ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Establishment of Sterilization Method for Emergent Quality of Sugarcane (*Saccharum officinarum* L.) In An Efficient Micropropagation System.

Avinash S Thorat[1, 2], Abhijeet B Muley[3], Prashant R Shingote[2], Virdhaval M Nalavade[2] and K Harinath Babu[1]*.

[1]Department of Botany, Shivaji University, Kolhapur, India 416004.
[2]Molecular biology and Genetic Engineering Section, Vasantdada Sugar Institute, Manjari (Bk), Pune, India 412307.
[3]Depatment of Food Technology, University Institute of Chemical Technology, North Maharashtra University, Jalgaon, India 425001.

ABSTRACT

The present investigation focuses on development of economical, reliable and effective sterilization protocol for micropropagation of sugarcane CoVSI 9805 with isolation and identification of contaminating microbes. Surface sterilization was carried out using different formulations of mercuric chloride, Bavistin™ and Streptomycin. Aseptic conditions in plant shoot generation medium were studied with use of different concentration of plant preservative mixture™ (PPM), sodium hypochlorite (NaOCl), chitosan (I and II). The isolated and identified contaminating microbes comprised of four fungi viz. *Alternaria, Aspergillus, Penicillium Fusarium* spp. and five bacteria viz. *E. coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa* respectively. The occurrence of bacterial contaminants in *in vitro* culture was higher than the fungal. After surface sterilization, maximum proliferation (55.33±1.45) with reduced necrosis (20±1.15) was obtained with combination of Bavistin (0.1%) + streptomycin (0.1%) at 25±1°C for 15 min. The sterilizing agents decontaminated bud surface and enhanced proliferation but single sterilant alone failed to control contamination. An increased response of axillary bud *in vitro* culture was achieved with PPM (0.5%), NaOCl (0.01%), chitosan I (0.01%) and chitosan II (0.03%). The improved control contamination strategy will result in mass multiplication of sugarcane by tissue culture and augment the proliferation to compete agriculture demands.

Keywords: Sugarcane, axillary buds, sterilization, proliferation, contamination, microbes.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.), one of the important cash crop cultivated in tropical and sub tropical climate is amongst world's top ten food crops contributing around 80% of the sugar produced globally. Along with sugar it is a potential source of economically essential by-products viz. pressmud, baggasse, molasses, etc. having wide applications. Sugarcane has occupied a prominent position as an agro industrial crop of India. Sugar industry is the second largest industry followed by cotton playing significant role to sustain world economy and India is the second largest producer of sugar after Brazil [1, 2]. Numerous small scale industries and factories are depended on raw materials provided by sugarcane and its by-products [3]. It has been recognized as one of the principle crop efficient in transformation of solar energy into valuable and applicable chemical energy harvestable as sucrose and biomass [4].

Traditionally, large scale sugarcane propagation was obtained by planting vegetative propagated setts (seed pieces containing three or four eye buds) but this method is prone to systemic diseases caused by several etiological agents like nematodes, fungi, bacteria and viruses leading to losses in production [1]. There are no physical or chemical methods to competently eliminate or eradicate the etiological agents [5]. To overcome the problems related to good quality plantlets for planting, tissue culture is the best approach as seed quality (in terms of genetic variations) is maintained uniform, disease free plants are produced with a comparatively short production time, low land requirement, acceleration of plant multiplication, etc. [6, 7]. But the *in vitro* culture establishments of plant growing in the field are highly susceptible to contamination and it is an important cause of *in vitro* culture losses [8]. Microbial contamination is a major hurdle in maintaining viable *in vitro* cultures of sugarcane. The major sources of contamination are endophytic and epiphytic microbes on/in plant tissue, air born contaminants in media, contaminants during poor handling practices in tissue culture work, etc. The *in vitro* culture medium is rich in nutrients supporting growth of microbes (both epiphytes and endophytes). Almost all contaminating microbes are developed in soil or to some extent in plant debris and hence there is a need to employ effective surface decontamination method to achieve sustainable micro propagation. The effective sterilizing agents used during surface sterilization must preserve tissue proliferation and vigor along with elimination of contaminating microbes [9].

The development of an aseptic tissue culture method is an important and critical step to avoid the invasion and growth of contaminating microbes. Microbial contamination can be avoided with surface sterilization by use of chemical disinfectants such as sodium hypochlorite, ethanol, hydrogen peroxide, mercuric chloride, bromine water, silver nitrate and antibiotics either individually or in combination [5]. However, surface sterilization is not effective every time as the internal contaminating agents are usually not removed by this method, especially when explants are brought form plants grown in field and transferred to in vitro culture [10, 11]. To overcome contamination problem while achieving *in vitro* growth in nutrient medium various anti-contaminating and growth promoting agents are been used in appropriate concentration. The agents widely used are sodium hypochlorite [7], PPM (Plant Cell Technology, Washington, D.C), Chitosan and its derivatives [12], broad spectrum antibiotics and fungicides [13], etc.

The most decisive step in tissue culture explants preparation for further processes is elimination of contaminants free living and healthy explants. Therefore the present investigation was focused to establish an efficient surface sterilization and maintain aseptic conditions in plant shoot generation medium (PSRM) for *in vitro* micropropagation through axillary bud culture in sugarcane CoVSI 9805 with application of disinfecting and sterilizing agents viz. $HgCl_2$, Bavistin™ and streptomycin, sodium hypochlorite (NaOCl), PPM and chitosan (CSN I and CSN II) along with isolation, identification and occurrence of the contaminating microbes during incubation.

MATERIAL AND METHODS

Materials

The sugarcane variety CoVSI 9805 grown in experimental field of VSI, Manjari (Bk.), Pune with standard practices was used for the study. CSN I and II (molecular weight 134560 ±1784.27 and 23116±365.8) were a gift from Tissue culture section, VSI, Manjari (Bk.), Pune. Bavistin™ (50% WP carbendazim, broad spectrum antifungal agent, BASF, India), $HgCl_2$ (Merck Chemicals, Mumbai, India), streptomycin (Duchefa Biochemicals, Netherland), PPM (Plant Cell Technology, Washington, DC), NaOCl (Himedia Lab. Pvt. Ltd.,

Mumbai, India) were used. All the media components were procured from reliable sources while reagents used were of high purity and of analytical reagent grade.

Plant material

The axillary buds used as explants were collected from 8-10 month old field grown sugarcane. Freshly collected sugarcanes were cleaned properly by washing under running tap water followed by 0.1% Tween-20 solution for 15 min. After choosing healthy axillary bud it was incised properly with the help of bud scooping machine.

Pre treatment of axillary buds

Buds were dissected properly (1 to 1.5 cm^2) with the help of scalpel and kept in antioxidant solution (0.1% citric acid and 0.1% Ascorbic acid) for 15 min followed by wash with sterile distilled water and then carried to the laminar hood for further sterilization.

In vitro cultivation of sugarcane

The axillary buds were inoculated in PSRM (MS basal medium supplemented with Sucrose (30 g/L), Casein hydrolysate (0.5 g/L), Polyvinyl pyrrolidone-40 (100 mg/L), Inositol (20 mg/L), Thymine (1 mg/L) and Benzylaminopurine (3 mg/L)) and incubated at 28±1°C with a 16 h photoperiod and contaminated cultures were used to isolate and identify contaminating microbes.

