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***In vitro* Testing of Common Disinfectants Used in Sericulture to Control the Growth of Fungi in Rearing Houses**

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ABSTRACT

Silkworms are susceptible to a number of diseases. For controlling diseases, disinfection is the best prophylactic measure. Present study aims to identify the load of fungal pathogen present in the rearing rooms before and after disinfection also the *in vitro* testing of disinfectants (Sanitech, Asthra, bleaching solution, 2% formalin and 70% alcohol) against the fungus culture was studied. In disinfected floor, wall and washed materials like stand, tray in all the models studied, swab germ load as number of colonies were reduced to some extent but not completely removed. Quantification of Colony Forming Unit Area (CFUA) indicated that in all three models showed that all colonies in floor, wall, tray and newspaper were reduced significantly after disinfection. Seven different fungal colonies were isolated and cultured on potato dextrose agar media and identified according to colour, shape and margin out of these 5 isolated fungal colonies (*Aspergillus tamarii*, *Aspergillus flavus*, *Aspergillus niger*, *Neurospora* spp. and *Beauveria bassiana*) were tested against 5 disinfectants. Bleaching solution had highest effect (25.250 mm) in controlling *Beauveria bassiana* (muscardine causing agent to silkworm) and significantly more ($p < 0.05$) than formalin (19.500 mm). Aspergilosis disease causing agent to silkworm *Aspergillus tamarii* was controlled by formalin significantly ($p < 0.01$) with highest effective zone among all the disinfectants used. *Aspergillus flavus* was controlled by bleaching solution with highest clear zone (20.875 mm) and non significant with Asthra (16.00 mm) and formalin (15.500 mm). Hence *in vitro* testing proved 2% bleaching solution with 0.3% slaked lime was best in effectively controlling the fungal diseases followed by 2% formalin solution.

Key words: Asthra, bleaching solution, disinfectant, formalin, fungal disease, muscardine, Sanitech, silkworm

INTRODUCTION

Silkworms are susceptible to a number of diseases caused by different infectious agents and mixed infections (Doreswamy *et al.*, 2004). The cocoon loss due to diseases in India is estimated to be about 15-20 kg per unit of 100 disease free layings (dfls) which account for about 30% of total loss (Selvakumar *et al.*, 2002). Information on investigations related to disease prevalence in silkworm in different parts of the country in different seasons is highly helpful to monitor, prevent and control (Babu *et al.*, 2009).

Environment in the silkworm-rearing house is dynamic and subject to continuous change. Despite the fact that the atmospheric air does not favour the growth of microorganism due to lack

of nutrients, the microorganisms are present in aerosol form (suspended in the air). The bio-aerosol consists of different types of microorganisms usually stuck to the particles of dust or suspended in tiny drop of water. Among them pathogenic viruses, bacteria and fungi are capable of causing diseases to the silkworm. Thus the rearing environment plays a great role in the onset and spread of infection because of multifactor causation. Therefore, routine microbiological monitoring is most essential. Further, it is necessary to study the epidemiology of infection as a multidisciplinary approach.

The high prevalence of diseases in sericultural areas may be due to persistence of the pathogen at high concentrations in the silkworm-rearing environment. These pathogens are not easily destroyed and can persist for long periods under congenial conditions. The diseases of silkworm are non-curable due to shorter larval period and associated with shorter incubation period of pathogen. The pathogens released by diseased silkworms easily accumulate and spread in the rearing environment through different routes.

Reddy and Rao (2009) surveyed and recorded Silkworm diseases viz., Grasserie, Flacherie and Muscardine. Among fungal diseases, white muscardine, green muscardine, yellow muscardine, aspergillosis caused by *Beauveria bassiana*, *Spicaria prasina* (*Nomuraea riyeli*), *Metarhizium anisopliae*, *Paecilomyces farinosus*, *Aspergillus flavus*, *A. oryza*, *A. ochraceus*, *A. tamarisii* etc. (Beena, 1996; Manochaya and Kumar, 2010). The fungal disease muscardine and Aspergillosis prevails during winter and rainy seasons (Verweij *et al.*, 1999; Manochaya and Kumar, 2010). They develop conspicuous macroscopic growth on the surface of their hosts. Kawakami *et al.* (1975) invented stamp agar method for easy detection of *Aspergillus* fungi in silkworm rearing houses. There are no curative methods for any of the silkworm diseases and they are best prevented than cured. However, recently silkworm disease management reported by probiotic application (Subramanian *et al.*, 2009), application of compounds from marine halophytes (Kumar *et al.*, 2009) and dichloromethane and methanol (1:1) extract of seaweed brown algae, *Turbinaria conoides* against *Beauveria bassiana* (Kumari *et al.*, 2011). There had been several attempts to develop specific measures against white muscardine. Different fungicides and chemicals (Rangaswamy *et al.*, 2003), botanicals (Sharma *et al.*, 2002; Sasidharan *et al.*, 2000; Chandrasekharan, 2009; Bimal, 2006; Kumar *et al.*, 2009) and bed disinfectants (Chandrasekharan *et al.*, 2004) were evaluated against *B. bassiana* by different workers. Integrated technology for controlling white muscardine disease were reported by Nataraju *et al.* (2002) and curative methods by feeding by Kumar *et al.* (2002) and Datta *et al.* (2003).

Disinfection is an integral part of healthy and successful silkworm rearing. Kuroiwa *et al.* (1969) studied the effect of some disinfectants upon the newly hatched larvae of silkworm, *Bombyx mori*. It aims at the total destruction of disease causing pathogens and not to harm silkworm. Of recent, Okpara *et al.* (2011) recommended UV and chlorination disinfection methods. Adoption of proper and effective methods of disinfection and stepwise maintenance of hygiene achieve this during rearing. Therefore, when the silkworms are reared under natural conditions it is essential to disinfect the surroundings thoroughly and maintain cleanliness. Visual cleanliness is a pre-requisite and is necessary to assess the germ load on the surface to determine the effectiveness of cleaning. It is necessary to know the degree of microbial contamination and effect of fungicidal disinfection in silkworm rearing room. The present study aims to identify the load of fungal pathogen present in the rearing rooms before and after disinfection. *In vitro* testing of the

disinfectants (Sanitech, Asthra, bleaching solution, 2% formalin and 70% alcohol) which are in regular use in sericulture industry against the fungus culture was studied.

MATERIALS AND METHODS

The present study was carried out from April 2009 to March 2010 at Silkworm Physiology Laboratory, Central Sericulture Research and Training Institute, Mysore.

Sample collection: Different rearing houses designated as Model-1 disinfected thrice first with 5% bleaching solution followed by Sanitech and Asthra. Model-2 was disinfected twice with 2% formalin and Model 3 disinfected first with 70% alcohol followed by formalin fumigation (250 g of KMnO_4 crystals+2.5 L of 36% Formalin for 10'×20' room).

Six samples from air, rearing stand, wall, used tray, used newspaper and floor were collected from two different rearing houses *i.e.*, model-1 and 2 and three samples from air, wall and floor were collected from model 3 before disinfection. After disinfection again six samples from air, rearing stand, wall, washed tray, new newspaper and floor were collected from model-1 and 2 and three samples of air, wall and floor were collected from model 3.

Techniques for isolation of fungal culture: Different techniques were used to isolate fungal culture before and after disinfection of rearing houses (Model 1-3) viz., Petri plate exposure method, Swabbing and Serial dilution. For serial dilution the obtained inoculums from b. wall, c. Stand, d. tray and e. newspaper were used for preparation of dilutions from 10^{-1} , 10^{-2} and 10^{-3} . Whereas for floor (a) 10^{-1} dilution obtained in first dilution itself was further diluted to 10^{-2} and 10^{-3} . Fungal colonies were observed before and after disinfection. Morphological characteristics such colour, texture and numbers of different colonies were examined in all the figures visual enumerations of the fungal colonies were made. Further, number of fungal spores/sq ft was calculated by multiplying number of fungal colonies mL^{-1} with the total volume of solvent (50 mL) used for collecting inoculum from per square foot area. Colonies isolated and prepare pure culture in slants. Colour and shape of pure colonies were observed and examined under Stereoscopic binocular microscope Nikon SMZ-0A (5×4.9). Microscopic examination conducted under student microscope Olympus (15×40).

***In vitro* testing of disinfectants:** *In vitro* testing for sensitivity of the effective concentration of commercially available disinfectants (Sanitech, Asthra, bleaching solution, formalin and alcohol) were carried out using potato dextrose agar as medium against 7 fungal cultures. Different disinfectants were prepared as standard methodology given by the manufacturer. The inhibition zone formed with disinfectants was studied following the paper disk method described by Thornberry (1950).

For the preparation of the solution of different disinfectants, following procedures are followed:

- **Sanitech:** The 25 mL Sanitech solution was added to 2.5 g activator crystals resulting in yellow colored solution. Twenty five million activated yellow colored Sanitech solution was added to 950 mL of water and prepared total 975 mL solution. Dissolved separately 5 g of slaked lime in 25 mL of water in a clean conical flask and mixed this solution with 975 mL of Sanitech solution. Stirred 1 L of the solution thoroughly

- **Asthra:** The 0.5 g of Asthra was dissolved directly in 1 L of water and allowed the prepared solution for 2 h for its activation
- **Bleaching solution:** The 2 g of bleaching powder and 300 mg of slaked lime were mixed in 100 mL of water
- **Formalin:** The 2% formalin solution was prepared using commercially available (36%) formalin
- **Alcohol:** The 70% used

Statistical analysis: Data were analysed by employing Student's t-test for testing a hypothesis on the basis of a difference between two sample means and for comparison of more than two data, the Analysis of variance (ANOVA test) was employed.

RESULTS

Air exposure: Overall fungal load in the air of rearing houses was detected by exposing the media to the air. Model 1, 2 and 3 revealed four, two and four types of fungal colonies before disinfection (Table 1) whereas after disinfection, the number of fungal colonies reduced to two, one and one types, respectively.

