50% Carbendazim)	2500 mgl^{-1}	10				
ST_3	Ethanol	70%	30 s	28.33	65.00	93.33	6.67
	Sodium hypochlorite	2.5%	10				
ST_4	Bavistin (50% Carbendazim)	1000 mgl^{-1}	30	23.33	43.33	66.67	33.33
	Water+Tween-20	2 to 3 drops	1				
	Sodium hypochlorite + Tween-20	4%	5				
	$HgCl_2$	0.1%	1				
ST_5	Ethanol	70%	30 s	20.00	40.00	60.00	40.00
	Sodium hypochlorite	2%	20				
	H_2O_2	5%	518				
	$HgCl_2$	0.1%	3				

2.3 Isolation, identification and antifungal activity of filamentous fungi

During micro propagation, fungal contaminants were transferred to a Petri dish containing potato dextrose agar (PDA) and stored at +4 °C for identification.

2.4 Morphological and cultural characteristics

The isolates were cultured on PDA agar for 4-5 days at 30 °C. Cultural features such as isolate morphology, colony characteristics, sporulation and spore pigmentation were observed. The mycelium and spores were observed under the microscope using lactophenol cotton blue staining technique and observed under low to high magnification microscope objectives. Microscopic observations (structure and arrangement of spores as well as mycelium types) were recorded for morphological characterization (Figure 3).