Isolation of contaminating microbes

The contaminating microbes were isolated from the infected plant tissue culture tubes on potato dextrose agar (PDA) in case of fungi and Luria Bertani (LB) agar for bacteria with repeated sub-culturing. The inoculated plates were incubated at 28±1° and 35±1°C for fungi and bacteria respectively.

Identification of contaminating microbes

The contaminating fungi were identified by colony characteristics (appearance, colour and pigmentation), morphology of vegetative hyphae and macroconidia produced by comparing with standards enlisted by Barnett and Hunter [14]. The bacteria were identified with morphological characters along with biochemical and physiological tests viz. Gram staining, motility, capsule staining, catalase test, starch and gelatin hydrolysis, citrate reduction, urease, oxidase test, indole, methyl red, Voges-Proskauer, nitrate reduction test. The characterized bacterial strains were compared with standard in Bergey's Manual of Bacteriology [15, 16].

Effect of surface sterilants on contamination, proliferation and necrosis of axillary buds

The axillary buds were treated with $HgCl_2$, Bavistin™ and streptomycin with various formulations for surface sterilization of sugarcane axillary bud set (as shown in Table 1) followed by three simultaneous washes with sterile distilled water under aseptic conditions. The buds were inoculated in PSRM and contamination, proliferation and necrosis of axillary buds were determined on 21 days after incubation.

Table 1: Combination of surface sterilants with varying temperature and time

Treatment	Sterilants and their combination	Temperature (°C)	Time (min)
T1	$HgCl_2$ (0.1%)	25±1	15
T2	$HgCl_2$ (0.1%)	25±1	30
T3	$HgCl_2$ (0.1%)	52±1	15
T4	$HgCl_2$ (0.1%)	52±1	30
T5	$HgCl_2$ (0.1%) + Bavistin (0.1%)	25±1	15
T6	$HgCl_2$ (0.1%) + Bavistin (0.1%)	25±1	30
T7	$HgCl_2$ (0.1%) + Bavistin (0.1%)	52±1	15

T8	$HgCl_2$ (0.1%) + Bavistin (0.1%)	52±1	30
T9	$HgCl_2$ (0.1%) + Streptomycin (0.1%)	25±1	15
T10	$HgCl_2$ (0.1%) + Streptomycin (0.1%)	25±1	30
T11	Bavistin (0.1%) + Streptomycin (0.1%)	25±1	15
T12	Bavistin (0.1%) + Streptomycin (0.1%)	25±1	30
T13	$HgCl_2$ (0.1%) + Bavistin (0.1%) + Streptomycin (0.1%)	25±1	15
T14	$HgCl_2$ (0.1%) + Bavistin (0.1%) + Streptomycin (0.1%)	25±1	30

Effect of PPM, NaOCl and Chitosan on proliferation and contamination of axillary buds

The effect of PPM (0.01, 0.03 and 0.05%), NaOCl (0.01, 0.03 and 0.05%), CSN I (0.005, 0.01 and 0.03 %) and CSN II (0.005, 0.01, 0.03%) on proliferation and contamination was studied by incorporating respective solution in PSRM. The pH was adjusted to 5.7±0.1 before autoclave and all cultures were incubated at 28±1°C with a 16 h photoperiod and observation recorded on 21st day. The proliferation and contamination effectivity (%) of inoculated axillary buds was determined as

$$\text{Proliferation effectivity (\%)} = \frac{\text{Number of buds proliferated}}{\text{Total number of buds inoculated}} \times 100$$

$$\text{contamination effectivity (\%)} = \frac{\text{Number of buds contaminated}}{\text{Total number of buds inoculated}} \times 100$$

Statistical Analysis

The data was analyzed using SPSS (software package version 16) and Microsoft Excel 2010. One way ANOVA was applied to test mean differences of all treatments while statistical significant difference between mean values was established at p ≤5% while Duncan's New Multiple Range Test was used. The results were expressed as mean± SE.

RESULTS AND DISCUSSION

In vitro cultivation of sugarcane

The *in vitro* cultivation of sugarcane variety CoVSI 9805 was carried out in PSRM supplemented with nutrients and hormones augmenting proliferation, growth and tissue morphogenesis. The healthy generated buds were fleshy and green colored, aseptic tissue growth was observed as the plant generated properly and medium was clear i.e. no turbidity was present (Fig. 1a). Few inoculated buds turned brownish black from creamish white and failed to proliferate during incubation as a result of tissue necrosis during pretreatment and preparation of axillary buds (Fig. 1b). During the growth of some buds the PSRM turned reddish brown colored due to secretion of poly-phenolics by damaged cells of growing tissue. This might be due to stressful conditions developed by chemical treatments during surface sterilization and tissue damage occurred during axillary bud scooping (Fig. 1c). Various normally germinated buds in early stages became dry on further incubation. This might be due to incompatibility of tissue with the nutrients in medium, tissue damage during pretreatment, axillary bud scooping and surface sterilization and moreover inability of cells to further redifferentiate into growing tissue (Fig. 1d). Along with prominent growth of *in vitro* shoots, contamination was a major hurdle in early as well as late stages of incubation due to growth of bacteria (Fig. 1e and Fig. 1f) and fungi (Fig. 1g and Fig. 1h) on tissue as well as in the medium.

