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Identification of most effective sterilization protocol(S) for establishment of *in vitro* culture of endangered medicinal plant *Skimmia laureola*

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Abstract

Skimmia laureola (DC) is an important endangered medicinal plant. The present research was conducted to investigate the effect of sterilizing procedure(s) for establishment of *in vitro* culture of *Skimmia laureola*. The explants were cultured on MS medium containing coconut water as growth promoter instead of any synthetic plant growth hormone. Mercuric chloride ($HgCl_2$) at 0.5 %concentration for 30 seconds and 1 minute was found effective sterilization procedure when used alone or in combination with fungicide (Ridomil) where 100 % contamination was controlled with 100 % plants survived. Sodium hypochlorite (NaOCl) alone and with fungicide at both concentrations i.e 3% and 5% remained less effective, however, NaOCl + fungicide at 3% concentration for 1 minute showed 20% contamination with 80% survived explants. After 28 days the survived plantlets in $HgCl_2$ treatment showed average 3.26 cm shoot length with average 5 number of leaves per explant. It has been concluded that $HgCl_2$ can be used as sterilant for establishment of *Skimmia laureola in vitro* cultures and further work is needed for standardizing $HgCl_2$ concentrations and time of exposure for sterilization of other endangered medicinal plants.

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Introduction

Skimmia laureola (*S. laureola*) (DC.) Decneis an aromatic shrub belongs to family Rutaceae. In Pakistan, *Skimmia laureola* grows at an altitude of 5500- 10000 feet. It is common in the Hazara region, Murree Hills and Kashmir, in Upper Swat and Shangla (Hamayun *et al.*, 2006; Ali and Qaiser, 2007) and Upper and Lower Dir (Barkatulah *et al.*, 2012).

Traditionally, thisplant is used as an antitussive, as veterinary anthelmintic, as an insecticide and pesticide. The garlands made from *Skimmia* fresh leaves are considered sacred and smoke of dried leaves is considered useful to ward off evils (Bhattari, 2006; Humayun, 2007). The smoke of the dried leaves is used to treat cold, fever and headache (Qureshi, 2009) and relieves cough (Baart *et al.,* 2004). The essential oils of this plant were used as antioxidant, antimicrobial and antifungal. The presence of high percentages of linalyl acetate and linalool in the oil have been reported for antibacterial and antifungal properties due to their ability to cross cell membranes, penetrating into the interior of the cell and interacting with critical intracellular sites (Trombetta *et al.*, 2005).

Nowadays, Tissue culture technology is used as a mean for germ-plasm conservation of endangered plants, for rapid mass propagation for large-scale re-vegetation, for genetic manipulation studies and for large scale production of high quality plant based medicines (Debnath, 2006; Yadav *et al.*, 2012).

Microbial contaminations are the major problem to initiate viable *in vitro* cultures. Losses due to contamination in *in vitro* cultures ranged between 3 to 15%. Plant tissues have various microorganisms on their surfaces which can grow in the culture medium, making the culture non sterile. In addition, they compete with the plant tissue for nutrition (CPRI, 1992). A successful tissue culture protocol starts with effective explants sterilization (Dodds and Roberts, 1985). Sterilization is the process of making explants contamination free before *in vitro* establishment of cultures. Various sterilization agents used for this purpose are also toxic to the plant tissues, hence proper concentration of sterilants, duration of exposure and the sequence of using these sterilants have to be standardized to minimize the injury to the explants (Goswami and Handique, 2013).

Sodium hypochlorite, ethanol, mercuric chloride and fungicides are common sterilants when using field grown tissues. The time of sterilization depends on tissue type (Funguomali *et al.*, 2013, Sharma and Nautiya, 2009). Nurul *et al.* (2012) reported that pre-sterilization using 0.2% Benomyl for 15 minutes reduces the contamination and mortality rate for leaf and nodal explants from *Aquilaria malaccensis*, especially when followed by surface sterilization with 0.1 % mercury chloride for 15 and 30 seconds.

There was no previous reports on *invitro* sterilization protocols for *Skimmia laureola* micropropagation. Therefore the present study was conducted to test the efficiency of various sterilization procedures against microbial contamination for the primary establishment of *in vitro* culture in order to standardize *in vitro* micropropagation protocol for *Skimmia laureola*.

Materials and methods

Plant material

The present study was conducted in the tissue culture laboratory at Hazara Agriculture Research Station Abbottabad during 2017. The *Skimmia laureola* was collected from naturally grown habitat of Nathia gali hills Abbottabad during the month of March. Plant was identified by using plant identification key 'Flora of Pakistan (Hassan ud din and Ghazanfar, 1980).