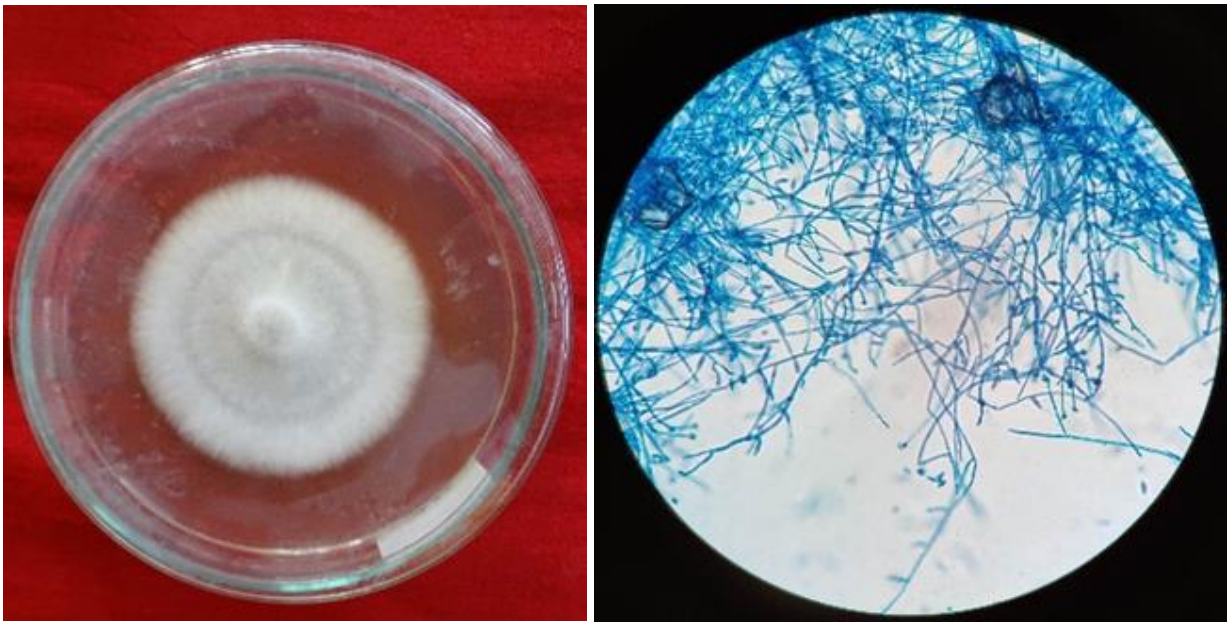

Fig 3: Morphological and cultural characteristics of fungal isolate

2.5 Molecular identification and characterization of selected fungal strains

Pure fungal strains were obtained by growing a single conidium of the respective isolates on PDA plates. The cultures were grown in potato dextrose broth (PDB) on a mechanical shaker at 150 rpm, 25±2°C, for 3-4 days. The mycelium growth was harvested after filtration and washing with sterile distilled water. The mycelium was ground to a fine dust with liquid nitrogen and transferred to a sterile 1.5 ml micro centrifuge tube. One milliliter of lysis buffer is added and the tube is incubated at 65°C in a water bath for lysis. Once the process was complete, an equal amount of saturated phenol was added to the lysate and centrifuged for 15 min at 14,000 rpm. The aqueous solution obtained from the centrifuge lysate is added to a new micro centrifuge tube and an equivalent amount of phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to it. The mixer was again centrifuged at 14,000 rpm for 15 min. After centrifugation, the aqueous phase was introduced into a new tube, then ¼ chloroform and 1/10 sodium acetate (pH 4.5) were added to the mixer. The mixer was centrifuged for 15 min at 14,000 rpm. From the centrifuged mixer aqueous phase was pipetted in new tube and 70 percent ethanol was added for precipitation of DNA and centrifuged at 12,000 rpm for 12 min at 4 °C and supernatant discarded without disturbing pellet. The pellet was air dried until smell of ethanol removed from the tube. The pellet was dissolved in 25 µl of sterile distilled water and DNA samples were stored at 4 °C till further analysis. 2µl DNase free RNase was added to the dissolved DNA stock and incubated in a water bath at 37 °C for 45 min followed by 65 °C for 10 minutes for enzyme inactivation. DNA purity was tested by agarose gel electrophoresis.

The ITS region is the most widely sequenced DNA region in molecular ecology of fungi. It has been useful for molecular systematics at the species level, and even within species. Sequence comparison of the ITS region is widely used in taxonomy and molecular phylogeny because it is easy to amplify even from small quantities of DNA due to high copy number of rRNA genes, and high degree of variation even between closely related species mostly standard ITS1+ITS4 primers were used and also several taxon-specific primers used that allow selective amplification of fungal sequences (Pitkaranta *et al.*, 2008) [15]. Internal transcribed spacer (ITS) refers to a piece of non-functional RNA situated between structural ribosomal RNAs (rRNA). Read from 5' to 3', this rRNA precursor contains 5' external transcribed sequence (5' ETS), 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA and 3' ETS.

2.5.1 DNA sequencing: Partial ITS *r*RNA gene sequencing was carried out for promising isolate and was performed using the terminator cycle sequencing kit on the genetic analyser. The ITS *r*RNA gene sequences were assembled using MEGA 4 software, compared with other isolates using NCBI BLAST analysis for identification purpose and comparison of homologies of isolated strains.

2.6 Antifungal activity of nanoparticles

Growth inhibitory effect of green synthesized AgNPs, Thymol Nano emulsion, sculpture and Zn nanoparticles was assessed on fungal and bacterial isolates from contamination observed during initiation stage by incorporation of different volume of synthesized nanoparticles which was procured from Nanotechnology Department. Antifungal tests were performed by the agar dilution method (Wiegand *et al.*, 2008) [20] with some modification. The autoclaved PDA media was mixed with 170 ppm AgNPs, 6% Thymol nanoemulsion, 30% Sulphur and Zn at different volume (2.5, 5.0, 7.5 ml) described in (Table 2 & fig. 4) and a solution was poured into the petri dishes (9 cm diameter). The fungi were inoculated after the PDA media solidified. A disc (1.4 cm) of mycelia material taken from the edge of 7 days old fungal cultures was placed in the center of each petri dish. The petri dish with inoculums was then incubated at 25 °C. After incubation, the mycelial growth in diameter (mm) of fungi was recorded at the end of incubation period. Three test plates for each treatment were used and results were recorded.