ISSN: 0975-8585

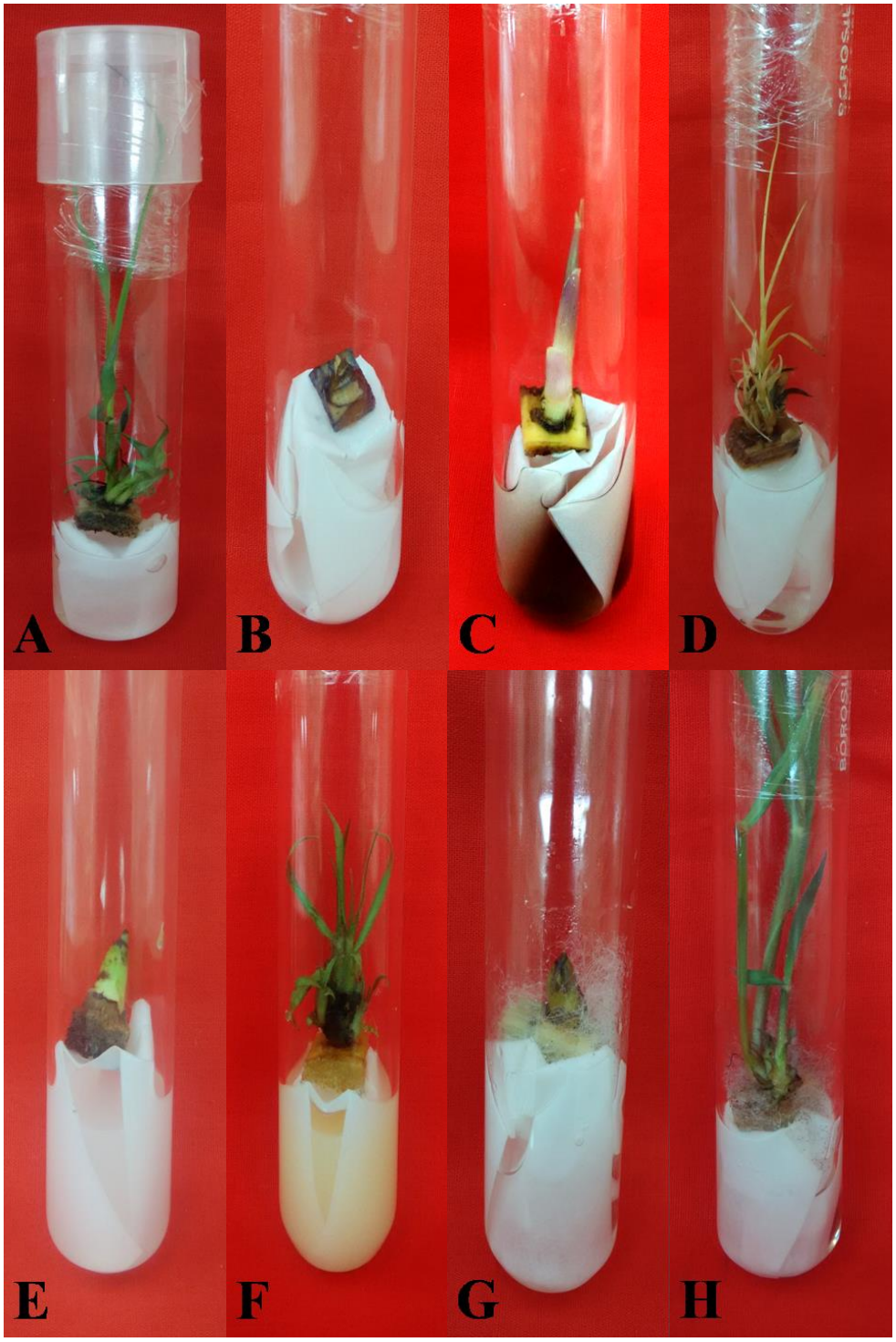


Figure 1: Aseptic, healthy and completely proliferated bud (A); Necrotized eye bud which failed to proliferate (B); Reddish brown PSRM due to secretion of poly-phenolics (C); Proliferated bud turning dry during incubation (D); Early bacterial contaminated bud (E); Late bacterial contaminated bud (F); Early fungal contaminated bud (G) and Late fungal contaminated bud (H)

Isolation and identification of contaminating microbes

The contaminating microbes were isolated in pure form and isolated colonies were maintained on PDA and LB plates. The identification of contaminating fungi was made with growth pattern on PDA plates, morphology of mycelia and macroconidial spores at Plant Pathology Division, Vasantdada Sugar Institute, Manjari (Bk.), Pune. The morphological characters of the isolated fungi are enlisted in Table 2. The

contaminating bacteria were identified with morphological, biochemical and physiological tests performed. The colony characters of isolated bacteria are given in Table 3 and the results of morphological, biochemical and physiological tests carried out are shown in Table 4.

Table 2: Morphological characters of isolated fungi

Isolate	Colony	Mycelia	Macroconidial spores
F1	Spreading, wooly appearing producing greenish black	Branched, septate and formed cob web like network	Brownish, ellipsoidal with conical beak at each end
F2	Spreading, cotton like black colored with white periphery	Branched, septate and spores radiating in all directions from the vesicle	Black colored, single celled with rounded apex and conidial head
F3	Spreading, flat, wooly, filamentous initially white but later producing grey green pigment	Branched and formed brush like structure	Grey and greenish, Single celled, Branched near apex and ovoidal
F4	Spreading with cotton like appearance and producing pink pigment	Branched and septate	Bent i.e. Curved and had pointed ends

Identified fungal isolate: F1: *Alternaria* Spp., F2: *Aspergillus* Spp., F3: *Penicillium* Spp. and F4: *Fusarium* Spp.

Table 3: Colony characteristics of isolated bacteria on LB agar

Isolate	Size (mm)	Shape	Colour	Margin	Elevation	Opacity	Consistency
B1	2-3	Circular	White	Entire	Raised	Translucent	Moist
B2	2-3	Circular	Yellow	Undulated	Flat	Translucent	Moist
B3	2-3	Circular	Yellow	Entire	Convex	Translucent	Moist
B4	3-4	Circular	Yellow	Entire	Convex	Translucent	Moist
B5	1-2	Circular	White	Undulated	Raised	Translucent	Moist

Table 4: Morphological, biochemical and physiological tests for identifying bacterial cultures

Test	B1	B2	B3	B4	B5
Gram Staining	Gram negative short rods	Gram positive rods	Gram positive cocci arranged in grapes like bunch	Gram negative short rods	Gram negative rods
Motility	Motile	Motile	Non motile	Motile	Motile
Endospore Staining	-	+	-	-	-
Capsule Staining	-	-	-	-	+
Catalase	+	+	+	+	+
Starch	-	+	-	+	-
Gelatin	-	+	-	-	+
Citrate	-	+	-	-	+
Urease	-	-	+	+	+
Oxidase	-	-	-	+	+
Indole	+	-	-	Not performed	-
Methyl red	+	+	+	-	-
Voges-Proskauer	-	+	+	Not performed	-
Nitrate reduction	+	+	+	Not performed	+

Identified bacterial isolate: B1: *Escherichia coli*, B2: *Bacillus subtilis*, B3: *Staphylococcus aureus*, B4: *Agrobacterium tumifaciens* and B5: *Pseudomonas aeruginosa*

ISSN: 0975-8585

From the growth pattern, morphological and biochemical studied, it was noticed that the contaminating fungi were *Alternaria, Aspergillus, Penicillium and Fusarium* spp. (Fig. 2) and the bacteria were *E. coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa* respectively (Fig. 3). The contamination might be caused from explants but the possibility from laboratory sources and handling the plant material cannot be discarded. The various sources of contamination in tissue culture laboratory includes endogenous microbes in plant tissue, exogenous microbes from preparative and incubation room, wet surfaces of air conditioners, human skin, indoor air, furniture, etc. [17, 18].