Media preparation

Murashige and Skoog (1962) basal medium containing 1 mgl^{-1}Ca-pentothenate, 100mgl^{-1}myoinositol with 30g/L sucrose and 6g/L agar was prepared. The coconut water @ 120ml/L was added to media instead of plant growth hormones. The pH was adjusted to 5.8 before autoclaving at 121 °C and 15 psi pressure for 15 minutes.

Explants surface sterilization

Each sterilizing agent at its respective concentration was tested for 3 different time periods.

There were twenty four experimental treatments (Table 1), each replicated 5 times. The fungicide (Ridomil) was used @ 0.1% (w/v).

After removing the leaves, *Skimmia* explants were washed under running tape water for 30 minutes. Then washed with mild detergent solution for 5 min and rinse thoroughly with distilled water.

The explants were cut into nodal segments. After applying the respective sterilization protocol(s) (Table 1) the nodal segments were rinsed 4 to 5 times with sterilized distilled water in order to remove any traces of sterilants. The test tubes were incubated in growth chamber at 20˚C under 16 h light and 8 h dark photoperiod for about 28 days.

The experimental design used was CRD. Data were analyzed by using computer software Statistix 8.1 and least significance difference test (LSD) at 95% level of significance was used to assess significant differences between various treatments.

Results and discussion

Sterilization is the process of making explants contamination free before establishment of cultures. The use of field grown plants as direct source for *in vitro* cultures presents a major challenge (Webster, *et al.*, 2003). The data of present study revealed that different sterilization protocols (Table 1) showed different effect on the establishment of in vitro culture and growth of Skimmia laureola.

Table 1. Different sterilization protocols used for *Skimmia* explants sterilization.

Treatments/Sterilization protocols	Sterilizing agents	Concentration%	Exposure time
T1	Mercuric chloride ($HgCl_2$)	0.1	30sec
T2			1min
T3			3min
T4	Mercuric chloride ($HgCl_2$)	0.5	30sec
T5			1min
T6			3min
T7	Sodium hypochlorite (NaOCl)	3	30sec
T8			1min
T9			3min
T10	Sodium hypochlorite (NaOCl)	5	30sec
T11			1min
T12			3min
T13	Sodium hypochlorite (NaOCl)+fungicide	3	30sec
T14			1min
T15			3min
T16	Sodium hypochlorite (NaOCl)+fungicide	5	30sec
T17			1min
T18			3min
T19	Mercuric chloride ($HgCl_2$)+fungicide	0.1	30sec
T20			1min
T21			3min
T22	Mercuric chloride ($HgCl_2$)+fungicide	0.5	30sec
T23			1min
T24			3min

Percent contamination

Data regarding the effectiveness of various sterilization procedures (Treatments) on percent contamination of explants after 7 days of culturing (Table 2) showed that mercuric chloride at both 0.1% and 0.5% was found significantly (P≤0.05) effective in reducing the contamination of cultured nodes (Table 2).

Table 2. Effect of sterilizing procedures on % contamination, % emergence and % survival rate of *Skimmia laureola* explants after 7 days of culturing.

Treatments	Sterilizing Agent	Concentration %	Time of exposure	Contamination %	Emergence %	Survival Rate %
T1	Mercuric choloride ($HgCl_2$)	0.1	30sec	0c	100a	100a
T2			1min	0c	100a	100a
T3			3min	0c	100a	100a
T4	Mercuric choloride ($HgCl_2$)	0.5	30sec	0c	100a	100a
T5			1min	0c	100a	100a
T6			3min	0c	100	100a
T7	Sodium hypochlorite (NaOCl)	3	30sec	0c	100a	100a
T8			1min	80ab	20c	20c
T9			3min	0c	100a	100a
T10	Sodium hypochlorite(NaOCl)	5	30sec	40b	60b	60b
T11			1min	0c	100a	100a
T12			3min	80a	20c	20c
T13	Sodium hypochlorite (NaOCl) +fungicide	3	30sec	0c	100a	100a
T14			1min	0c	100a	100a
T15			3min	0c	100a	100a
T16	Sodium hypochlorite (NaOCl) +fungicide	5	30sec	40b	60b	60b
T17			1min	100a	0c	0c
T18			3min	20bc	80ab	80ab
T19	Mercuric choloride ($HgCl_2$)+ Fungicide	0.1	30sec	0c	100a	100a
T20			1min	0c	100a	100a
T21			3min	0c	80ab	80ab
T22	Mercuric choloride ($HgCl_2$)+ fungicide	0.5	30sec	0c	100a	100a
T23			1min	0c	100a	100a
T24			3min	0c	100a	100a

Means in a column expressed with different alphabets are significantly different (P≤0.05).