Swab: Swab is the procedure to get overall load of fungi on the surface of the material used for silkworm rearing. Floor of model-1 revealed three types of fungal colonies before disinfection and four types after disinfection whereas in model-2, two types of fungal colonies before disinfection and four types after disinfection were observed. In model-3 five types of fungal colonies were observed before disinfection which was reduced to four after disinfection. In the wall of model-1, three fungal colonies were found before disinfection which was reduced to two, in model-2 five fungal colonies before disinfection were reduced one type of fungal colony after disinfection. Similarly in model-3 initially three types of colonies were found which after disinfection reduced completely. In stand four types of fungal colonies were found before disinfection which was reduced to three in model-1 and in model-2 five fungal colonies, were observed before disinfection and three colonies after disinfection. Before washing the tray, two types of fungal colonies were observed, whereas after disinfection four types of fungal colonies were found in both model-1 and 2. Used newspaper before

Table 1: Comparison of fungal colonies by air exposure before and after disinfection of model 1-3

Sl No.	Before disinfection			After disinfection		
	Colour of colony	Texture	Identified fungus	Colour of colony	Texture	Identified fungus
Model 1						
1	Olive green	Velvety	<i>Cladosporium</i> spp.	Light green	Velvety	<i>A. flavus</i>
2	White	Cottony	<i>Rhizopus</i> spp.	White	Cottony	<i>Rhizopus</i> spp.
3	Pink	Fluffy	<i>B. bassiana</i>			
4	Yellow	Cottony	<i>A. niger</i>			
Model 2						
1	Olive green	Velvety	<i>Cladosporium</i> spp.	White	Cottony	<i>Rhizopus</i> spp.
2	White	Cottony	<i>Rhizopus</i> spp.			
Model 3						
1	Olieve green	Velvety	<i>Cladosporium</i> spp.	White	Cottony	<i>Rhizopus</i> spp.
2	White	Cottony	<i>Rhizopus</i> spp.			
3	White	Leathery	<i>B. bassiana</i>			
4	Light green	Leathery	<i>A. flavus</i>			

disinfection contained three and four types of fungal colonies, respectively in the models 1 and 2. New newspaper in model-1 showed two colonies and model-2 showed only one colony of fungi after disinfection.

Serial dilution: Dust of the floor of model-1 after serial dilution, 10^{-2} showed three types of 33 fungal colonies and in 10^{-3} dilution, three types of 7 fungal colonies before disinfection whereas no colonies were observed after disinfection in both the dilutions (Table 2). In model-2, 10^{-2} dilution showed two types of 9 fungal colonies before disinfection which was reduced non-significantly to 7 colonies of two types (Table 3). In 10^{-3} dilution there was non-significant difference in the fungal colonies before (4) and after disinfection (3). Model-3 revealed three types of 6 and 3 colonies, respectively in 10^{-2} and 10^{-3} dilutions before disinfection and three types and no colony was found after disinfection in both the dilutions (Table 4). In the wall of model-1 significant reduction ($p < 0.001$) in three types of fungal colonies to one in 10^{-2} dilutions after disinfection (83 and 1, respectively) and in 10^{-3} , three types of 54 colonies were reduced to zero after disinfection (Table 2). In model-2 and 3, no fungal colony was observed before and after disinfection in both 10^{-2} and 10^{-3} dilutions (Table 3 and 4). In model-1, stands after 10^{-2} dilution showed two types of 68 fungal colonies before disinfection which was reduced ($p < 0.001$) to 15 fungal colonies of one type (Table 2) whereas in 10^{-3} dilution there was one type of two colonies reduced to zero after

Table 2: Comparison of fungal colonies after serial dilution before and after disinfection of model 1

Material	Dilution factor	Before disinfection			After disinfection				
		Colour of colony	Texture	Identified fungus	No. of colonies	Colour of colony	Texture	Identified fungus	No. of colonies
Floor	10^{-2}	Olive green	Velvety	<i>Cladosporium</i> spp.	31	-	-	-	-
		White	Cottony	<i>Rhizopus</i> spp.	1	-	-	-	-
		Yellow	Cottony	<i>A. niger</i>	1	-	-	-	-
	10^{-3}	Olive green	Velvety	<i>Cladosporium</i> spp.	1	-	-	-	-
		White	Cottony	<i>Rhizopus</i> spp.	5	-	-	-	-
		Yellow	Cottony	<i>A. niger</i>	1	-	-	-	-
Wall	10^{-2}	Light pink	Fluffy	<i>B. bassiana</i>	15	Olive green	Velvety	<i>Cladosporium</i> spp.	1
		White	Cottony	<i>Rhizopus</i> spp.	4	-	-	-	-
		Olive green	Velvety	<i>Cladosporium</i> spp.	64	-	-	-	-
	10^{-3}	Light pink	Fluffy	<i>B. bassiana</i>	3	-	-	-	-
		Light green	Velvety	<i>A. flavus</i>	1	-	-	-	-
		White	Fluffy	<i>B. bassiana</i>	50	-	-	-	-
Stand	10^{-2}	Olive green	Velvety	<i>Cladosporium</i> spp.	30	Light green	Velvety	<i>A. flavus</i>	15
		White	Fluffy	<i>B. bassiana</i>	38	-	-	-	-
	10^{-3}	White	Cottony	<i>Rhizopus</i> spp.	2	-	-	-	-
Tray	10^{-2}	Orange	Powdery	<i>Neurospora</i> spp.	30	Orange	Powdery	<i>Neurospor</i> spp.	1
		Orange	Powdery	<i>Neurospora</i> spp.	1	-	-	-	-
	10^{-3}	White	Fluffy	<i>B. bassiana</i>	1	-	-	-	-
News paper	10^{-2}	Olive green	Velvety	<i>Cladosporium</i> spp.	1	Yellow	Cottony	<i>A. niger</i>	1
		White	Fluffy	<i>B. bassiana</i>	37	-	-	-	-
	10^{-3}	Olive green	Velvety	<i>Cladosporium</i> spp.	2	-	-	-	-
		White	Fluffy	<i>B. bassiana</i>	21	-	-	-	-

Table 3: Comparison of fungal colonies after Serial dilution before and after disinfection of model 2

Material	Dilution	Before disinfection				After disinfection			
		Colour of colony	Texture	Identified fungus	No. of colonies	Colour of colonies	Texture	Identified fungus	No. of colonies
Floor	10 ⁻²	Light green	Velvety	<i>A. flavus</i>	4	Light green	Velvety	<i>A. flavus</i>	3
		White	Cottony	<i>Rhizopus</i> spp.	5	Orange	Powdery	<i>Neurospora</i> spp.	4
	10 ⁻³	White	Cottony	<i>Rhizopus</i> spp.	3	Olive Green	Velvety	<i>Cladosporium</i> spp.	2
		Yellow	Cottony	<i>A. niger</i>	1	Orange	Powdery	<i>Neurospora</i> spp.	1
Wall	10 ⁻²	-	-	-	-	-	-	-	-
	10 ⁻³	-	-	-	-	-	-	-	-
Stand	10 ⁻²	-	-	-	-	-	-	-	-
	10 ⁻³	-	-	-	-	-	-	-	-
Tray	10 ⁻²	Pale green	Velvety	<i>A. tamarii</i>	1	-	-	-	-
	10 ⁻³	-	-	-	-	-	-	-	-
News paper	10 ⁻²	Pale green	Velvety	<i>A. tamarii</i>	3	-	-	-	-
		Orange	Powdery	<i>Neurospora</i> spp.	2	-	-	-	-
	10 ⁻³	-	-	-	-	-	-	-	-

-: No fungal colony was observed

Table 4: Comparison of fungal colonies after Serial dilution before and after disinfection of model 3

Material	Dilution	Before disinfection				After disinfection			
		Colour of colony	Texture	Identified fungus	No. of colonies	Colour of colonies	Texture	Identified fungus	No. of colonies
Floor	10 ⁻²	Yellow	Cottony	<i>A. niger</i>	2	-	-	-	-
		White	Cottony	<i>Rhizopus</i> spp.	1	-	-	-	-
		White	Fluffy	<i>B. bassiana</i>	3	White	Fluffy	<i>B. bassiana</i>	1
	10 ⁻³	White	Cottony	<i>Rhizopus</i> spp.	1	-	-	-	-
		Light green	Velvety	<i>A. flavus</i>	1	-	-	-	-
		White	Fluffy	<i>B. bassiana</i>	1	-	-	-	-
Wall	10 ⁻²	-	-	-	-	-	-	-	-
	10 ⁻³	-	-	-	-	-	-	-	-

-: No fungal colony was observed

disinfection. In model-2, no fungal colony was observed before and after disinfection in both 10⁻² and 10⁻³ dilutions (Table 3). In trays of model-1, significant reduction (p<0.001) in one type of 30 fungal colonies in 10⁻² to one type of one colony after disinfection (Table 2). In 10⁻³ dilution, two different colonies before disinfection were reduced to zero after disinfection (Table 2). In model-2, 10⁻² dilution showed single fungal colony before disinfection however no colony was found after disinfection. Similarly in 10⁻³ no colony was found before and after disinfection (Table 3). In used newspaper of model-1, there was significant (p<0.001) reduction in 38 no. of two types of fungal colonies to single colony in 10⁻² and in 10⁻³ dilutions before disinfection 23 no. of two types of fungal colonies reduced to zero after disinfection (Table 2). In model-2, 10⁻² dilution showed two

Table 5: Comparison of total fungal spores quantification in a unit area by serial dilution before and after disinfection