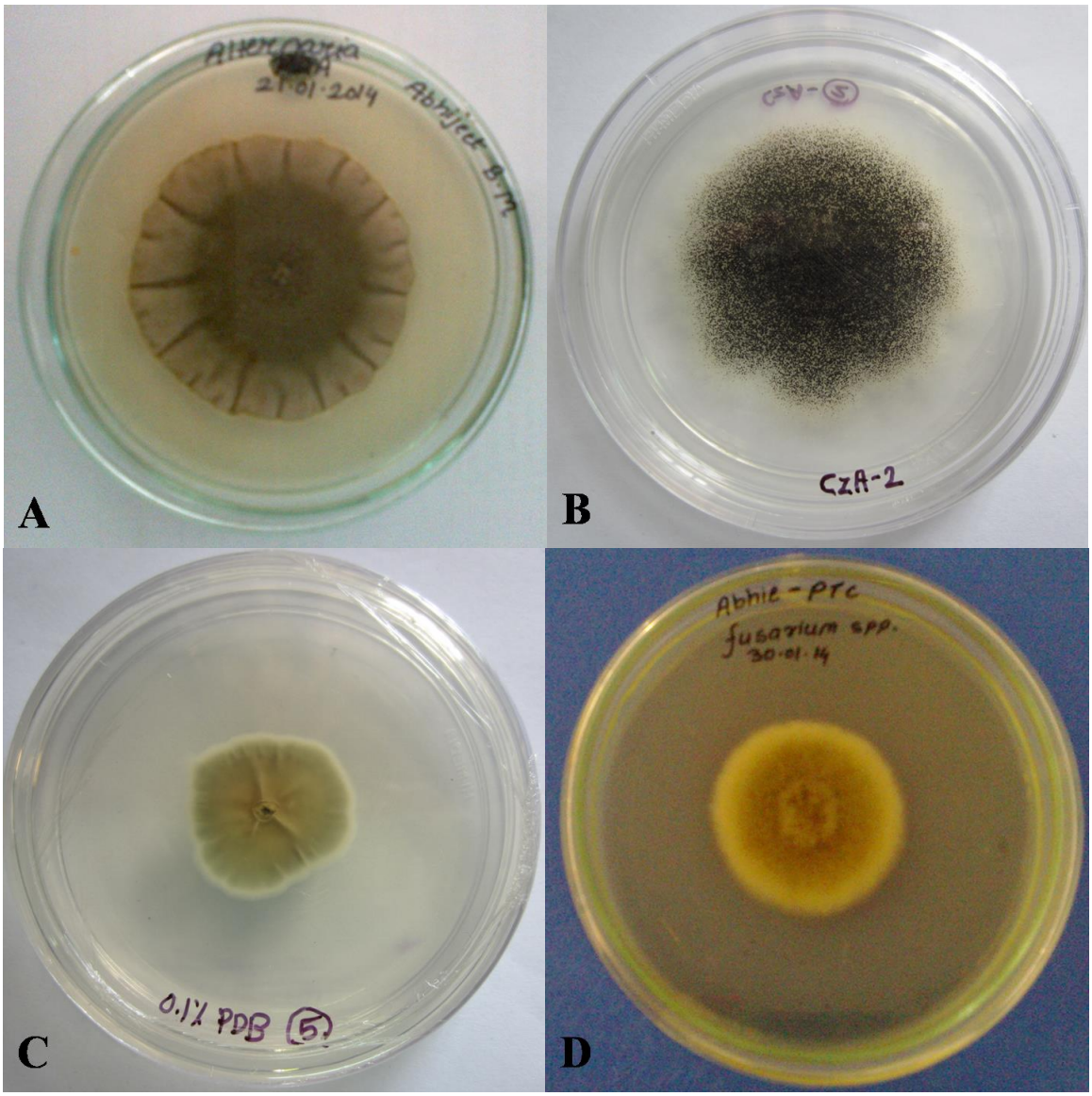


Figure 2: Isolated pure cultures of *Alternaria* (A), *Aspergillus* (B), *Penicillium* (C) and *Fusarium* Spp (D)

ISSN: 0975-8585

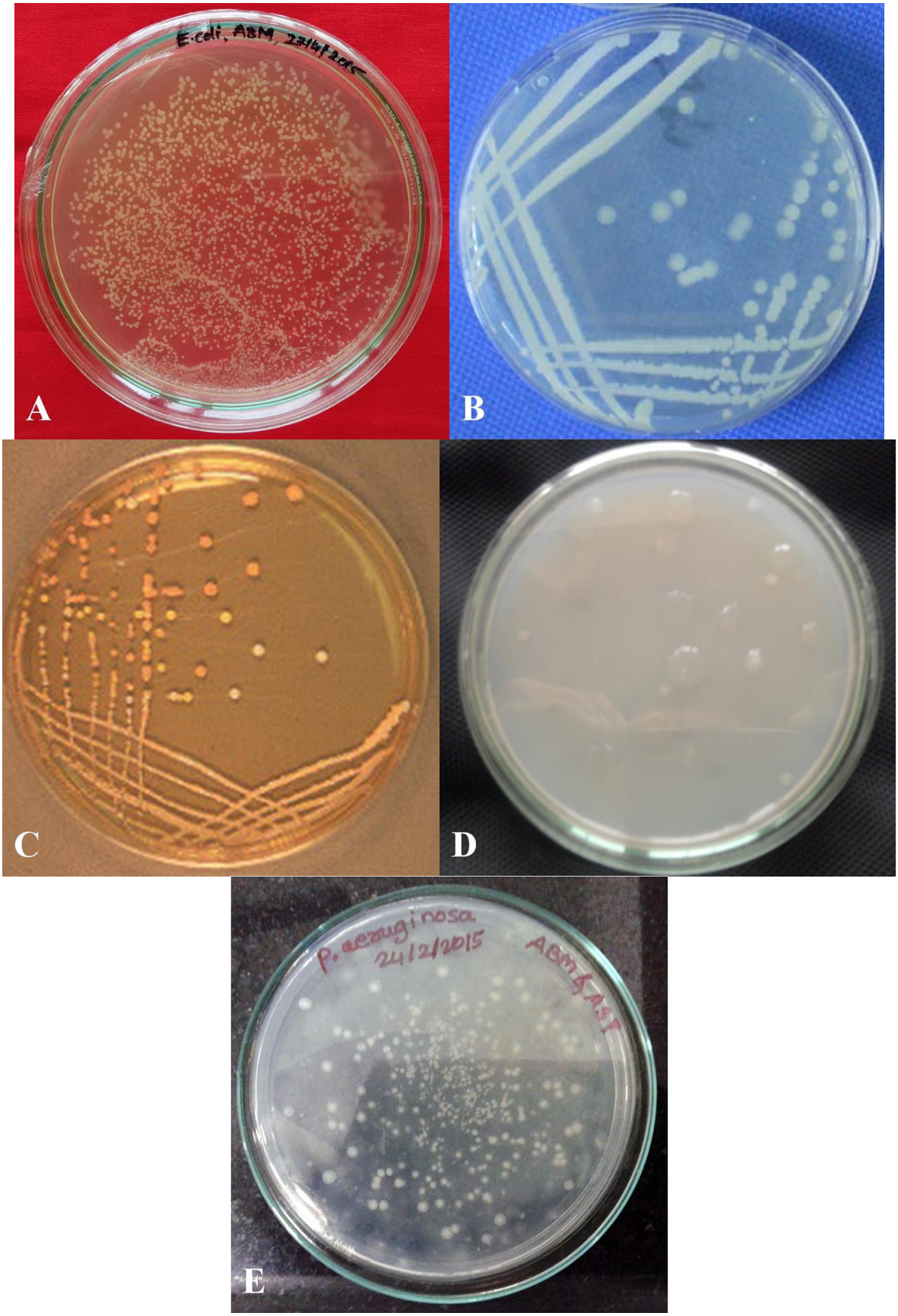


Figure 3: Isolated pure cultures of *E. coli* (A), *Bacillus subtilis* (B), *Staphylococcus aureus* (C), *Agrobacterium tumefaciens* (D) and *Pseudomonas aeruginosa* (E)

ISSN: 0975-8585

Occurrence of contaminating microbes during incubation

The contamination was determined visually and divided as early (upto 7 days) and late (after 10 days) depending on occurrence of turbidity and microbial growth in medium during incubation. The contaminating microbes were determined by microscopic and biochemical studies. The rate of occurrence of contaminating microbes in early and late phases of incubation is shown in Fig. 4. It was noticed that in early phases of incubation the bacteria *S. aureus* was most prominent 35% followed by *A. tumefaciens* (26%) and lowest with *P. aeruginosa* (8%) while fungi *Aspergillus* (41%) and *Penicillium* Spp. (28%) were more common. During late phase of incubation the bacteria *B. subtilis* (31%) and *P. aeruginosa* (28%) while fungi *Penicillium* (31%) and *Fusarium* Spp. (27%) were common contaminants during sugarcane micropropagation.