Such response might be due to bleaching action of two chloride atoms and also ions that combines strongly with proteins and causing the death of organisms. There are many reports of surface sterilization in plant tissue culture using $HgCl_2$ (Naika and Krishna, 2008; Preethi *et al.*, 2011; Sen *et al.,* 2013).

The efficacy of $HgCl_2$ solution as surface sterilant in *in vitro* cultures of *A. paniculata* had also been reported by Martin, 2004; Purkayastha *et al.,* 2008; Kataky and Handique, 2010) who reported that 0.1% solution of $HgCl_2$ was sufficient in establishing the explants in culture medium. Whereas sodium hypochlorite at 3% concentration for 30 seconds (T7) and at 5 % concentration for 1 minute (T11) showed 0% contamination. After 14 days of culturing (Table 3) the sterilization procedures (T1, T2, T3, T4, T5, T6) also showed 0 % contamination while in the treatments 3% and 5% NaOCl (T7) and (T10) each for 30 seconds the contamination increased to 100% (Fig. 1).

In our study the sterilizing agent NaOCl at both concentration i.e 3% and 5% showed contamination for all the tested time periods. Daud *et al.* (2012) reported that NaOCl was ineffective on *A. malaccensis* for leaves and nodal segments explants and concluded that this effect is because the plant materials came directly from natural surroundings and even if the surface is effectively sterilized, the contaminants could come from the inner tissues after dissection of plant materials into small explants after the surface sterilization. After 28 days of inoculation (Table 4) similar trend was observed.

Table 3. Effect of sterilizing procedures on % contamination, % emergence, % survial rate, % death, % discoloration, shoot length and no of leaves of *Skimmia laureola* explants after 14 days of culturing.

Treatments	Sterilizing Agent	Concentration %	Time of exposure	Contamination %	Emergence %	Survival Rate %	Death %	Discoloration %	Shoot length (cm)	No of leaves
T1	Mercuric Chloride($HgCl_2$)	0.1	30sec	0e	80cd	80cd	20ab	20a	0.12cde	0.4fg
T2			1min	0e	60abc	60abc	40bcd	40bc	0.6bcde	2.2abcd
T3			3min	0e	20ab	20ab	80cd	80cd	0.32cde	1.8abcdef
T4	Mercuric Chloride($HgCl_2$)	0.5	30sec	0e	100a	100a	0d	0d	2.24a	3ab
T5			1min	0e	100a	100a	0d	0d	0.7bcde	2abcde
T6			3min	0e	40bcd	40bcd	80ab	80a	0.4cde	0.8defg
T7	Sodium hypochlorite(NaOCl)	3	30sec	100a	0d	0d	100a	0d	0e	0g
T8			1min	80ab	20cd	20cd	80ab	0d	0.32cde	0.8defg
T9			3min	20de	80ab	80ab	20cd	0d	0.48cde	2.4abc
T10	Sodium hypochlorite(NaOCl)	5	30sec	100a	0d	0d	100a	0d	0e	0g
T11			1min	60bc	20cd	40bcd	60abc	0d	0.1de	1cdefg
T12			3min	80ab	80ab	20cd	80ab	0d	0.4cde	0.6efg
T13	Sodium hypochlorite(NaOCl) +fungicide	3	30sec	100a	0d	0d	100a	0d	0e	0g
T14			1min	20de	80ab	80ab	20cd	0d	0.8bcd	2.4abc
T15			3min	80ab	20cd	20cd	80ab	0d	0.08e	0.4fg
T16	Sodium hypochlorite(NaOCl) +fungicide	5	30sec	100a	0d	0d	100a	0d	0e	0g
T17			1min	100a	0d	0d	100a	0d	0e	0g
T18			3min	100a	0d	0d	100a	0d	0e	0g
T19	Mercuric Chloride($HgCl_2$)+ Fungicide	0.1	30sec	0e	100a	100a	0d	0d	0.92bcde	3.2a
T20			1min	0e	60abc	60abc	40bcd	40bc	0.84bcde	1.6bcdef
T21			3min	20de	40bcd	40bcd	60abc	40bc	0.3cde	1cdefg
T22	Mercuric Chloride($HgCl_2$)+ fungicide	0.5	30sec	0e	100a	100a	0d	0d	1.3b	3ab
T23			1min	0e	80ab	80ab	40bc	40bc	0.7bcde	1.4cdefg
T24			3min	0e	40bcd	40bcd	80ab	80a	0.6bcde	1.2cdefg

Means in a column expressed with different alphabets are significantly different (P≤0.05).