Materials	Dilution	Model 1				Model 2				Model 3			
		BD	AD	T-value	Sig.	BD	AD	T-value	Sig.	BD	AD	T-value	Sig.
Floor/	10 ⁻²	33×10 ²	Nil	-	-	9×10 ²	7×10 ²	2.45	NS	6×10 ²	Nil	-	-
sq. ft	10 ⁻³	7×10 ³	Nil	-	-	4×10 ³	3×10 ³	1.22	NS	3×10 ²	Nil	-	-
Wall/	10 ⁻²	415×10 ³	5×10 ³	707	***	Nil	Nil	-	-	Nil	Nil	-	-
sq.ft	10 ⁻³	27×10 ⁵	Nil	-	-	Nil	Nil	-	-	Nil	Nil	-	-
Stand 1'	10 ⁻²	34×10 ⁴	75×10 ³	45.7	***	Nil	Nil	-	-	-	-	-	-
x 3/4	10 ⁻³	1×10 ⁵	Nil	-	-	Nil	Nil	-	-	-	-	-	-
dia. rod													
Tray/	10 ⁻²	15×10 ⁴	5×10 ³	25	***	5×10 ³	Nil	-	-	-	-	-	-
sq.ft	10 ⁻³	5×10 ⁴	Nil	-	-	Nil	Nil	-	-	-	-	-	-
Newspaper/	10 ⁻²	19×10 ⁴	5×10 ³	31.9	***	25×10 ³	Nil	-	-	-	-	-	-
sq.ft	10 ⁻³	115×10 ⁴	Nil	-	-	Nil	Nil	-	-	-	-	-	-

***Significant at p<0.1% 8, NS: Non significant, BD: Before disinfection, AD: After disinfection, Sig. Significant), Nil: No fungal colony was observed -:Statistical analysis cannot be done, presented

types of 5 fungal colonies before disinfection whereas after disinfection no colony was found. Similarly in 10⁻³ no colony was found before and after disinfections (Table 3).

Quantification: Quantification of germ load was made for 10⁻² and 10⁻³ serial dilution plates (Table 5). In floor it was found that in 10⁻² dilution, one sq.ft. area of floor contained 33×10², 9×10² and 6×10² fungal cells, respectively before disinfection in model-1, 2 and 3 which were reduced to zero in model-1 and 3 whereas in model-2 non-significant reduction to 7×10² after disinfection was observed. In 10⁻³ dilution, model-1 showed reduction from 7×10³ to zero after disinfection whereas non-significant difference was observed in model-2 before (4×10³) and after (3×10³) disinfection. Model-3 showed reduction from 3×10² to zero after disinfection. One sq. ft. area of wall in 10⁻² dilution contained 415×10³ fungal cells before disinfection which was significantly reduced to (p<0.01) 5×10³ in model-1. In model-1 10⁻³ dilution showed 27×10⁵ fungal spores before disinfection reduced to zero after disinfection. In model-2 and 3 with dilution 10⁻² and 10⁻³ before and after disinfection no fungal load was observed. One foot long rod of ¾" diameter of iron stand in 10⁻² dilution contained 34×10⁴ fungal cells before disinfection significantly reduced to (p<0.001) 75×10³ cells after disinfection in model-1. In model-1 10⁻³ dilution showed 1×10⁵ fungal spores before disinfection, this reduced to zero after disinfection. In model-2 with 10⁻² and 10⁻³ dilution, no fungal load was observed before and after disinfection. In model-1, one sq. ft. area of the used tray in 10⁻² dilution contained 15×10⁴ fungal cells before disinfection which after washing significantly reduced (p<0.001) to 5×10³ cells. In model-2, 5×10³ cells were reduced to zero after disinfection. In 10⁻³ dilution, model-1 showed reduction from 5×10⁴ to zero after disinfection whereas no germ load was observed in model-2 before and after disinfection. It was found that in 10⁻² dilution, one sq. ft. area of old newspaper contained 19×10⁴ fungal cells before disinfection in model-1 which was significantly (p<0.001) reduced to 5×10³ in new newspaper whereas in 10⁻³ dilution 115×10⁴ fungal cells reduced to zero. In 10⁻² dilution, old newspaper contained 25×10³ fungal cells before disinfection in model-2 which was reduced to zero in new newspaper. There was no germ load observed in model-2 before and after disinfection in 10⁻³ dilution.

Isolation and observation of pure culture: Under Stereoscopic binocular Nikon SMZ-10A (24.5x), 7 types of the fungal colonies were identified based on their morphological features as advocated by Gilman (1971). Sporangioophore/conidiophores and mycelia of fungi were stained with lacto phenol cotton blue and observed under student microscope. The fungi identified were *Aspergillus tamarii*, *A. flavus*, *A. niger*, *Neurospora* spp., *Beauveria bassiana*, *Rhizopus* spp. and *Cladosporium* spp. (Fig. 1-7). Among these fungi *Aspergillus tamarii*, *A. flavus* and *Beauveria bassiana* are reported to be pathogenic to silkworm.

In vitro testing of the disinfectants against the fungal culture: The effectiveness of disinfectants (Sanitech, Asthra, bleaching solution, formalin and alcohol) was assessed by *in vitro* testing based on the size of the zone of inhibition. The size of the zone depends on the interaction between the inoculum and the type and diffusibility of the disinfectant which was presented in Table 6, Fig. 8-14.



Fig. 1: Isolated fungal colony of *Aspergillus tamarii* under stereoscopic binocular (24.5x) in (a-d) Petri-plates and slants and (e) under student microscope (600x)

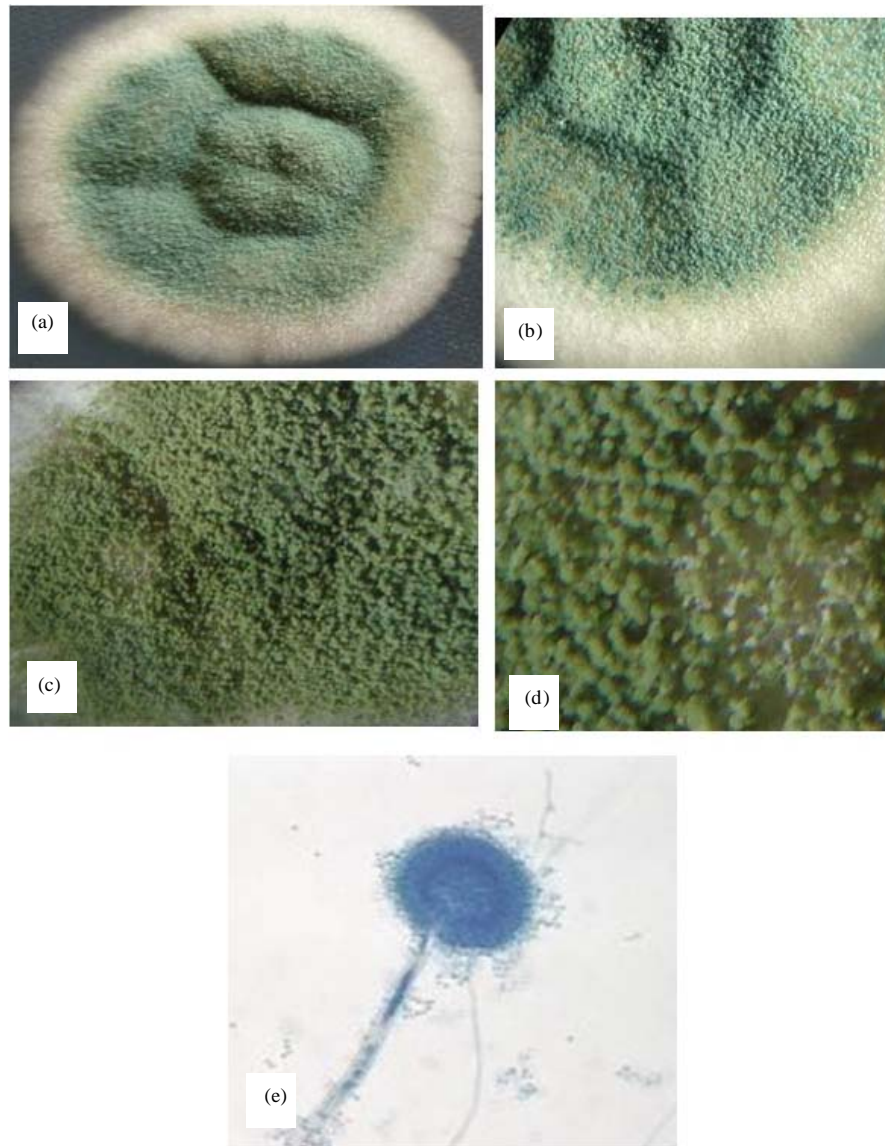


Fig. 2: Isolated fungal colony of *Aspergillus flavus* under stereoscopic binocular (24.5x) (a-d) in Petri-plates and slants and (e) under student microscope (600x)

- ***Aspergillus tamarii***: The 24 h after inoculation, colony was white in colour with clear zone of effectiveness (Fig. 8a). After 48 h, colony matured by producing bottle green spores with clear zones (Fig. 8b). For *Aspergillus tamarii* four disinfectants viz., Asthra, bleaching solution, formalin and alcohol were effective. Out of these, alcohol was least effective (6.000 mm) significantly ($p < 0.01$) less than other three disinfectants. Asthra showed 9.500 mm clear zone which was significantly ($p < 0.01$) less effective than bleaching (14.375 mm) and formalin (22.875 mm). Formalin had significantly ($p < 0.01$) highest effective zone from all the disinfected used (Table 6, Fig. 8a,b).