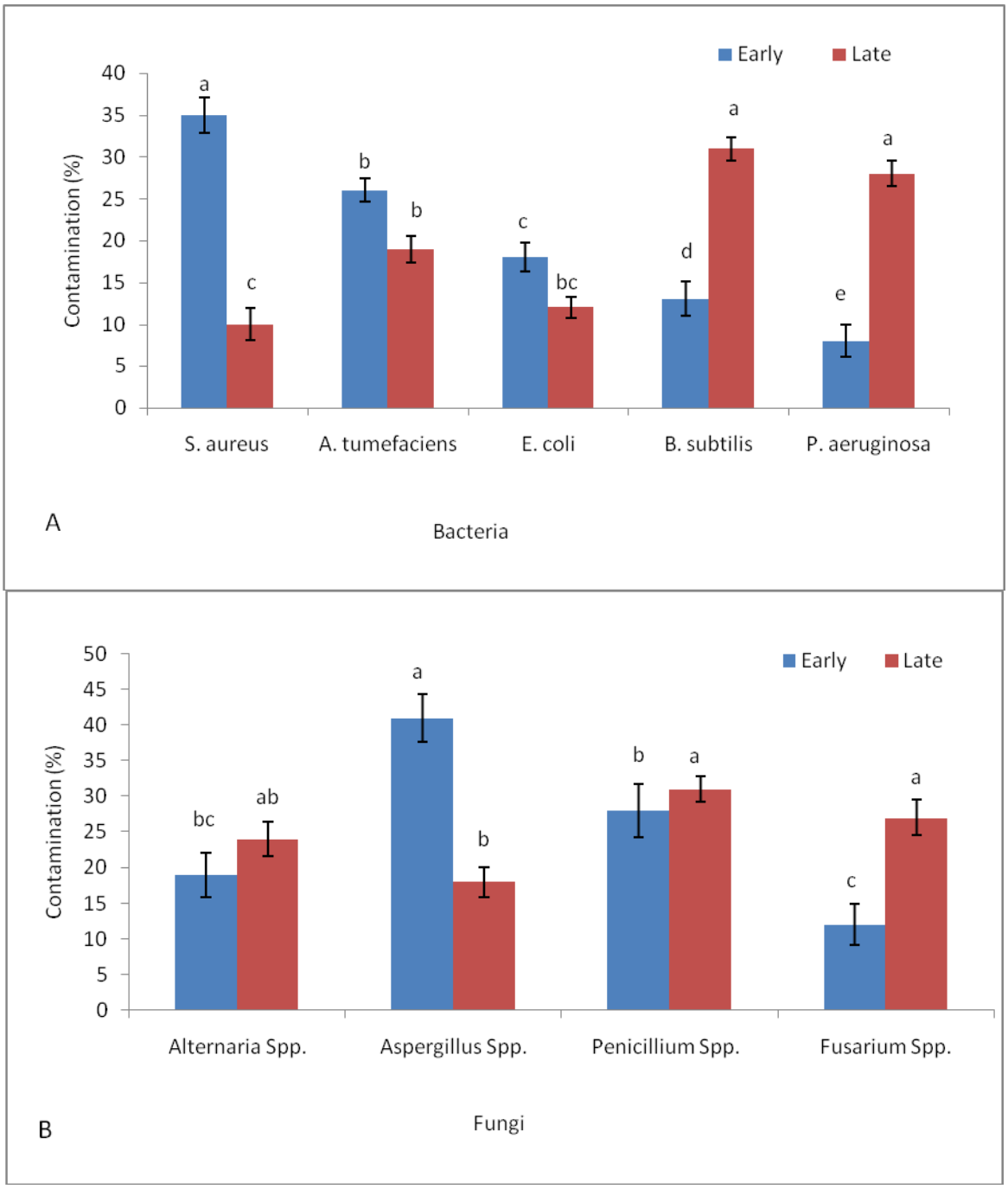


Figure 4: The occurrence of Bacteria (A) and Fungi (B) contamination in sugarcane tissue culture

It was observed that the rate of occurrence of bacterial contaminants in sugarcane tissue culture was much higher as compared to the fungal contaminants. Similar results were reported by in tissue culture of *Hibiscus cannabinus* and *Telfaria occidentalis* [18]. The occurrence of early contamination was due to microbes that survived after surface sterilization. Late contamination resulted because of growth of endophytic microbes remaining latent in sugarcane tissues, resistant to surface sterilization and emerging days after inoculation in medium [19, 20].

Growth response of axillary buds after surface sterilization

The growth response (in terms of proliferation and necrosis) and contamination of axillary buds grown in PSRM varied with exposure time and temperature of sterilizing agent (Table 5). Significantly superior proliferation (55.33 ±1.45%) and reduced necrosis (20.00±1.15) was found on surface sterilization with Bavistin (0.1%) + Streptomycin (0.1%) for 15 min at 25±1°C (T_{11}). Lowest contamination (13.33 ±4.41%) was recorded with combined treatment of $HgCl_2$ (0.1%) + Bavistin (0.1%) + Streptomycin (0.1%) at 25±1°C for 30 min (T_{14}). The $HgCl_2$ (0.1%) at 52±1°C for 15 and 30 min also had considerably reduced levels of contamination (14.67±1.86 and 15.67±4.70) as compared to other treatments (T_3 and T_4). It was observed that prolonged treatment (30 min) with sterilants (either individually or in combination) reduced the bud proliferation but contamination and tissue necrosis was higher. Single sterilant individually failed to control the microbial contamination at 25±1°C but it had less impact on tissue growth as proliferation was good and necrosis was reduced due to comparatively low chemical toxicity in contrast to sterilant combinations. Babaei et al. [21] reported the exposure time of $HgCl_2$ (0.1%) to be 5 min for minimal toxicity to shoot tips. Tiwari et al. [5] stated a short exposure time and lower concentration of $HgCl_2$ for significant sugarcane tissue *in vitro* growth. Similar results were noticed by Sawant and Tawar [7].