Shoot emergence

After seven days of inoculation (Table 2) maximum shoot emergence(100%) was observed in treatment T1, T2, T3, T4, T5 and T6 containing 0.1 % and 0.5 % $HgCl_2$ alone and 0.1 % and 0.5 % $HgCl_2$ with fungicide (T19, T20, T21, T22, T23, T24) for three respective time period i.e 30 seconds, 1 minute and 3 minutes (Fig. 2). Similarly Treatment T11(5% NaOCl for 1 minute), treatment T7 and T9 having 3% NaOCl for 30 seconds and 3 minute respectively and treatment T13, T14 , T15 (3% NaOCl + fungicide) for three different time periods also showed 100% shoot emergence followed by Treatment T18 (5% NaOCl + fungicide) and T21 (0.1 % $HgCl_2$+fungicide) for 3 minutes showed 80% emergence.

The minimum shoot emergence i.e 20% was observed in treatment T8 and T12 (NaOCl 3% and 5 % for 1 and 3 minutes, respectively) (Table 2).

Results after 14 days (Table 3) showed that the sterilization procedure 0.5 % $HgCl_2$ (T4,T5) for 30 seconds and 1 minute and $HgCl_2$ + fungicide (T19, T22) at both concentration (0.1 % and 0.5 %) for 30 seconds showed 100% shoot emergence while treatment T9 (3% NaOCl for 3 minutes), treatment T1 (0.1 % $HgCl_2$), treatment T14 (3% NaOCl + fungicide) and treatment T23(0.5 % $HgCl_2$+fungicide 0.5 %) for 1 minute showed 80 % shoot emergence. However, NaOCl treatments T7, T10, T13, T16, T17 and T18 showed 0% shoot emergence after 28 days of inoculation (Table 4).

Kiani *et al.*, (2017) obtained maximum percentage of germination (81.33%) of *Melissa officinalis* L. after treating with 2.5% Sodium Hypochlorite for 3 min and (32.66%) at 0.2% Mercuric Chloride for 6 minutes.

Survival rate

Effect of sterilizing agents on explants survival after seven days of inoculation (Table 2) showed 100% survival rate in $HgCl_2$ treatments. Results of present study is in agreement with other reports (Alam, 2016) who reported that $HgCl_2$ at 0.1% for 2 min time caused 55.55% of cultures of *Cucumis sativus*

remained survived.0.1% $HgCl_2$+fungicide (Ridomil) showed 0% contamination after 7 days of culturing but after 28 days 20% contamination and 80% survival rate was observed at minimum time period but on increasing time of exposure i.e 3 min, contamination rate remain 20% but survival rate was reduced to 40% (Table 4).

Table 4. Effect of sterilizing procedures on % contamination, % emergence, % survival rate, % death, % discoloration, shoot length and no of leaves of *Skimmia laureola* explants after 28 days of culturing.