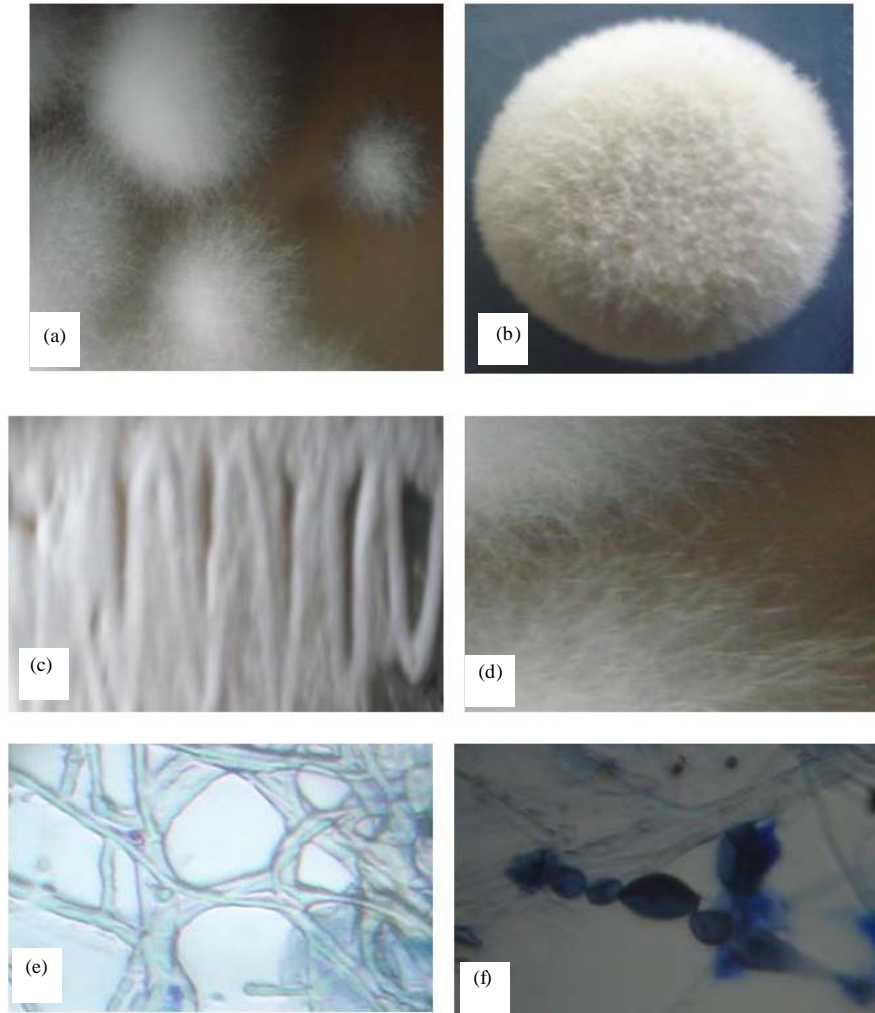


Fig. 3: Isolated fungal colony of *Beauveria bassiana* under stereoscopic binocular (24.5x) (a-d) in Petri-plates and slants and (e-f) under studentmicroscope (600x)

Table 6: *In vitro* assessment of different disinfectants against different fungal colonies

Disinfectants (zone diameter in mm)

Sl no.	Organism inoculated	Sanitech	Asthra	Bleaching	Formalin	Alcohol	SE±	CD at 5%	CD at 1%
1	<i>Aspergillus tamarii</i>	Nil	9.5008**	14.375**	22.875**	6.000	0.447	1.865	2.889
2	<i>Aspergillus flavus</i>	Nil	16.000	20.875**	15.500	9.625	1.694	7.068	10.949
3	<i>Aspergillus niger</i>	6.625	10.875**	10.375**	11.375**	Nil	0.528	2.204	3.414
4	<i>Neurospora</i> spp.	Nil	17.500*	16.250	15.875	11.625	1.158	4.829	7.481
5	<i>Beauveria bassiana</i>	Nil	17.375	25.250**	19.500*	13.250	1.371	5.718	8.859
6	<i>Rhizopus</i> spp.	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
7	<i>Cladosporium</i> spp.	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

* Significant at 5%, ** Significant at 1%, Nil: No. clear zone detected

- ***Aspergillus flavus***: Inoculated colony was white in colour with clear zone of effectiveness at 24 h (Fig. 9a). After 48 h, colony matured by producing pale green spores with clear zones

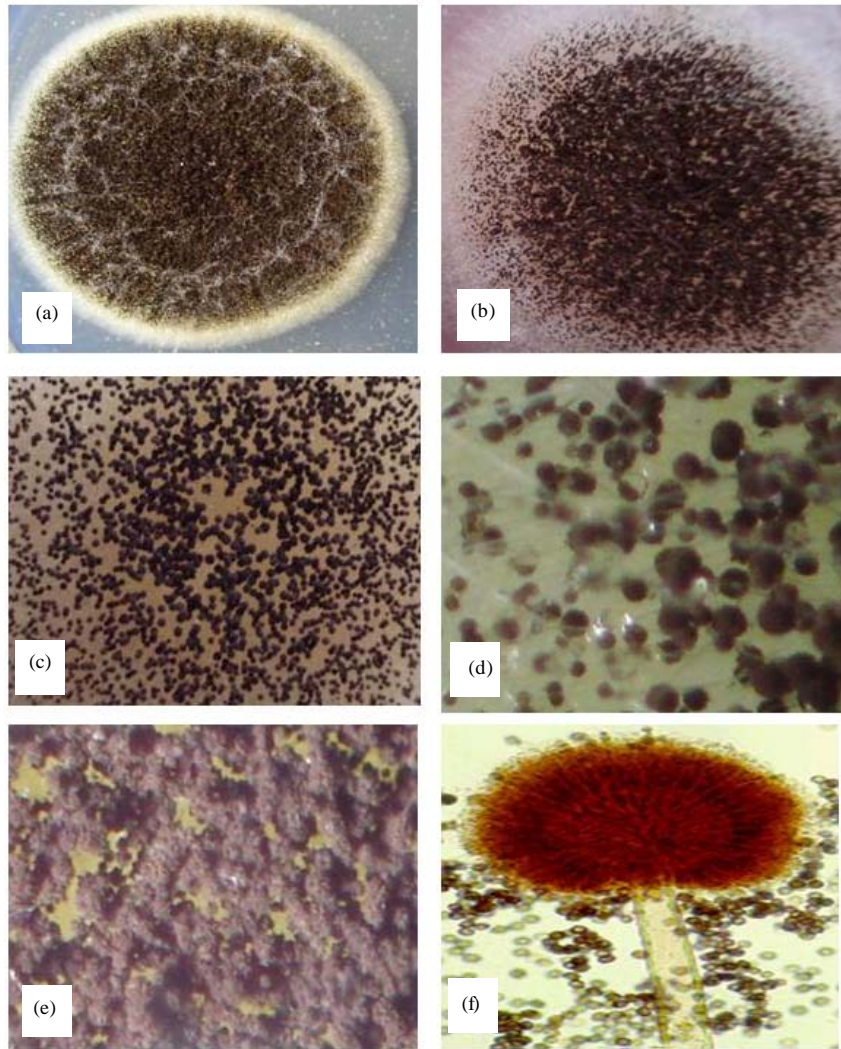


Fig. 4: Isolated fungal colony of *Aspergillus niger* under stereoscopic binocular (24.5x) in (a-e) Petri-plates and slants and (e) under student microscope (600x)

zones (Fig. 9b). Four disinfectants viz., Asthra, bleaching solution, formalin and alcohol were effective in controlling *A. flavus*. Out of four, alcohol had least zone (9.625 mm) and had non-significant difference with Asthra and formalin whereas, it showed significantly less ($p < 0.01$) than bleaching solution. Bleaching solution had highest clear zone (20.875 mm) which was non-significant with Asthra (16.000 m) and formalin (15.500 mm) (Table 6, Fig. 9a,b).

- ***Aspergillus niger***: The 24 h after inoculation, colony was not visible whereas, after 48 h colony developed by producing black spores with clear zones (Fig. 10). In this fungal colony four disinfectants viz., Sanitech, Asthra, bleaching solution and formalin were effective. Sanitech had least effect (6.625 mm) and significantly less ($p < 0.01$) than other three disinfectants. Formalin showed highest clear zone (11.375 mm) which was non-significant with Asthra (10.875 mm) and bleaching solution (10.375 mm) (Table 6, Fig. 10).

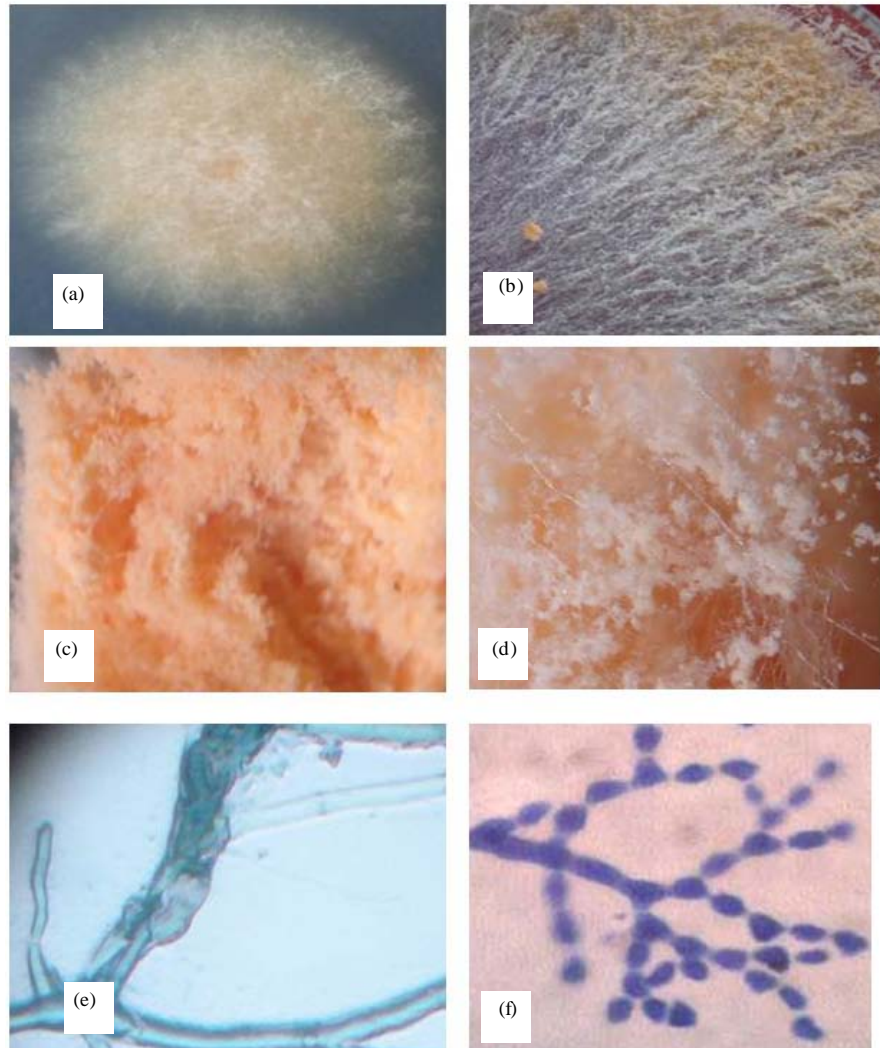


Fig. 5: Isolated fungal colony of *Neurospora* spp. under stereoscopic binocular (24.5x) in (a-d) Petri-plates and slants and (e-f) under Student microscope (600x)