It was noticeable that contaminats specifically reduced with use of $HgCl_2$ alone but at the same time there was adverse effect on tissue growth due to increased necrosis. Additionally, it was also reported that use of Bavistin (0.1%) in combination with streptomycin (0.1%) was the best combination as there was maximum shoot proliferation and least necrosis as well s comparatively moderate level of contamination. This combination can be solely used as less harmful and more effective surface sterilant.

Table 5: Proliferation response and contamination of axillary buds after surface sterilization

Treatment	Proliferation (%)	Contamination (%)	Necrosis (%)
T1	43.33±1.20^{cd}	34.00±2.08^{e}	22.67±1.20^{ab}
T2	41.67±2.40^{d}	33.00±2.08^{ef}	25.33±3.84^{abc}
T3	47.00±6.11^{abcd}	**15.67±4.70^{a}**	37.33±1.45^{e}
T4	51.67±2.03^{abc}	**14.67±1.86^{a}**	33.67±1.45^{de}
T5	46.00±4.36^{abcd}	24.00±1.73^{bcd}	30.00±2.65^{bcde}
T6	45.67±3.48^{abcd}	24.67±1.45^{bcd}	29.67±2.03^{bcde}
T7	50.00±0.58^{abcd}	20.00±3.21^{abc}	30.00±3.61^{bcde}
T8	48.33±1.45^{abcd}	18.67±3.18^{abc}	33.00±4.16^{cde}
T9	47.67±1.20^{abcd}	29.33±1.45^{def}	23.00±1.15^{ab}
T10	45.00±1.53^{bcd}	26.00±1.15^{cde}	29.00±2.08^{bcd}
T11	**55.33±1.45^{a}**	24.67±1.45^{bcd}	**20.00±1.15^{a}**
T12	54.00±1.73^{ab}	21.67±1.76^{abcd}	24.33±1.45^{ab}
T13	52.67±2.33^{abc}	17.33±1.45^{ab}	30.00±2.08^{bcde}
T14	53.33±4.41^{ab}	**13.33±4.41^{a}**	33.33±1.67^{de}

The presence of contamination at early stages indicated incomplete removal of epiphytic microbes from the sugarcane bud surface. The variations in bud proliferation and necrosis were due to impact of sterilants on the growing tissue and damage during preparation of sugarcane bud chip. The appearance of contamination two weeks after culture initiation suggests possibility of endophytic microbes. These endophytes may not be apparent immediately after culture initiation but can emerge weeks later and persist

in culture leading to losses [19, 20]. Some endophytes are resistance to surface sterilization as they remain latent within plant tissue and may grow in early as well as late phases of incubation [22]. Thus incorporation of physical thermostatic treatment in sterilization to augment decontamination frequency with mercuric chloride is effective against as endophyte contamination. $HgCl_2$ at lower concentration can be ineffective in eliminating contamination, but higher concentration and combination of fungicides can control contamination due to epiphytic and endophytic microbes on/in the bud tissue. Tiwari et al. [6] stated that clonal micro propagation of sugarcane via tissue culture has been severely affected by both epiphytic and endophytic microbes. Vazquez-Molina et al. [23] applied 0.1% $HgCl_2$ for seven minutes to achieve 56.3% decontamination frequency and double treatment to gain 70% decontamination. Treatment of explants with $HgCl_2$ and heat were more effective than single sterilization but the proliferation rate was affected due higher necrosis. Increasing the exposure time with $HgCl_2$ causes severe necrosis indicating phytotoxicity [24]. $HgCl_2$ has a significant effect impact on tissue growth and biomass accumulation as it leads to oxidative stress in cucumber seedlings [25].

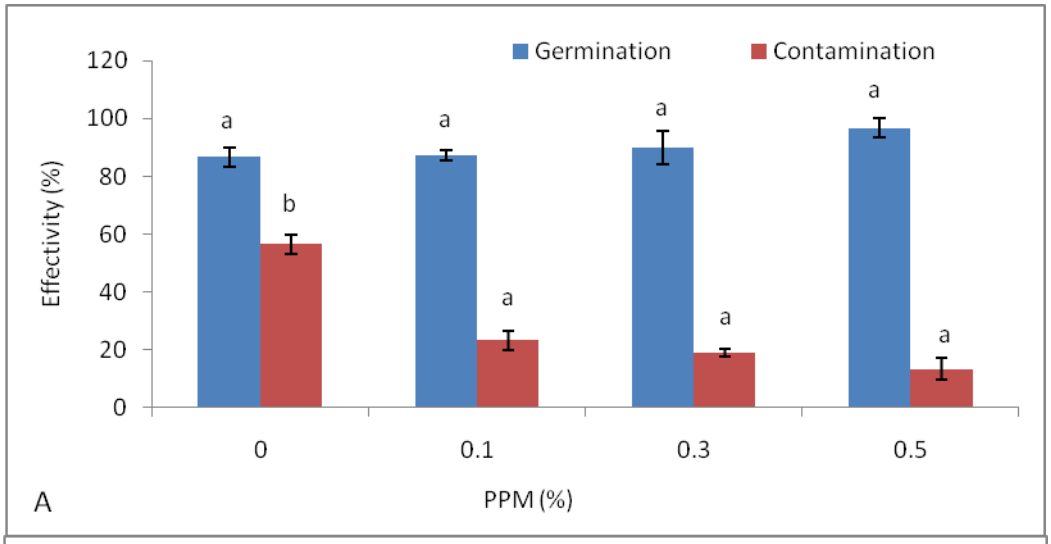
Growth response of axillary buds incorporated with sterilizing agents

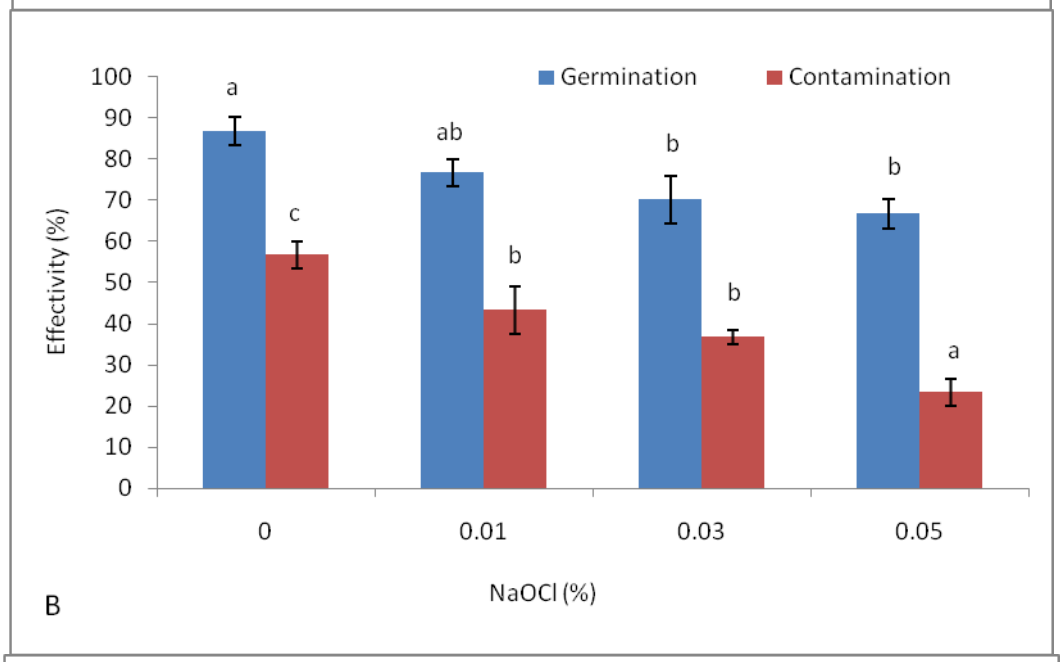
The rate of proliferation i.e. growth response and contamination varied according to the concentration of agent used to control contaminants in media and endophytic microbes. There was no superior difference in proliferation but contamination was significantly controlled with PPM as compared to the control. The explants had highest proliferation (96.67±3.33%) and least contamination (13.33±0.96%3.33) with PPM (0.5%) (Fig. 5a). PPM was found to play distinct role in sugarcane eye bud proliferation as well as controlling contamination and similar findings were reported by Rihan et al. [26] in development, growth and proliferation of artificial seeds with controlled contamination. There was no negative impact of PPM on growth and development of European beach callus and the concentration for controlling contamination without affecting tissue growth depends on optimum concentration and plant species [27]. The contamination in only upper surface was noticed with PPM supplemented medium, Babaie et al. [21] reported similar results as direct contact of medium supplemented with PPM could significantly control growth of contaminants.