Treatments	Sterilizing Agent	Concentration %(w/v)	Time of exposure	Contamination %	Emergence %	Survival Rate %	Death %	Discoloration %	Shoot length(cm)	No of leaves
T1	Mercuric choloride ($HgCl_2$)	0.1	30sec	20c	60cd	60cd	40a	20a	0.16fg	0.60ef
T2			1min	40bc	60abc	60abc	40bcd	0d	0.88bcdefg	3.4abcd
T3			3min	20c	20abc	20abc	100bcd	80cd	0.52defg	2.4bcdef
T4	Mercuric cholorid e($HgCl_2$)	0.5	30sec	0c	100a	100a	0d	0d	3.26a	4.6ab
T5			1min	0c	100a	100a	0d	0d	1.74ab	4.6ab
T6			3min	0c	40bcd	40bcd	80ab	80a	0.58defg	1.2def
T7	Sodium hypochlorite (NaOCl	3	30sec	100a	0d	0d	100a	0d	0g	0f
T8			1min	80ab	20cd	20cd	80ab	0d	0.4efg	1.2def
T9			3min	40bc	60abc	60abc	40bcd	0d	0.68cdefg	2.8abcde
T10	Sodium hypochlorite(NaOCl	5	30sec	100a	0d	0d	100a	0d	0g	0f
T11			1min	60abc	40bcd	40bcd	60abc	0d	0.66cdefg	3.6abcd
T12			3min	80ab	20cd	20cd	80ab	0d	0.5efg	1.2def
T13	hypochlorite(NaOCl) +fungicide	3	30sec	100a	0d	0d	100a	0d	0g	0f
T14			1min	20c	80ab	80ab	20cd	0d	1.2bcde	3.8abc
T15			3min	80ab	20cd	20cd	80ab	0d	0.30fg	0.8ef
T16	hypochlorite(NaOCl) +fungicide	5	30sec	100a	0d	0d	100a	0d	0g	0f
T17			1min	100a	0d	0d	100a	0d	0g	0f
T18			3min	100a	0d	0d	100a	0d	0g	0f
T19	Mercuric choloride($HgCl_2$)+ fungicide	0.1	30sec	20c	80ab	80ab	20cd	0d	0.86bcdefg	3.4abcd
T20			1min	20c	40bc	40bc	60abc	40bc	0.76cdefg	2.6abcde
T21			3min	20c	40bcd	40bcd	60abc	40bc	0.60defg	1.60cdef
T22	Mercuric choloride($HgCl_2$)+ fungicide	0.5	30sec	0c	100a	100a	0d	0d	1.50abc	5.00a
T23			1min	0c	60abc	60abc	40bcd	40bc	1.40abcd	3.6abcd
T24			3min	0c	20cd	20cd	80ab	80a	0.92bcdef	2cdef

Means in a column expressed with different alphabets are significantly different (P≤0.05).

Results similar to present study was also reported by Sen *et al.* (2013) who found that at high concentration of $HgCl_2$ with fungicide control contamination but cause corrugation to explants.

Similarly Treatment T11, T7 and T9 and treatment T13, T14, T15 having 0.1% NaOCl and 3% NaOCl+ fungicide respectively showed 100% shoot survival rate.

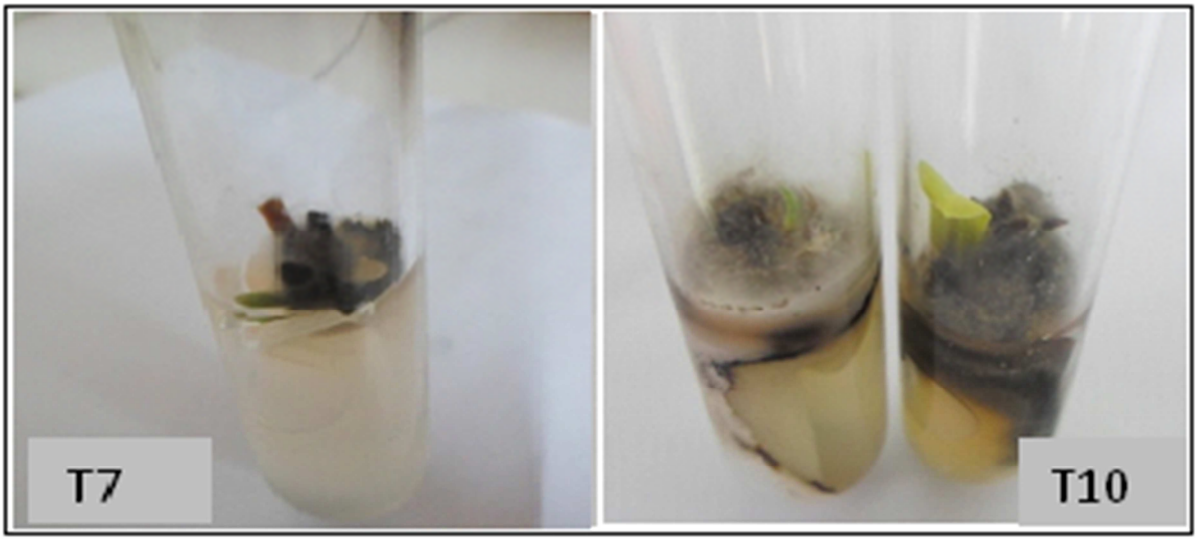

Fig. 1. Treatment T7 (3%NaOCl), T10 (5% NaOCl) showing fungal contamination after 14 days of culturing.

Results after 14 days (Table 3) showed that treatment T1 (0.1 % $HgCl_2$), T9 (3% NaOCl for 3 minutes), T14 (3% NaOCl + fungicide) and T23 (0.5% $HgCl_2$+fungicide for 1 minute) showed 80 % survival rate.