Table 2: Antifungal activity of Thymol nanoemulsion, Sulphur, Zn and AgNPs

Treatments	Growth inhibition (mm) Thymol nanoemulsion		
	3rd day	5th day	7th day
Control	43	69	89
Carbendazim - 50% (500mgl^{-1})	12	12	13
10% Thymol nanoemulsion	12	52	60
20% Thymol nanoemulsion	12	22	32
50% Thymol nanoemulsion	12	12	18
Growth inhibition (mm) silver nanoparticles			
Control	43	59	75
Carbendazim - 50% (500mgl^{-1})	11	12	14
2.5ml AgNPs	20	32	52
5.0ml AgNPs	14	23	48
7.5ml AgNPs	12	14	24
Growth inhibition (mm) sulphur nanoparticles			
Control	42	60	80
Carbendazim - 50% (500mgl^{-1})	12	14	14
2.5ml sulphur nanoparticles	26	38	48
5.0ml sulphur nanoparticles	20	35	45
7.5ml sulphur nanoparticles	15	27	35
Growth inhibition (mm) Zn nanoparticles			
Control	43	58	84
Carbendazim - 50% (500mgl^{-1})	12	14	14
2.5ml Zn nanoparticles	24	36	36
5.0ml Zn nanoparticles	14	24	24
7.5ml Zn nanoparticles	12	14	24

2.7 Experiment 2: Effect of Thymol nanoemulsion and Silver nanoparticles on contamination and axenic culture establishment in Black ginger

After antimicrobial activity, nanoparticles were used as a Sterilant. Rhizomes explants were washed thoroughly under running tap water and rinsed in Tween-20 for 10 minutes. Explants were then treated with Thymol Nano emulsion with different concentration (10%, 50% and 100%) for 10 minutes in case of NP_1, NP_2 and NP_3 treatments, respectively. In case of NP_4, NP_5 and NP_6 treatments rhizomes were treated with 100% thymol Nano emulsion with varying time exposure (15, 20, 25 min). In case of NP_7 treatment explants were treated with diluted $AgNo_3$ Nanopaticles for 60 minutes and in NP_8 treatment rhizomes were treated with $AgNo_3$ Nanopaticles for 60 minutes then deep in BAP (150 mgl^{-1}) for 30 minutes and washed thoroughly with sterilized distilled water thrice in laminar air flow cabinet and inoculated on MS basal media (Table 3).

Table 3: Different Surface sterilants and nanoparticles for sterilization of explants

Treatments	Surface sterilants	Concentration	Time (minute)	Contamination			Axenic cuture
				Bacterial	Fungal	Total	
NP_1	Tween 20	2 to 3 drops	10	18.33	81.67	100.00	0.00
	Thymol Nanoemulsion	10%	10				
NP_2	Tween 20	2 to 3 drops	10	10.00	78.33	83.33	11.67
	Thymol Nanoemulsion	50%	10				
NP_3	Tween 20	2 to 3 drops	10	10.00	70.00	80.00	20.00
	Thymol Nanoemulsion	100%	10				
NP_4	Tween 20	2 to 3 drops	10	10.00	36.67	46.67	53.33
	Thymol Nanoemulsion	100%	15				
NP_5	Tween 20	2 to 3 drops	10	1.00	26.67	36.67	63.33
	Thymol Nanoemulsion	100%	20				
NP_6	Tween 20	2 to 3 drops	10	5.00	23.33	28.33	71.67
	Thymol Nanoemulsion	100%	25				
NP_7	Tween 20	2 to 3 drops	10	10.00	40.00	50.00	50.00
	$AgNo_3$Nanopaticles	50%	60				
NP_8	Tween 20	2 to 3 drops	10	5.00	5.00	10.00	90.00
	$AgNo_3$Nanopaticles	100%	60				

3. Results and Discussion

3.1 Disinfection

Experiment 1: Effect of Streptocycline, Kanamycin, Bavistin, Aureofungin, Ethanol, Sodium hypochlorite, Hydrogenperoxide and $HgCl_2$ for axenic culture establishment in Black ginger