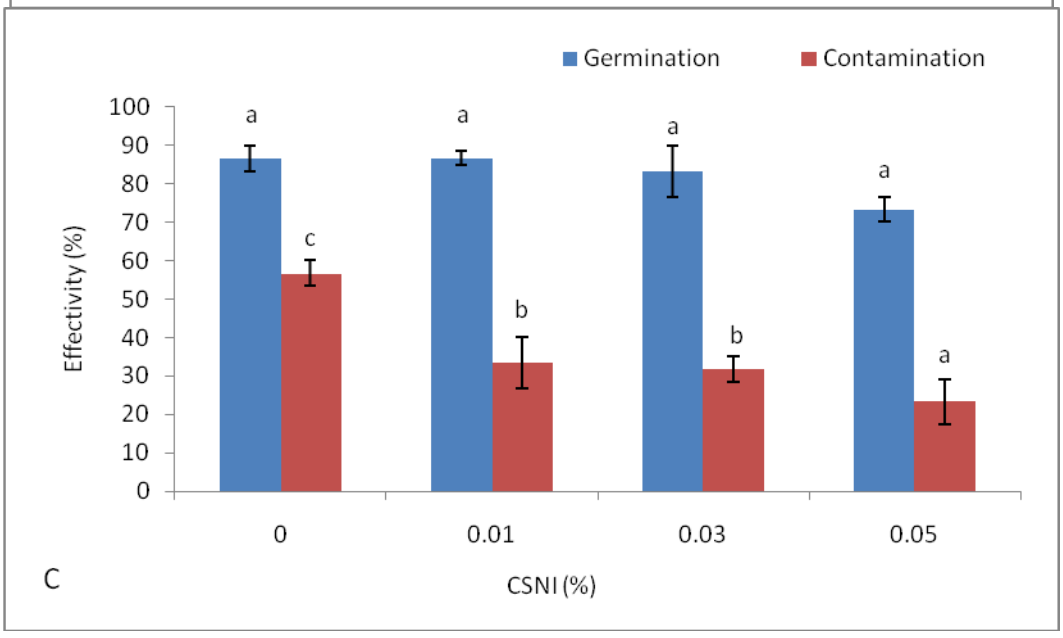
NaOCl (0.05%) had least contamination (23.33±3.33%) but proliferation was drastically reduced with respect to control (Fig. 5b). The decrease in proliferation may be due to contamination and impact of NaOCl on morphogenesis, as described previously by Tiwari et al. [7] during sugarcane micropropagation. Teixeira et al. [28] reported that NaOCl at lower concentration to be successful in maintaining aseptic conditions in nutrient medium and growth of banana, eucalyptus, pineapple and orchid explants along with beneficial impact on shoot generation. Sawant and Tawar [7] noticed reduced contamination and effective proliferation of sugarcane shoot and root with 0.01-0.05% NaOCl. Aseptic conditions in micropropagation of sugarcane can be maintained using chlorine disinfectants without thermal treatment (i.e. autoclaving) of medium.

CSN I (0.03%) prominently controlled contamination (23.33±3.33%) but notable proliferation (86.67±3.33%) was observed at 0.005% as compared to the control (Fig. 5c). Similar results were reported for CSN II (Fig. 5d) i.e. enhanced proliferation (93.33±3.33%) and restricted contamination (16.67±3.33%). Comparably similar proliferation was noticed between control, CSN I and CSN II incorporated medium as chitosan might have helped in minerals accumulation and plant growth. Similar results were reported by Chatelain et al. [29] in accumulation of macro and micro minerals in *Phaseolus vulgaris* by application of chitosan. Chitosan triggers the production of secondary metabolites in cell suspension and callus culture of several plant species and improves *in vitro* quality of plantlet. Chitosan and its derivatives have inhibition spectrum against a wide range of fungi and bacteria, acting as an elicitor of plant defense mechanisms [9, 12].

ISSN: 0975-8585







ISSN: 0975-8585

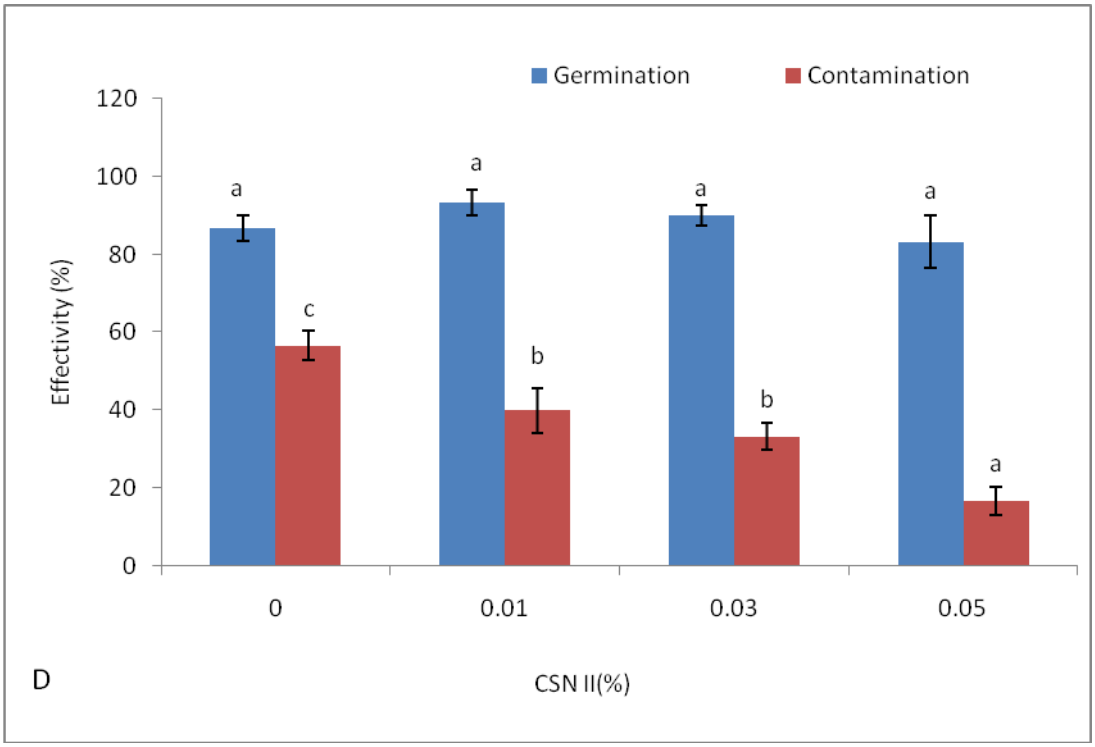


Figure 5: Effect of various sterilants on *in vitro* shoot proliferation and contamination (%) after 21 days of incubation