However, results after 28 days of inoculation (Table 4) revealed that most of the treatments containing NaOCl as sterilizing agent showed 0% survival rate. According to Ahmed, *et al.*, (2014) considerable reduction in contamination of explant was obtained by treating the explants with sodium hypochlorite 5% for 10 minutes after rapid rinsing in 70% ethanol for 30 seconds.

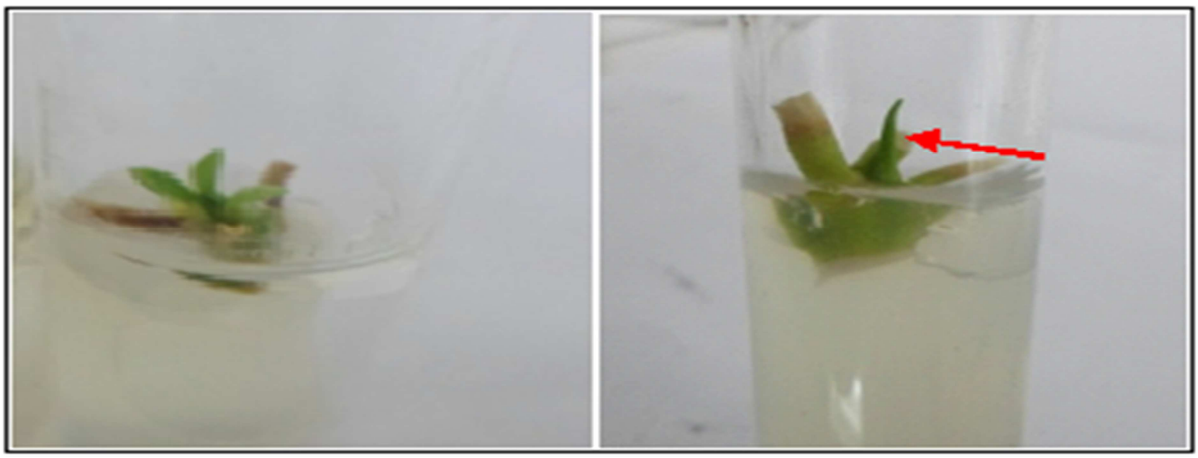

Fig. 2. *Skimmia laureola* explants showing shoot emergence in treatment T4 (0.5 % mercuric chloride) as after 7 days of culturing.

In the present study 3% NaOCl along with fungicide for 1 minute also showed 20% contamination and 80% survived explants even after 28 days of culturing.

Death percentage

Data regarding death percentage of explants after 14 days of culturing (Table 3) showed that sterilization procedure T4, T5 (0.5 % $HgCl_2$ for 30 seconds and 1 minute, respectively) and T19, T22 having 0.1 % and 0.5 % $HgCl_2$+fungicide for 30 seconds gave significantly ($P \leq 0.05$) lowest percentage of explants death i.e 0%. While treatment T7 (3 % NaOCl), treatment T10 (5 % NaOCl), T13 (3% NaOCl + fungicide) for 30 seconds showed 100% death of explants (Fig 3). Sterilization protocols T3 (0.1 % $HgCl_2$ for 3 minute), T12 (5% NaOCl), T15 (3% NaOCl + fungicide), T6 and T24(0.5 % $HgCl_2$ for 3 minutes) showed 80% death of explants.