- ***Neurospora* spp.:** As *Neurospora* is fast growing colony, inoculated colony developed within 24 h with clear zone of effectiveness (Fig. 11a) whereas, after 48 h colony over grown and all clear zones were covered by mycelia and its spores (Fig. 11b). Four disinfectants viz., Asthra, bleaching solution, formalin and alcohol were effective in controlling fungal colony temporarily. Least zone (11.625 mm) was observed in alcohol-disinfected plate which was significantly ($p < 0.05$) less than Asthra. Asthra had highest zone (17.500 mm) whereas non-significant difference with bleaching solution (16.250 mm) and formalin (15.875 mm) was observed (Table 6, Fig. 11a,b).
- ***Beauveria bassiana*:** Colony was not visible upto 24 h after inoculation, whereas after 48 h colony developed by producing white fluffy mycelium with clear zones (Fig. 12). Four disinfectants viz., Asthra, bleaching solution, formalin and alcohol were effective in controlling fungal colony. Alcohol is found to be least effective (13.250 mm), non significant with Asthra (17.375 mm), significantly ($p < 0.05$) less effective than formalin (19.500 mm) and highly

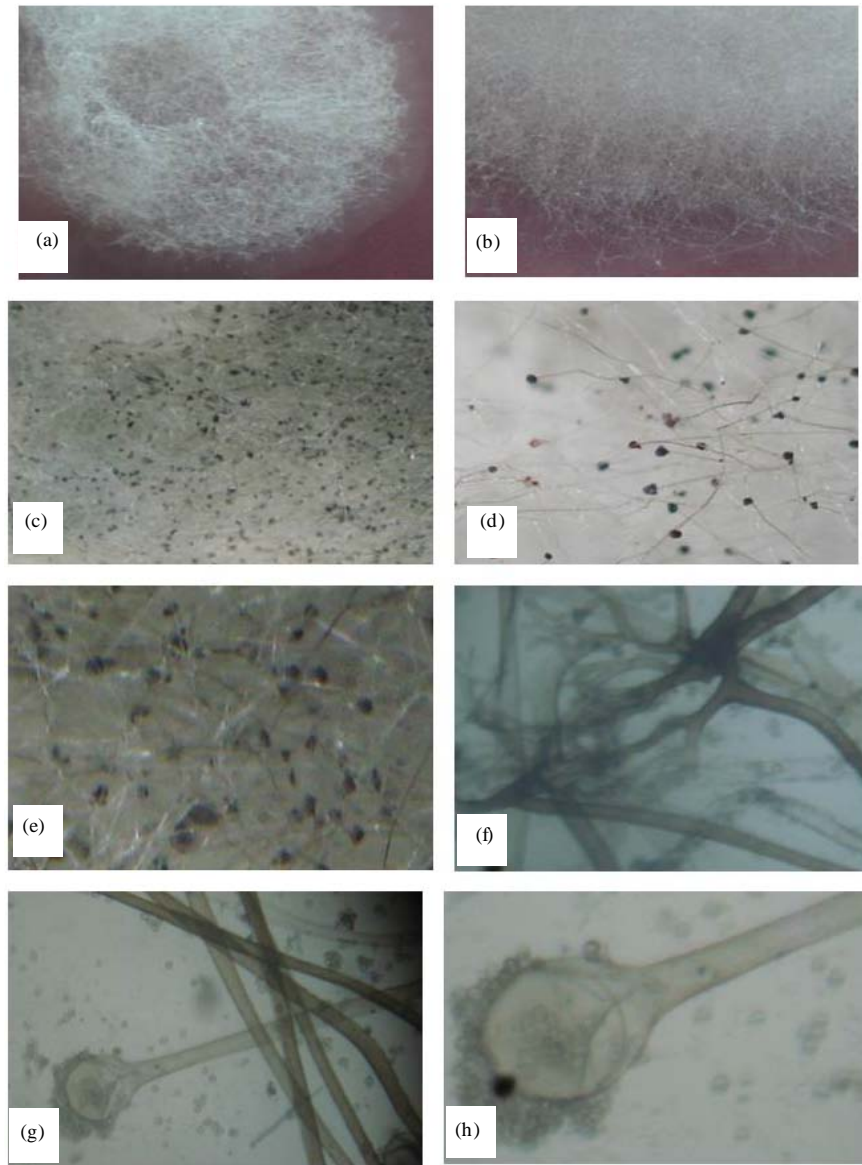


Fig. 6: Isolated fungal colony of *Rhizopus* spp. under stereoscopic binocular (24.5x) in (a-e) Petri-plates and slants and (f-h) under Student microscope (600x)

significantly less ($p < 0.01$) than bleaching solution (25.250 mm). Asthra was non significant in controlling the fungi as compared to formalin and less effective ($p < 0.05$) than bleaching solution. Bleaching solution had highest effect in controlling *Beauveria bassiana* and significant more ($p < 0.05$) than formalin (Table 6, Fig. 12).

- ***Rhizopus* spp.:** The 24 h after inoculation, colony was not visible whereas, after 48 h, colony developed rapidly and overgrew throughout the media covering the disc. Faint zones of inhibition were observed in bleaching and formalin treated disc but it was covered with white cottony mycelium so that measurement of zones could not be taken up (Table 6, Fig. 13).
- ***Cladosporium* spp.:** The plates inoculated with *Cladosporium* did not show any growth even after 7 days of inoculation (Fig. 14). So no data can be recorded for this fungal colony.



Fig. 7: Isolated fungal colony of *Cladosporium* spp. under stereoscopic binocular (24.5x) in (a-e) Petri-plates and slants and (f) under Student microscope (600x)

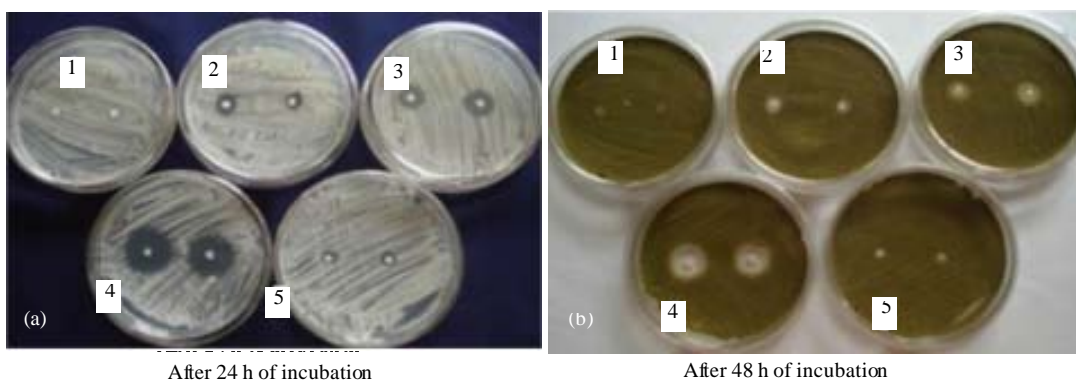


Fig. 8: *In vitro* testing of different disinfectants 1: Sanitech, 2: Asthra, 3: Bleaching soln, 4: Formalin 5: Alcohol against fungal colony *A. Tamarii*

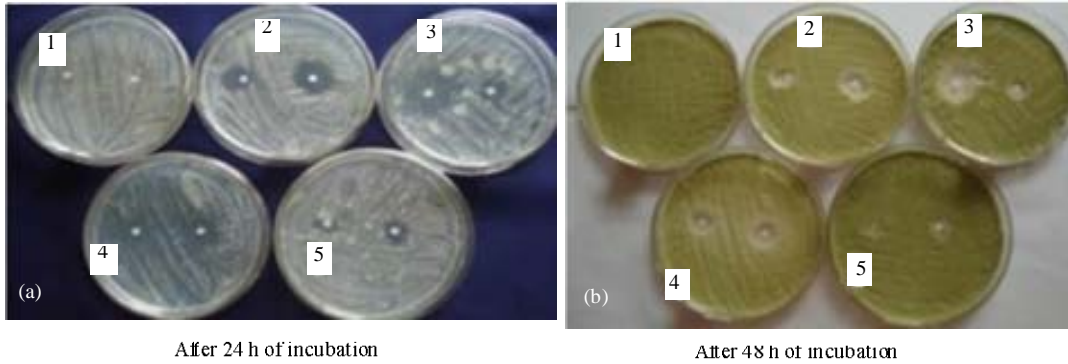


Fig. 9: *In vitro* testing of different disinfectants 1: Sanitech, 2: Asthra, 3: Bleaching soln, 4: Formalin, 5: Alcohol against fungal colony *A. flavus*