CONCLUSION

The study indicated that surface sterilization of sugarcane axillary buds with a single sterilizing agent is not sufficient to combat contamination problems. Sterilants used in high concentration leads to tissue necrosis and have adverse effects on bud proliferation. Promising surface contamination can be achieved by either $HgCl_2$ (0.1%) at 52±1°C for 15 or 30 min and $HgCl_2$ (0.1%) + Bavistin (0.1%) + Streptomycin (0.1%) at 25±1°C for 30 min. The highest proliferation (%) with least bud tissue necrosis can be achieved by application of 0.1% Bavistin + 0.1% streptomycin at 25±1°C for 15 min. These findings provide a good base for effective and quick surface sterilization of sugarcane explants especially when they are procured from field grown plants. The early and late contamination during *in vitro* culture can be avoided by addition of 0.5% PPM without affecting proliferation and tissue growth. Direct shoot regeneration from of sugarcane is a faster and rapid way of plant multiplication. Thus, the application of this surface sterilization in combination with media sterilants (after inoculation) will be useful for most favorable tissue culture conditions. Hence, it can be successfully utilized to avoid contamination and produce sugarcane plantlets on large scale to compete the increasing demands in agriculture.

Research Highlights:

- The recurrent contaminating microbes during sugarcane micropropagation were isolated and identified.
- During *in vitro* culture of axillary bud, the occurrence of bacterial contamination was much higher than fungal.
- The prevalence of early and late contaminating microbes was determined.
- Significantly superior surface sterilization with maximum proliferation and reduced necrosis was achieved with combination of Bavistin and streptomycin at 25±1°C for 15 min.
- An improved response of *in vitro* culture of axillary bud with lower contamination and higher proliferation was achieved with PPM, NaOCl and chitosan.
- The use of surface sterilization (before inoculation) in combination with media sterilants (after inoculation) is useful for optimum tissue culture conditions.

ACKNOWLEDGEMENTS

The authors gratefully thank Shri. Shivajirao Deshmukh, Director General, VSI, Manjari (Bk.), Pune, for generous help, encouragement and availing all the required facilities to carry out the research work.

REFERENCES

[1] Thorat AS, Pal RK, Shingote PR, Kharte SB, Nalavade VM, Dhumale DR, Pawar BH, Babu KH. J Pure Appl Microbio 2015; 9(1): 319-327.
[2] Wagh AD. Scholars world-IRMJCR 2015; 3(1): 111-117.
[3] Yadav RL, Solomon S. Sugar Tech 2006; 104-111.
[4] Wang J, Nayak S, Koch K, Ming R. Front Plant Sci 2013; 4: 1-6.
[5] Tiwari AK, Tripathi S, Lal M, Mishra S. Sugar Tech 2012; 14 (4): 364-369.
[6] Tiwari S, Arya A, Kumar S. Research Journal of Botany 2012; 7(1): 1-7.
[7] Sawant RA, Tawar P N. Sugar Tech 2011; 13(1): 27-35.
[8] Omamor IB, Asemota AO, Eke CR, Ezia EI. Afr J Agric Res 2007; 2: 534-537.
[9] Goñi MG, Moreira MR, Viacava GE, Roura SI. Carbohydrate Polymers 2013; 92: 817–823.
[10] Hennerty MJ, Upton ME, Harris DP, Eaton RA, James DJ. Acta Hortic 1988; 225: 129-139.
[11] Savela ML, Uosukainen M. J Appl Bacteriol 1994; 76: 398-376.
[12] Ghatge S, Kudale S, Dalvi S, Dixit G. 2014. Journal of Chitin and Chitosan Science 2014; 2 (1): 62-69.
[13] George MW, Tripepi RR. Horticulture Science 2001; 36: 768-769.
[14] Barnett HL, Hunter BB. Illustrated genera of imperfect fungi. Burgress Publishing Company, Minneapolis MN, USA 241, 1972.
[15] Bergey DH, Holt JG, Krieg NR, Sneath PHA. Bergey's Manual of Determinative Bacteriology, 9th ed., (Breed RS, Murray EGD and Smith NR, eds.) Williams and Wilkims, Baltimore, 1994.
[16] Buchanan RE, Gibbons NE. Bergey's Manual of Determinative Bacteriology. Science Publisher, 8th ed., Beijing, 1984.
[17] Odutayo OI, Amusa NA, Okutade OO, Ogunsanwo YR. Afr J Agric Res 2007; 2(3): 067-072.
[18] Odutayo OI, Oso RT, Akinyemi BO, Amusa NA. Afr J Biotechnol 2004; 3(9): 473-476.
[19] Miyazaki J, Tan BH. 2010. Plant Cell Tissue Organ Cult 2010; 102: 365-372.
[20] Thorpe TA, Harry IS. Special problems and prospects in the propagation of woody species. In: Plant aging: Basic and applied approaches. New York: Plenum Press, 186, 1990, pp. 67-74.
[21] Babaei N, Abdullah NAP, Saleh G, Abdullah TL. J Med Plants Res 2013; 7(8): 448-454.
[22] Barrett C, Cassells AC. 1994. Plant Cell Tissue Organ Cult 1994; 36: 169-175.
[23] Vazquez-Molina DE, DeLosSantos A. Biologia Plantarum 2005; 49(4): 481-485.
[24] Puerner NJ, Siegel SM. Physiologia Plantarum 2006; 26(3): 310-312.
[25] Cargnelutti D, Tabaldi AL, Spanevello RM, Jucoski G de Oliveira, Battisti V, Redin M, Linares CEB, Dressler VL, Flores ÉM de Moraes, Nicoloso FT, Morsch VM, Schetinger MRC. Chemosphere 2006; 65(6): 999-1006.
[26] Rihan HZ, Al-Issawi M, Al-swedi F, Fuller MP. Scientia Horticulturae 2012; 141: 47-52.
[27] Kraj W, Dolnicki A. Acta Scientiarum Polonorum 2003; 72: 303-307.
[28] Teixeira SL, Ribeiro JM, Teixeira MT. Plant Cell Tissue Organ Cult 2006; 86: 375-378.
[29] Chatelain PG, Pintado ME, Vasconcelos MW. Plant Science 2014; 215: 134-140.