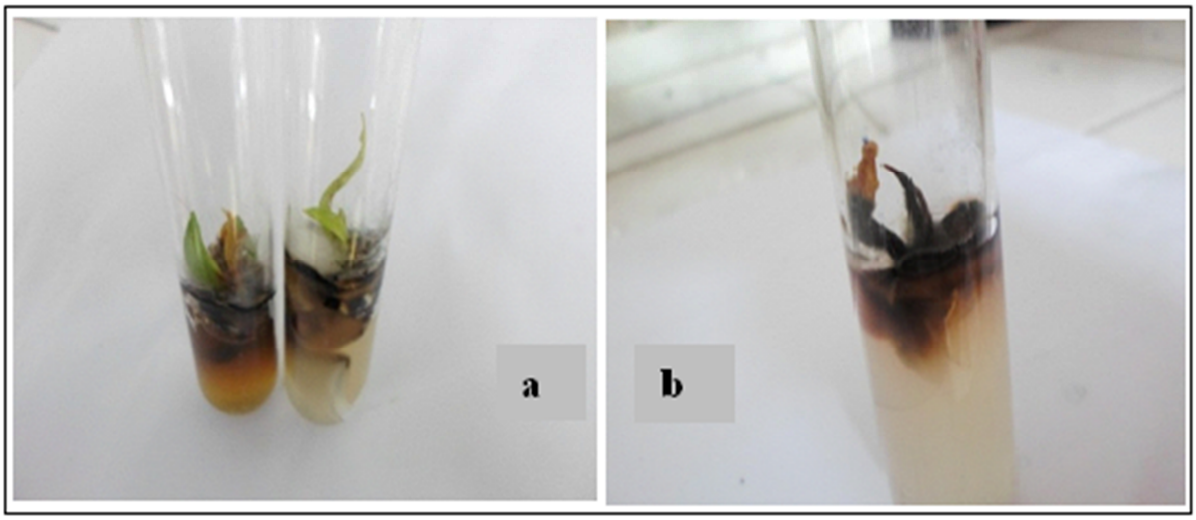

Fig. 3. Fungal contamination on *Skimmia laureola* explants (a), causing explant death (b).

After 28 days of inoculation (Table 4) same trend was observed except treatment T3 (0.1 % $HgCl_2$ for 3 minute) which showed 100% explants death. Results similar to present study was also reported by Sen *et al.,* (2013) who found that at high concentration of $HgCl_2$ with fungicide controls contamination but cause corrugation to explants.

Percent discoloration

It was observed that the sterilization protocols NaOCl, NaOCl + fungicide at both concentration (3 % and 5%) for all time periods (30 seconds, 1minute and 3 minutes) gave significantly ($P \leq 0.05$) lowest discoloration percentage of explants (0%) (Table 4).

Similarly 0.5% $HgCl_2$ for two time period (30 seconds and 1 minute) and 3 % $HgCl_2$+ fungicide for 30 seconds also showed 0% discoloration. While significantly maximum discoloration i.e 80 % was observed in 0.1% $HgCl_2$ for 3 minutes (T3) (Fig. 4a), 0.5% $HgCl_2$ (T6) and 0.5% $HgCl_2$+fungicide treatments for 3 minute (Fig. 4b).

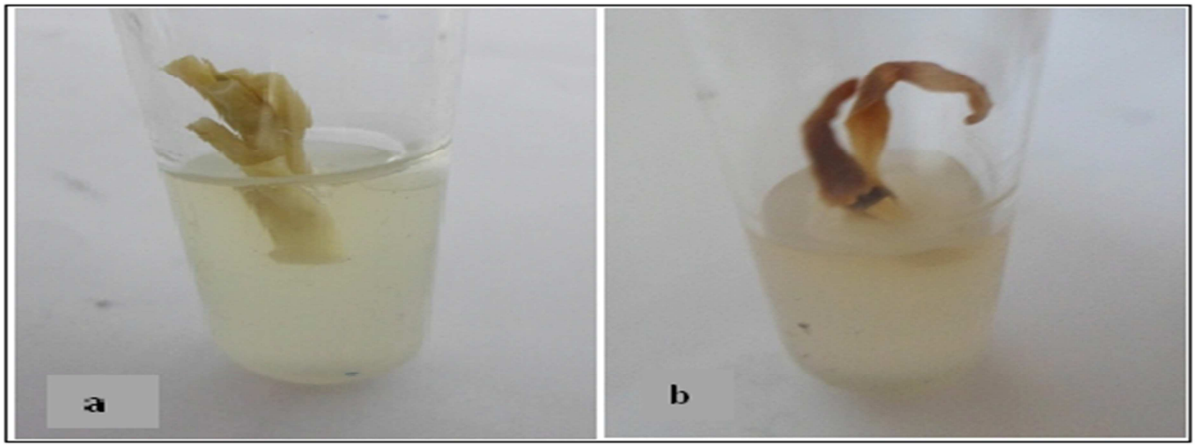

Fig. 4. Treatments T1 (a), and T24 (b) showing discoloration of explants after 14 days of culturing.

Our results are in accordance with the findings of Danso, *et al.*, (2011) who reported that exposure to $HgCl_2$ may have negative effects on survival rate of explants leads to browning and death of explants. The present study also showed deleterious effect of $HgCl_2$ at high concentration and long exposure.

Shoot length

The effect of sterilizing agents on growth of explants was recorded after 14 days of culturing (Table 3). The significantly ($P \leq 0.05$) maximum shoot length (2.24cm) was observed in treatment T4 (0.5% $HgCl_2$ for 30 seconds) (Fig. 5) followed by treatment T22 (0.5 % $HgCl_2$ + fungicide) 1.3 cm shoot length.