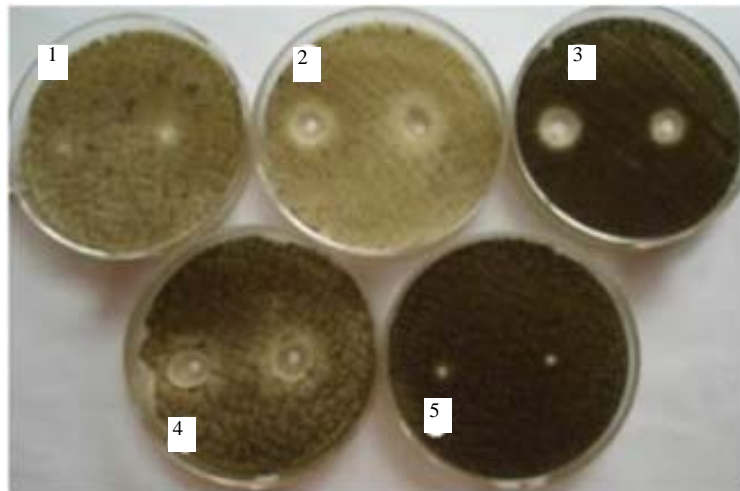


Fig. 10: *In vitro* testing of different disinfectants after 48 h of incubation 1: Sanitech, 2: Asthra, 3: Bleaching soln, 4: Formalin, 5: Alcohol against fungal colony *A. niger*

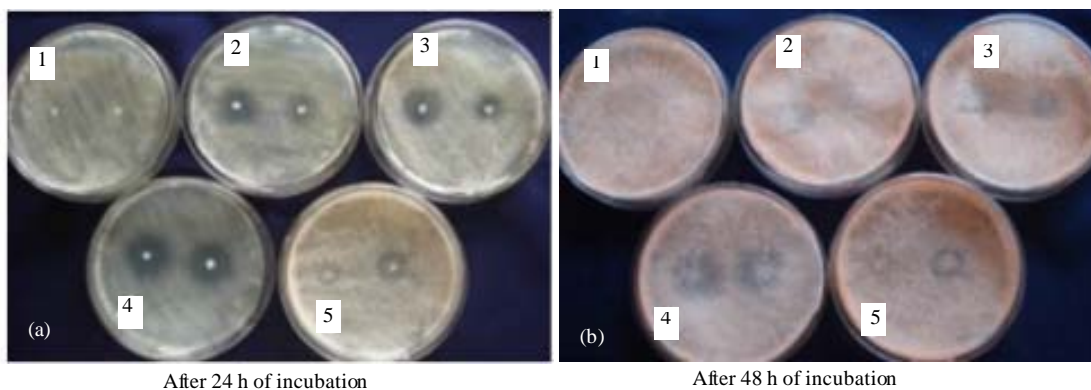


Fig. 11: *In vitro* testing of different disinfectants 1: Sanitech, 2: Asthra, 3: Bleaching soln, 4: Formalin, 5: Alcohol against colony *Neurospora* spp.

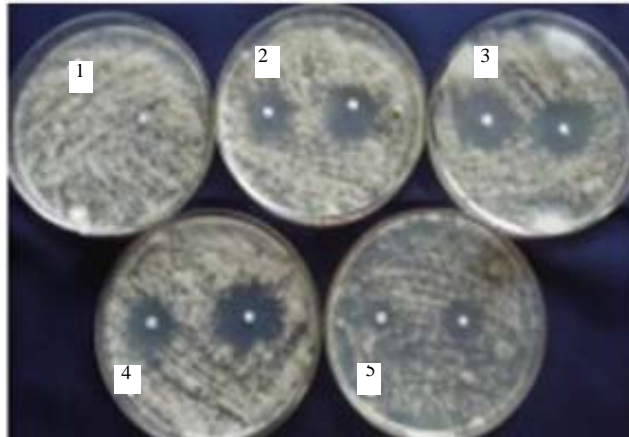


Fig. 12: *In vitro* testing of different disinfectants after 48 h of incubation: 1: Sanitech, 2: Asthra, 3: Bleaching soln, 4: Formalin, 5: Alcohol against fungal colony *Beauveria bassiana*

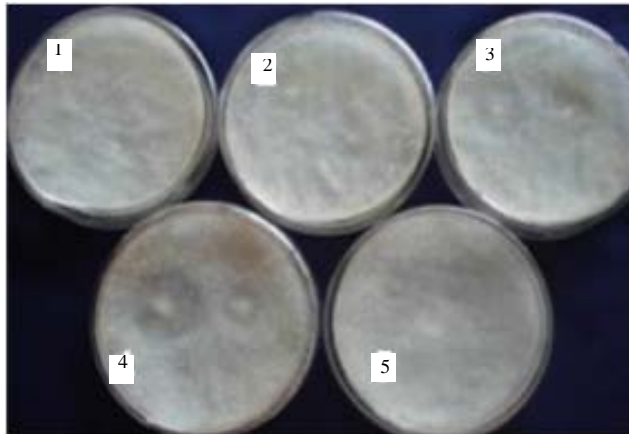


Fig. 13: *In vitro* testing of different disinfectants after 48 h of incubation: 1: Sanitech, 2: Asthra, 3: Bleaching soln, 4: Formalin, 5: Alcohol against fungal colony *Rhizopus* spp.

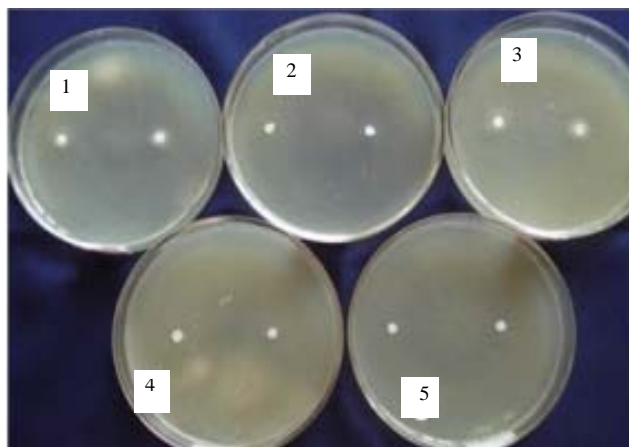


Fig. 14: *In vitro* testing of different disinfectants after 48 h of incubation: 1: Sanitech, 2: Asthra, 3: Bleaching soln, 4: Formalin, 5: Alcohol against fungal colony *Cladosporium* spp.

DISCUSSION

Most of the microorganisms present in rearing houses are non-pathogenic. But there is every possibility of pathogens present in a lower concentration in rearing houses despite no disease occurrence (Susan and Jones, 1981). For controlling the disease, rearing shed and appliances should be disinfected thoroughly to destroy the fungal spores. Large number of fungicides has been screened as topical and systemic application against *Beauveria bassiana* and *Aspergillus* species of fungus (Kumar *et al.*, 2002; Kumar *et al.*, 2003; Singh *et al.*, 2002; Nataraju *et al.*, 2005). Disinfection is the reduction of contaminants to an acceptable level. A wide variety of active chemical agents (biocides) are found as antiseptics and disinfectants, many of which have been used for hundreds of years, including alcohols, phenols, iodine and chlorine (McDonnell and Russell, 1999).

In the present study fungal germ load was assessed by surveillance for air born pathogen from three silkworm-rearing houses before and after disinfection, by sedimentary sampling method (Table 1). Multiple plates were kept and results are based on overall assessment. In the air trap culture *Aspergillus* spp., *Cladosporium* spp, *Rhizopus* spp. and *Beauveria bassiana* were identified. The distribution of microbes particularly fungi in the air in quantitative and qualitative terms varies among area and also influenced by season and climatic factors such as temperature, air humidity, wind speed and direction, human activity and type of ventilation. According to Kawakami (1982) air sampling method by the bio-test RCS air centrifugal sampler was more effective than sedimentary sampling method. Difficulty of establishing reliable method to measure the exposure fungal spores in the air is one of the main impediments in clarifying the real impact of exposure in the development of fungal infection. There is a need to monitor the differences between automatic passive sedimentation techniques and automated technique, although efficiency in quantitative analysis is also of limited use because they require noisy equipment with constant power supply. The passive sedimentation technique is also limited because it does not permit adequate quantitative analysis. But it is still widely recommended in literature for the use as a microbiological alert (Cordeiro *et al.*, 2010).

According to Kawakami (1982) fungi ranging from 10/100 liter to $7.5 \times 10/100$ liter were trapped by RCS air sampler from rearing room and it contained *Penicillium* and *Aspergillus* in high frequency besides the colonies of *Cladosporium*, *Alternaria*, *Paecilomyces*, *Cephalosporium*, *Rhizopus* or mucor were also detected. While *Penicillium*, *Alternaria*, *Paecilomyces* and *Cephalosporium* were not detected in present study. Kangu (2006) reviewed the air borne 40 species of *Cladosporium*. *Cladosporium* spp. was not pathogenic except to immuno-compromised sericulturists. Spore concentrations at elevated levels can elicit chronic allergy and asthma. The spores at concentrations of 3000/cu m of air are taken as the threshold concentration for clinical significance. Chandrasekharan (2009) collected air samples from different sericulture farmer houses and found the presence of conidia of *B. bassiana* inside and out side the rearing house was less during summer and it increased in rainy and winter.

All the three rearing houses were disinfected differently and after disinfection fungal germ load was assessed. It was found that air borne fungal colony in the model 1-3 reduced comparatively after disinfection (Table 1). In model-1, chlorine dioxide and formalin in model 2 were used as a major disinfectant. This clearly indicates that chlorine dioxide and formalin are effective. In few cases new fungus was observed after disinfection which can be due to sampling difference in the place of collection before and after disinfection. In general, chlorine dioxide has been determined to be equal to or superior to chlorine on a mass-dose basis. Quantitative data published as early as

1940s demonstrates the efficacy of chlorine dioxide (Anonymous, 1999; Tian *et al.*, 1996) on pathogens of silkworm *Bombyx mori*.