Surface sterilization of explants is an important precondition in any tissue culture experiment to minimize the chances of contamination. Since, surface sterilization requires the use of chemicals that are, toxic to microorganism but nontoxic to plant material preferably at low concentrations, and in order to find an optimized protocol for sterilization of a specific tissue, three factors were to be taken into consideration *viz*., sterilant, its concentration and the treatment duration. After observing the inoculated explants for 21 days on bacterial contamination, fungal contamination, total contamination and

survival of cultures, it was observed that Surface sterilization of rhizome explants in this study does not found any effective treatment with antifungal and antibacterial for contamination. Increasing time and concentration showed adverse effect on explants.This results agree with (Eed *et al.* 2010) [5], also found no significant differences in contamination while using bavistin (0 and 0.1%) and three streptomycin sulphate concentrations (0.1, 0.5 and 1.0%) in *Citrus limonia* Osbeck. Due to higher dose of the Sodium hypochlorite, explants were damaged and resulted in to discoloration of explants. Similar findings for sodium hypochlorite were reported by (Hamirah *et al.* 2010) [7] in which the higher number (40%) of damaged explants of red ginger (*Zingiber montanum* Koenig) were resulted due to high Clorox concentration which is commercially available bleach solution containing 7.5% NaoCl, detected by discoloration of explants. The explants turned whitish or reddish after 5-6 days. Even after the treatments, contamination was observed, so identified other treatments for surface sterilization. Sodium hypochlorite being mild sterilizing agents provided more percentage of infection. Increasing concentration and time of sterilization with NaOCl, showed almost negligible reduction in contamination (Khairudin *et al.* 2020) [9] also used different concentrations (40, 50, 60, 70 and 80%) of Clorox with few drops of Tween 20 for surface sterilization of *K. parviflora*, concluding that application of 50% Clorox produced the highest percentage of explants, above which decrease in percent survival of explants was observed. Solanki *et al.* (2014) also surface sterilized the ginger tubers with 0.5% (v/v) NaOCl for 15 minutes followed by dipping in 70% (v/v) ethyl alcohol for 30 seconds and finally treating with 0.1% (w/v) $HgCl_2$ solution for 12 minutes which resulted into low contamination. Similarly, (Srivastava *et al.* 2010) [17] also found that increasing time and concentration significantly reduces contamination but shows adverse effect on explants of *Aconitum heterophyllum*, while using Mercuric Chloride ($HgCl_2$), Sodium Hypochlorite (NaOCl) and Hydrogen Peroxide (H_2O_2) for surface sterilization.

3.2 Isolation of Fungal strain

After 2-3 weeks of culture, fungus were visible as a cloudiness around the base of the rhizome explants in each *in vitro* culture of black ginger. Pinkish white fungal colonies were observed on the surface of the explants embedded in the medium. These fungal contaminants could not be eradicated by sterilants. Therefore, the fungal colonies were isolated by placing the contaminated plantlets directly ontoPDA by serial dilution and spread plate technique. Fungal isolates were obtained during initiation. Isolated fungus was used for molecular analysis.

3.3 Molecular identification of fungal isolate: The molecular characterization of fungal isolate (BG-1) was carried out by targeting 18S region of fungi and with the help of ITS-1 and ITS-4 universal primers.ITS-1 and ITS-4 produced an amplicon of 540 bp for BG-1. Amplified products were analyzed on 2 percent agarose gel. The PCR products were purified and sequenced, the results were aligned and analyzed in the NCBI nucleotide BLAST to find out the similarity of native fungal sequenced samples to the GenBank databases and matched at the diverse global similarity. A BLAST search for similarities of isolate BG-1 identification showed 100% with *Fusarium oxysporum* sequences.

Fusarium oxysporum

TTAAGTTCAGCGGGTATTCCTACCTGATCCGAGGTCA
ACATTCAGAAGTTGGGGTTTAACGGCGTGGCCGCGA
CGATTACCAGTAACGAGGGTTTTACTACTACGCTAT
GGAAGCTCGACGTGACCGCCAATCAATTTGAGGAAC
GCGAATTAACGCGAGTCCCAACACCAAGCTGTGCTT
GAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCC
AGAATACTGGCGGGCGCAATGTGCGTTCAAAGATTC
GATGATTCACTGAATTCTGCAATTCACATTACTTATC
GCATTTTGCTGCGTTCTTCATCGATGCCAGAACCAAG
AGATCCGTTGTTGAAAGTTTTGATTTATTTATGGTTT
TACTCAGAAGTTACATATAGAAACAGAGTTTAGGGG
TCCTCTGGCGGGCCGTCCCGTTTTACCGGGAGCGGG
CTGATCCGCCGAGGCAACAAGTGGTATGTTCACAGG
GGTTTGGGAGTTGTAAACTCGGTAATGA