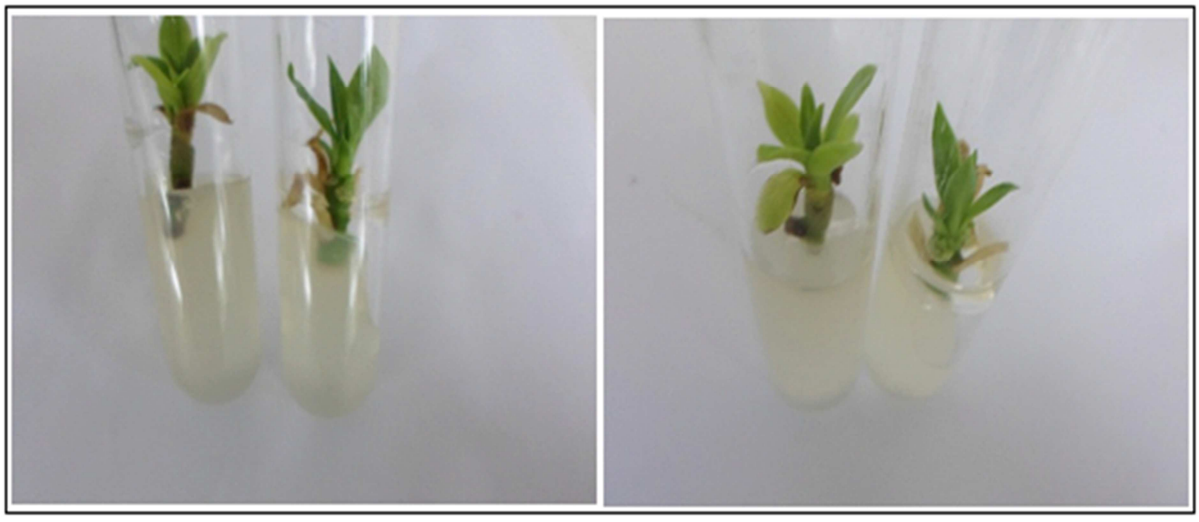

Fig. 5. *Skimmia laureola* explants showing shoot growth.

It was reported by Mahmoud and Nabeel, (2016) that at concentration (0.1%) mercuric chloride gave (90%) decontamination percentage and (60%) of viable nodal segments showing shoot development.No shoot growth was recorded in NaOCl treatments.

Results after 28 days of culturing (Table 4) showed an increase in shoot growth (3.26cm) in treatment T4 (0.5% $HgCl_2$for 30 seconds) whereas treatment T5 (0.5% $HgCl_2$ 0.5% for 1 minute) and treatment T22 showed 1.74cm 1.50 cm respectively of shoot length. Our results are in line with the findings of Sen *et al*., (2013)who found that at minimum concentration (0.1%) with the shortest duration of treatment (1 min), $HgCl_2$ was efficient as sterilizing agent giving 22.19% of *A. aspera* seed culture to be contamination free with a vigorous growth of plantlets of 5.67 cm in length was recorded.

Number of leaves

Data collected after 14 days (Table 3) showed that treatment T19 (0.1% $HgCl_2$ + fungicide for 30 seconds) showed maximum mean number of leaves i.e 3.2 followed by T4 and T22 in which 3 leaves per growing plantlets were observed. An increase in average leaf number was observed after 28 days of culturing (Table 4).This may be due to the fact that as the maximum number of plants remained survived in $HgCl_2$+fungicide treatment at both concentrations for 30 seconds (Table 3), these plants also showed growth in terms of shoot length and leaf number. However in literature direct effect of sterilizing agents on leaf number has not been reported.

Conclusion

Among the different sterilization protocols tested for the successful establishment of *in vitro* culture of *Skimmia laureola*, it has been concluded that mercuric chloride ($HgCl_2$) at 0.5 % concentration is most effective sterilizing agent when used alone or in combination with fungicide for controlling 100 % contamination with 100% survival rate for 30 seconds and 1 minute. However, 0.5 % $HgCl_2$ for 3 minutes showed 80 % discoloration and death of explants. It has been recommended that $HgCl_2$ alone or in combination with fungicide for short time of exposure can be used as sterilant for sterilization of important medicinal plants and further work is needed for standardizing $HgCl_2$ concentrations and time of exposure for establishment of *in vitro* culture of endangered medicinal plants.

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