In model 1 and 3 germ load reduced (Table 2 and 4). In Model 2, tray, wall, stand and newspaper revealed significant reduction in germ load except floor after the disinfection with formaldehyde. Floor contains different types of fungal germloads which were not controlled by formalin disinfection (Table 3). Probably this can be due to differential selection of sampling area. However, Kobayashi *et al.* (1968) found the disinfecting ability of the mixture of bleach powder and formalin. Rasowo *et al.* (2007) evaluated the effects of different disinfectants viz., formaldehyde, sodium chloride, potassium permanganate and hydrogen peroxide. Model-3 was disinfected with alcohol revealed significant reduction in germ load (Table 4). According to Price (1950) ethyl alcohol is inexpensive, readily obtainable and harmless to the skin. Its drawback lies in its inability to kill spores with 100% alcohol. Its fungicidal power varies with the concentration of the alcohol solution. According to traditional opinion, 50% by volume is best. After experimentation, it was found that 60 to 70% by volume is the optimum concentration (Price, 1950). In swab method disinfected floor, wall and washed materials like stand, tray in all the models, germ load and number of colonies were reduced but not completely removed. It should be below the level required to cause disease. This means, viable microorganisms are still present (http://www.lbl.gov/ehs/biosafety_Mannual/html/decontamination.shtml). Similarly Susan and Jones (1981) took 36 different cleaning materials of 6 poultry and 2 pig houses to assess the cleanliness of the building. They found that varnished plywood retained less germ load than by blockboard due to retention of less dirt. Harry and Hemsley (1964) showed that when a building is left empty, the germ load would reduce logarithmically so that in 6 weeks only 20% of original load will remain. It is uneconomical however, to leave rearing houses unoccupied for long period. So cleaning routinely is necessary. Initially the gross dirt should be removed as pointed out by Walters (1967) that hard surface should be thoroughly cleaned and disinfected to reduce the microbial load by 5% followed by fumigation of 1%. Effect of disinfection will be there for a few days after disinfection. As the effect of disinfectant keeps on decreasing, the fungal germ will revive again due to its rapid multiplication rate. Some fungi survive a chemical attack; they give rise to the next generation. Thus they may be able to develop resistance to hostile chemicals (Kodama, 2001).

Quantification: Quantification of Colony Forming Unit Area (CFUA) indicated that in model 1 disinfected with chlorine dioxide (Asthra and Sanitech) and bleaching solution showed that all colonies of floor observed in 10^{-2} and 10^{-3} dilutions before disinfection are destroyed. Hence no colony observed after disinfection and germ load reduced. Whereas, germ load of wall, stand, tray and newspaper were reduced significantly ($p < 0.01$) after disinfection in 10^{-2} dilution. In 10^{-3} dilution after disinfection, no fungal colonies revived (Table 5). Model 2 which were disinfected by formalin, fungal contamination was not reduced significantly in floor (10^{-2} and 10^{-3}) whereas, wall and tray (10^{-2} and 10^{-3}) showed no fungal growth before and after disinfection, tray and used news paper showed fungal contamination at 10^{-2} dilution which was not revived (Table 5). In model 3 disinfected with alcohol and formalin fumigation, floor contained fungal germ load before disinfection (10^{-2} and 10^{-3}) that was not revived after disinfection (Table 5). Results clearly indicated that all the disinfectants used significantly reduced the fungal germload. According to www.inspectapedia.com/sickhouse/Mold-Standards.htm CFUA is a colony of microorganism referred to a mass of individual cells of same organism, growing together. In the present work, quantification was carried out per square feet area of each item. Colony forming units can be

measured as CFU per unit weight, CFU per unit area or CFU per unit volume depending on the type of sample tested (Kung'u, 2006). During summer season, average CFU inside the rearing house was 0.67×10^4 while it was 1.57×10^4 outside the rearing house (Chandrasekharan, 2009).

Isolation and observation of pure culture and microscopic examination: In the present study, fungal colonies were cultured on potato dextrose agar media, identified and isolated according to colour and texture of colony. For all the isolates, prepared slides and identified and confirmed the species (www.entomology.wisc.edu/mbcn/kyf410.html). Harmful fungi isolated were *Aspergillus tamaris* (Fig. 1), *Aspergillus flavus* (Fig. 2) and *Beauveria bassiana* (Fig. 3). *Aspergillus* spp., causing aspergillosis disease adversely affected the hatching of silkworm eggs, causing death to hatched and early instar larvae. *Aspergillus tamaris* (Fig. 1) was identified by its pale green colour and velvety texture in the swab of used news paper and tray of model 2, whereas, *Aspergillus flavus* (Fig. 2) was light green and velvety in texture was found in the air (After disinfection), swab of wall (10^{-8} dilution before disinfection) and stand (10^{-2} dilution after disinfection) of model 1, floor of model 2 (10^{-2} dilution before and after disinfection) and floor (10^{-8} dilution) and air of model 3 before disinfection. Muscardine causing *Beauveria bassiana* was identified as white-light pink fluffy colony (Fig. 3) isolated from air (model 1 and 3 before disinfection). Dilution at 10^{-2} and 10^{-3} of all the materials showed *Beauveria bassiana* before disinfection. The same was not detected after disinfection demonstrating that the disinfection pattern followed (5% Bleaching+ClO₂) aids in reducing the pathogen load. *Beauveria bassiana* was not found in model 2, whereas, in model 3 it was found in swab and serial dilution of floor and wall before disinfection which was again detected in floor sample even after disinfection because alcohol disinfection is least effective to control this fungus. Other cultures identified and isolated *Aspergillus niger* (Fig. 4), *Neurospora* spp. (Fig. 5), *Rhizopus* spp. (Fig. 6) and *Cladosporium* spp. (Fig. 7) were non-pathogenic to silkworm and found to be the common inhabitants of the silkworm rearing houses.

In vitro testing of the disinfectants against the fungal culture: Fungal spores isolated from the system and cultured in the nutrient media provide the nutrients needed for the cells to survive. Following Thornberry (1950) method these spores/hyphae were exposed to disinfectants and each disinfectant was analysed to understand its effectiveness similar to Devleeschouwer and Dony (1981). In the present study 7 fungal colonies were tested (Table 6). Out of these 5 isolated fungal colonies viz., *Aspergillus tamaris* (Fig. 8), *Aspergillus flavus* (Fig. 9), *Aspergillus niger* (Fig. 10), *Neurospora* spp. (Fig. 11) and *Beauveria bassiana* (Fig. 12), were tested against commercially recommended dose of 5 disinfectants and observations were recorded after 24 and 48h of incubation. Sanitech and Asthra are trade names of commercially available chlorine dioxide in liquid and dry form, respectively. One colony of *Rhizopus* spp. over-grew within 24 h due to its rapid nature of growth could not be considered (Fig. 13) and one colony of *Cladosporium* spp. did not grow upto 7 days of inoculation (Fig. 14). Out of 5 fungal cultures tested, Sanitech was effective in controlling only one colony (*A. niger*), Alcohol in all four colonies except *A. niger* and Asthra, bleaching and formalin in all the 5 colonies. Bleaching solution controlled 2 pathogenic colonies (*Aspergillus flavus* and *Beauveria bassiana*) of silkworm significantly. Whereas, formalin significantly controlled one colony (*Aspergillus tamaris*). Organism in hand may be divided into transients and residents (Price 1938). Transients organisms are those which do not grow on the skin and can be removed more easily whereas resident grow on the skin and are more difficult to

be removed. *Aspergillus tamarii* that contaminate artificial diet may be a transient type of organism contaminated while handling. 24 h after inoculation, *Aspergillus tamarii* colony was white in colour with clear zone of effectiveness (Fig. 8a). After 48 h, colony matured by producing pale green spores with clear zones (Fig. 8b). Formalin, bavistan, mancozeb, zineb and benzoic acid were found effective against *A. tamarii* by Peter and Devaiah (1999), similarly in the present study formalin was proved as the best disinfectant against *A. tamarii* followed by bleaching solution. Similar growth pattern was observed in *Aspergillus flavus* (Fig. 9, Table 6) also and growth was controlled by bleaching solution with highest clear zone. In the present study conidia are used as inocula for the *in vitro* susceptibility testing of *Aspergillus* spp. Since the MIC (minimum inhibitory concentration) is defined on the basis of visible mycelial growth, conidia should germinate and produce sporelings (germinated conidia) for monitoring of the growth inhibition and fungicidal activity of a disinfectant. If a disinfectant is capable of inhibiting germination of conidia while affecting or not affecting the growth of the organism, the MIC obtained will be the concentration of the disinfectant required for the inhibition of conidial germination but not necessarily that required for inhibition of the growth of the organism. According to Manavathu *et al.* (1999), susceptibility of germinated and ungerminated conidia of *Aspergillus fumigatus* to antifungal agents in 24 h exposure and found that MICs of various antifungal agents for germinated conidia were almost identical to those obtained for ungerminated conidia. These results suggest that either germinated or ungerminated conidia could be used as inocula for *in vitro* susceptibility studies of *A. tamarii* and *A. flavus*. According to Tortorano *et al.* (2005) limitation of *in vitro* methods is that they test activity of biocides against conidia and not against hyphae which might be present in some substrates. In *A. tamarii* and *A. flavus* both mycelia and conidial growth was controlled by disinfectants. Whereas in *Aspergillus niger* no visible inhibition zone was detected at 24 h. However, it pre-dominated only at 48 h. Colony developed by producing black spores with clear zones (Fig. 10). This indicated that in the mycelial growth was not inhibited whereas conidial growth was inhibited. In this fungal colony formalin showed highest clear zone which was non-significant with Asthra and bleaching solution (Table 6, Fig. 10). According to Kumar *et al.* (2009) growth of fungal pathogens of *Aspergillus niger* was highly inhibited by marine plant extract viz., *S. isoetifolium* (15 mm dia) and *Padina tetrostomatica* (13 mm dia) respectively. 25 isolates of *Aspergillus* spp. *in vitro* tested by Espinel-Ingroff *et al.* (1995) and found that all the isolates produced clearly detectable growth within 1 to 4 days at 35°C. Present study revealed that some fungal colonies grow at 25°C within 24h, some after 24, 48, 72 h even after 7 days. 35°C temperature for the experiment was not taken up. Based on data from several studies (Espinel-Ingroff and Kerkering, 1991; Espinel-Ingroff *et al.*, 1995; Espinel-Ingroff *et al.*, 1997) recommended incubation at 25°C for 24 h (*Rhizopus* spp.) and 48 h (*Aspergillus* spp.). In the present study *Aspergillus tamarii* and *Aspergillus flavus* are not formaldehyde resistant strain as reported by Saijo (1970) and Yanagita, 1980a, Yanagita, 1980b, Yanagita, 1980c. Walker *et al.* (2002) indicated variations in the degree of efficacy of certain commercial disinfectants in similar conditions in clinical isolates of *Aspergillus terreus* as compared to Voriconazole. *Neurospora* is fast growing colony, inoculated colony developed within 24 h with clear zone of effectiveness (Table 6, Fig. 11a) whereas, after 48 h colony over grown and all clear zones were covered by mycelia and its spores (Fig. 11b). *Beauveria bassiana* (Fig. 12, Table 6) was not detectable at 24 h, whereas it pre-dominated only at 48 h colony. Bleaching solution had highest effect in controlling *Beauveria bassiana* and significantly more ($p < 0.05$) than formalin. Similarly Kumari *et al.* (2011) reported *in vitro* inhibitory effect against *Beauveria bassiana* increased with increased concentration (1000