3.4 Screening for Antifungal Activity of Various Nanoparticles: Fungal contamination is a serious problem in plant tissue culture procedures. An experiment was conducted to evaluate the potential of nano particles (NP) to remove fungal contaminants of rhizome explants. Growth inhibition against the fungal isolates was observed for AgNPs, Thymol Nano emulsion, sulphur and Zn nanoparticles. The fungal isolates showed higher susceptibility against thymol nano emulsion and AgNPs at higher dilution and fungal isolates shows sensitivity against silver nanoparticles (Table 2) & (figure 4). Similar to our findings different researchers also used AgNPs for antifungal activity. (Devi *et al.* 2014) [4] and (Hassan *et al.* 2019) [8] investigated the antifungal activity against *Candida albicans*, *Candida parapsilosis* and *Aspergillus niger* was using antifungal drug-Nystatin as a comparable control. Different concentrations such as 10, 20, 30 and 40 µl were checked for antifungal activity. AgNPs revealed higher antifungal activity with inhibition zone of 24, 26 and 30mm. (Akpinar *et al.* 2021) [2] and (Khan *et al.* 2021) [10] investigated antifungal effects of silver nanoparticles (AgNPs) against phytopathogenic *Fusarium oxysporum* with different concentrations (12.5-100 ppm). Mycelium growth abilities were decreased about 50%, 75% and 90% by AgNPs treatment with 25 ppm, 37.5 ppm and 50 ppm concentrations, respectively.

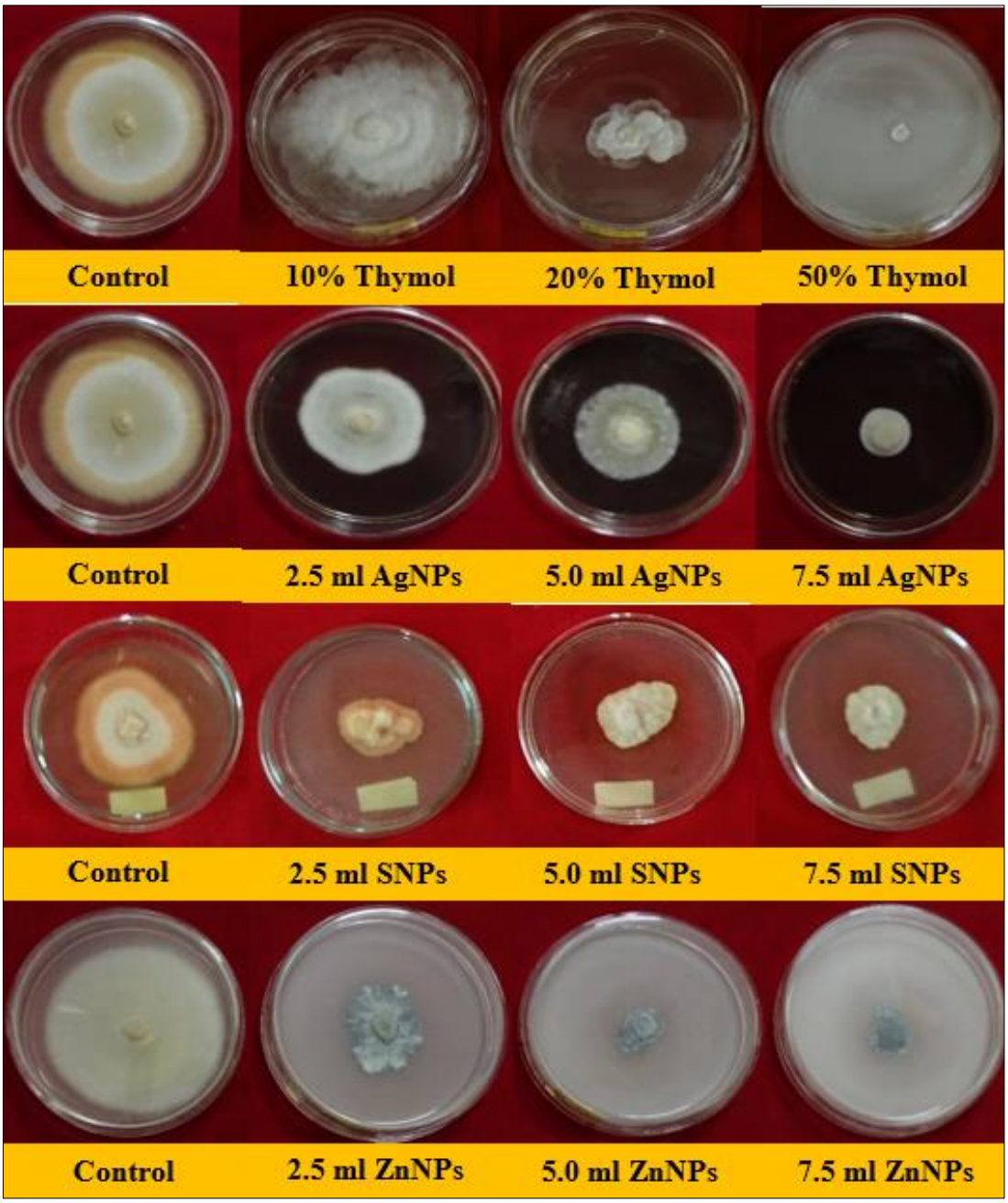

Fig 4: Screening of Antifungal activity of Thymol nanoemulsion, AgNPs, Sulphur and Zn nanoparticles

3.5 Experiment 2: Effect of Thymol nano emulsion and Silver nanoparticles on contamination and axenic culture establishment in Black ginger

In this experiment, the effect of thaymolnanoemulsion and silver nanoparticles on the explant contamination and viability were assessed. The least bacterial, fungal and total contamination was recorded as 5.0% after ($AgNo_3$Nanopaticles for 60 minutes) and (100% Thaymolnanoemulsion for 25 min) treatments; while, the highest axenic culture were recorded 90% after NP_8 ($AgNo_3$Nanopaticles for 60 minutes) treatment. Similar to our findings, silver nanoparticles (AgNPs) was reported as surface sterilant in black ginger. (Park *et al.* 2021) utilized 100 mgl^{-1}AgO NPs for 60 min eliminated contamination without affecting the survival of black ginger explants. (Tung *et al.* 2021) [19] used 200 and 300 ppm (AgNPs) for surface disinfection in *Begonia tuberous*. Explants derived from AgNPs sterilization did not show any abnormalities in somatic embryogenesis and eliminated contamination. (Abdi *et al.* 2008) [1] also utilized 100 mgl^{-1} of nano solution for 60 min resulted in the highest percentage (89%) of disinfected explants of valerian. In contrast to our observations, (Gouran *et al.* 2014) [6] reported silver nanoparticles on grapevine leaf explants showed low rates of burned explants in nano-silver treatments and had moderate effect for fungi contamination control.

4. Conclusion

In case of surface sterilization of the rhizome explants of Black ginger, very huge amount fungal, bacterial and total contamination was observed because numerous microbes are attached to the underground surface of explants. Molecular identification of endogenous fungus that contaminate *in vitro* plant tissue cultures requires a reliable and specific detection method. The tested treatments were found to be effective for the control of fungal and bacterial contamination. From the

results it was observed that treatment NP_8 having 100% $AgNo_3$Nanoparticles was effective for controlling overall contamination. Thus, our study provides a technique to identify and resolve fungal contamination during *in vitro* conservation of black ginger germplasm.

5. Author Contribution statement: PB: Acquisitions of the research work and drafted manuscript. GP: Overall supervision of the research work. AP, MM, and RR: Provided research material. AP, RP, and BG: Assisted in research work.

6. Acknowledgement
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7. Conflict of interest
The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

8. Data availability
The datasets generated during and/or analysed during the current study are given in the manuscript.

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