to 1500 $\mu\text{g mL}^{-1}$) of algal *Turbinaria conoides* crude extract. Ignoffo and Dutky (1963) observed the effect of sodium hypochlorite on the viability and infectivity of *Beauveria* spores. However, many of them are not in practice due to incomplete curative effect or impracticability in sericulture. Balavenkatasubbaiah *et al.* (1994) tested salicylic acid, paraformaldehyde, benzoic acid, slaked lime, formalin, bleaching powder and asiphor in different concentrations to compare their efficacy against *B. bassiana*. Except slaked lime, all were found effective against the latter. Four concentrations of asiphor (alkyl phenoxy polyglycol) were tested against the silkworm pathogen (Reddy *et al.*, 1990) and found that 2% asiphor considerably reduced muscardine incidence. (Byrareddy, 1986; Byrareddy *et al.*, 1991; Byrareddy *et al.*, 1993) tested different disinfectants including bleaching powder, potassium permanganate, dithane M-45 and formalin for controlling *B. bassiana* infecting *Bombyx mori*. Formalin (1.5%) was the most effective treatment while the least effective was 1.5% neem extract. *In vitro* studies on the efficacy of Disfect-S (poly alkyl monohydric phenol) by Samson *et al.* (1995) indicated its efficacy against *B. bassiana*. Chandrasekharan (2009) used chemicals, antifungal antibiotic and plant extracts against *B. bassiana*. He observed that all antibiotics are effective to inhibit the growth of *B. bassiana*. Ketacanazole showed highest inhibition (11.6 mm) and nystatin lowest inhibition (8.4 mm). 5 chemicals were listed for fully controlling the *B. bassiana*. Extract of 5 plants viz., *Cassia*, *Garlic*, *Mango bark*, *Mehindi* and *Tridax* were proved promising in controlling the disease. Sutton *et al.* (1999) studied *in vitro* amphotericin B resistance varied interaction by different disinfectants and different fungal colonies based on differences in inhibition zones. Russell (2003) observed considerable variation in the response of different microorganisms to biocides. Pakdaman *et al.* (2002) tested the antifungal activities of five herbicides *in vitro*. 2,4-D, desmedipham and diclofop-methyl showed the broad antifungal effects than of other herbicides tested. Effect of fungicides (Carbendazim, Mancozeb, Sulphur and conjoint Carbendazim and Mancozeb) against *Fusarium oxysporum* was tested by Shah *et al.* (2006) and found that all the treatments significantly reduced the growth. Gasparini *et al.* (1995) studied a new acid peroxygen system based disinfectant (Virkon), in order to assess its *in vitro* efficacy.

The problem is that, how well *in vitro* tests predict the response of the disinfectants to be used to disinfect the silkworm rearing houses. Efficacy of disinfectants depends upon the concentration of pathogen load present in rearing houses. According to Nightingale (1987), factors affecting the reliability of *in vitro* testing systems include the limitations in interpreting MIC data. *In vitro* test conditions cannot duplicate the host environment. He further explained that this is due to the variability of testing media and the limiting effect of protein binding on an antimicrobial agent. Similarly Boughaleb *et al.* (2006) evaluated four fungicides for their effectiveness against *Phytophthora cactorum* both *in vitro* and *in vivo*, found that *in vitro* testing is more effective to reduce mycelium growth. *In vitro* testing systems do not consider the pharmacokinetics of the antimicrobial agent or the post-antibiotic effect, whereby microbial growth is suppressed even when the antibiotic concentration falls below the MIC. Despite the limitations of currently available systems, *in vitro* testing of the susceptibility of microorganisms can be an invaluable tool in selecting antimicrobial therapy.

In vitro testing proved that 2% bleaching with 0.3% slaked lime solution is the best disinfectant in controlling pathogenic fungal followed by 2% formalin. Similarly Reddy and Rao (2009) found an improvement of 11.16 kg cocoons/100 dfls in the batches of PM \times CSR2 dfls reared in rearing houses disinfected with 2% bleaching powder. In India, nearly 90% of the rearing houses are not suitable for disinfection with formalin as they are open type and rearing-cum-dwelling houses.

Formalin has pungent and irritating odour and required to protect against inhaling. The protective devices are not readily available. Formalin is effective at high humid condition (>70%), a constraint in tropical conditions. Formalin is reported to be carcinogenic and its use needs to be avoided (Beena, 1996). Balavenkatasubbaiah *et al.* (1994), Balavenkatasubbaiah *et al.* (1996) suggested bleaching solution as alternate to formalin for disinfection and maintenance of rearing hygiene. Though 2% formalin is quite effective against all the silkworm pathogens, it takes longer duration to completely inactivate certain germs. The test of bleaching powder, 30% available chlorine solution in different concentrations (1-5%) against different pathogens indicated its effectiveness at all concentrations. Therefore 2% bleaching+0.3% slaked lime solution can be used to control pathogenic fungal diseases in silkworm rearing houses. Since an effective and alternative disinfectant is the need of the h for wide spectrum usage, other industries are also working on this line. Herruzo-Cabrera *et al.* (1999) found that N-duopropenide (NDP) alone or in alcoholic solution is good alternative to glutaraldehyde in hospital instruments disinfection. Ultraviolet light is currently a more preferable method for disinfection. It has some inherent advantages over all other disinfection methods viz., no chemical consumption, no transportation and handling, no harmful by-products and low energy requirements (Oparaku *et al.*, 2011).

Regular detection, quantification and characterization of all fungus and moulds in living and working environments are essential for exposure risk assessment to safe guard the health of sericulturists. Thus, monitoring fungal spore concentration in indoor environments is important for indoor air quality control. Some disinfectant and methods recommended on curative and controlling aspects of the disease are not popular among farmers. It was felt that being a devastating disease and the heavy loss, very effective, user and eco friendly disinfectant should be developed for management of fungal diseases. Further, wide range of disinfectants and concentrations can be tested *in vitro* against pathogenic fungal culture. Longevity of effectiveness of each disinfectant is to be assessed.

CONCLUSION

Silkworms are affected by a number of fungal diseases caused by *Beauveria bassiana*, *Spicaria prasina* (*Nomuraea riyeli*), *Metarhizium anisopliae*, *Paecilomyces farinosus*, *Aspergillus flavus*, *A. oryzae*, *A. ochraceus*, *A. tamarii* etc. For controlling diseases, disinfection is the best prophylactic measure. In the present study fungal germ load was assessed from three silkworm-rearing houses before disinfection. It was found that air borne fungal colony in the model 1 and 3 reduced comparatively after disinfection, whereas in model 2 which was disinfected twice with formalin, there was no reduction in the germ load. This may be due to differential area of sampling. In disinfected floor, wall and washed materials like stand, tray in all the models studied, swab germ load as number of colonies were reduced to some extent but not completely removed. CFUA indicated that in all three models, all colonies in floor, wall, tray and newspaper were reduced significantly after disinfection. 7 different fungal colonies were isolated, cultured and identified according to colour, shape and margin. Out of these, one colony of *Cladosporium* spp. did not grow upto 7 days of inoculation, one colony of *Rhizopus* spp. overgrew within 48 h due to its rapid nature of growth could not be considered. 5 isolated fungal colonies (*Aspergillus tamarii*, *Aspergillus flavus*, *Aspergillus niger*, *Neurospora* spp. and *Beauveria bassiana*) were tested against 5 disinfectants viz., Sanitech, Asthra, bleaching solution, formalin and alcohol. Out of 5 fungal cultures tested, Sanitech was effective in controlling only one colony (*A. niger*), Alcohol in all four

colonies (*Aspergillus tamaritii*, *Aspergillus flavus*, *Neurospora* spp. and *Beauveria bassiana*) except *A. niger* and *Asthra*, bleaching and formalin in all the 5 colonies. *In vitro* testing indicated clearly that bleaching solution had highest clear zone in controlling *Beauveria bassiana* and *Aspergillus flavus*. Further, it is proved that 2% bleaching solution with 0.3% slaked lime was best in effectively controlling the fungal diseases followed by 2% formalin solution.

Considering the presence of the microorganism with pathogenic potential monitoring the rearing environment before the commencement of rearing is essential to help, prevent the infections. Among the preventive measures more frequent mechanical removal of dust, better cleaning and maintenance, correction of room humidity and better training regarding threats of infection by microorganisms should be considered.

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