Effect of sodium hypochlorite on control of *in vitro* contamination and seed germination of *Ficus religiosa*

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Abstract

Ficus religiosa has great mythological, religious, and medicinal importance in the culture of India and Nepal since times immemorial. The present paper was done to evaluate the potential of sodium hypochlorite in controlling the contamination of *F. religiosa* micro-propagation and seed germination of *F. religiosa*. In this study, a factorial experiment based on a completely randomized design with 18 treatments including six different sodium hypochlorite concentrations (0, 5, 10, 15, 20, and 25 %) and three soaking time of explants (5, 10 and, 15 min) with three replications was conducted. The seeds were inoculated on one-tenth strength of MS (Murashige and Skoog, 1962) medium. The lowest rate of contamination (0%) was obtained in treatments containing 20% Sodium hypochlorite at 10 and 15 min immersion and 25% Sodium hypochlorite at 5, 10 and 15 min immersion. The highest seed germination (63.33%) was observed in treatments including 10% Sodium hypochlorite at 5 and 10 min immersion.

Keywords: contamination; *Ficus religiosa*; germination; medicinal plant; micro-propagation

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Introduction

Family Moraceae comprises over 50 genera and nearly 1400 species distributed in tropical and subtropical regions of America, Africa, Asia and Australia (Zerega et al., 2005). *Ficus*, the fig genus, alone consists of over 800 species and is one of about 40 genera of the mulberry family Moraceae (Gill and siwach, 2009). *Ficus religiosa* Linn (Moraceae) is a deciduous tree native of the sub-Himalayan tract, Bengal and central India. It has been extensively distributed worldwide through cultivation (Deshpande et al., 1998). As its name implies, is one of the most venerated trees in the world, regarded as holy by millions of people - Buddhists as well as Hindus - in southeast Asia since ancient times (Parasharami et al., 2014). Different parts of this plant have been used traditionally in the treatment of a variety of disorders. It has been reported as an antibacterial (Pawar and Nabar, 2010), anticonvulsive (Patil et

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al., 2011), anti-diabetic (Kirana et al., 2009), and antinephropathic plant (Ballabh et al., 2008). It is also widely cultivated as a street or park tree in tropical and sub-tropical countries worldwide (Singh et al., 2011a). *F. religiosa* can be propagated by seeds or by vegetative methods (Singh et al., 2011a). The best propagation method is one of the uncertainties for planting on large scale. Although the direct seeding method can result in lower survival rate, the plants have the potential to be more tolerant to abiotic and biotic (Singh et al., 2011a). Conventionally, the tree is propagated by seeds, which remain viable for a few months. Also the vegetative propagation by cutting is not efficient under varied climatic conditions. Because of its medicinal importance and potential, there is a need to carry out rapid, mass propagation of the species.

The *in vitro* culture techniques can be an alternative for the continuous provisions of the woody plantlet stocks for large scale field cultivation (Gill and siwach, 2009). Different parts of *in vitro*-grown plants are often used for *in vitro* regeneration of various plants (Pierik, 1987). Therefore, the development of a protocol for seed sterilization and germination of this medicinal plant is very important.

Contamination with microorganisms such as fungi, bacteria, viruses, and yeast is considered as the single most important reason for losses during *in vitro* culture of plants (Omamor et al., 2007). Generally, there are four possible sources of contamination, namely, the plant which may be internal or external, the nutrient media that may be insufficiently sterilized, the air, and the research worker who may be inaccurate (Onwubiko et al., 2013). Explant contamination is a function of several plant and environmental related factors such as plant species, age, explant source, and prevailing weather condition (Singh et al., 2011b).

Maintaining an aseptic or sterile condition has been identified as essential in successful tissue culture procedure (Badoni and Chauhan, 2010). The desire of every researcher in tissue culture studies is to eliminate or prevent contaminations. Unfortunately contamination cannot be eliminated totally but can only be managed to reduce both the frequency of occurrence and the seriousness of its consequences, and this can be achieved through chemical sterilization (Pierik, 1987). Chemicals used in sterilization of materials for tissue culture should be effective, cheap, available, and non-toxic. An effective chemical (or sterilant) is one that is strong enough to inhibit the growth of disease causing microorganisms even in small quantity and at the same time will not injure the explants (Onwubiko et al., 2013). Hence, it is important to determine the appropriate concentration of the sterilant and exposure time in order to standardize the sequence of using sterilants to minimize explants injury for better result. Therefore, this study was set up to determine the appropriate concentration of sodium hypochlorite (commonly known as bleach) and exposure time for best sterilization protocol in *in vitro* culture of *Ficus religiosa* seeds.

Materials and Methods

This study was carried out in the Tissue Culture Laboratory of Department of Horticulture science, Ramin University of Agriculture and Natural Resources, Khuzestan, Iran. The fruits of *F. religiosa* L. were collected from 45-50 years old *F. religiosa* mother plants in a field grown at campus of Ramin Agriculture and Natural Resources University, Khuzestan, Iran. The fruits were washed under running water for 30 min and five to six times rinse with tap water and then with liquid soap solution followed by washing with tap water. Further, surface sterilization treatment was conducted in a laminar air flow chamber. The seeds were surface sterilized with 70% aqueous ethanol for 10 seconds and dipped into six different levels of concentrations of sodium hypochlorite (0%, 5%, 10%, 15%, 20%, and 25%) and exposure time (5, 10, and 15 minutes), giving a total of 18 treatment combinations., and then washed 3 times in sterilized distilled water. The seeds were inoculated on one-tenth strength of MS (Murashige and Skoog, 1962) medium. The media were supplemented with 3% w/v sucrose, adjust 5.8 pH (1N HCl or 1N NaOH) and solidified with 0.6% agar before autoclaved at 121.5 °C for 20 min and poured into culture bottles (2 cm in

Table 1
Analysis of variance of contamination and seed germination under the effect of various concentrations of Sodium hypochlorite and different immersion times

Treatments	Contamination (%)	Seed Germination (%)
Sodium hypochlorite		
0%	93.33 a	6.66 de
5%	52.22 b	47.77 b
10%	32.22 c	61.11 a
15%	16.66 d	35.55 c
20%	1.11 e	12.22 d
25%	0.00 e	1.11 e
Time		
5 min	35.55 a	30.00 a
10 min	32.77 ab	27.77 ab
15 min	29.44 b	24.44 b
Sodium hypochlorite× Time		
0%× 5min	93.33 a	6.66 fgh
0%× 10min	93.33 a	6.66 fgh
0%× 15min	93.33 a	6.66 fgh
5%× 5min	56.66 b	43.33 cd
5%× 10min	53.33 b	46.66 bcd
5%× 15min	46.66 b	53.33 abc
10%× 5min	36.66 c	63.33 a
10%× 10min	33.33 cd	63.33 a
10%× 15min	26.66 cde	56.66 ab
15%× 5min	23.33 de	46.66 bcd
15%× 10min	16.66 ef	36.66 d
15%× 15min	10.00 fg	23.33 e
20%× 5min	3.33 g	16.66 ef
20%× 10min	0.00 g	13.33 efg
20%× 15min	0.00 g	6.66 fgh
25%× 5min	0.00 g	3.33 gh
25%× 10min	0.00 g	0.00 h
25%× 15min	0.00 g	0.00 h
p-Value		
Sodium hypochlorite	<0.001	<0.001
Time	0.0116	0.0603
Sodium hypochlorite× Time	0.6393	0.0529

Different letters within columns indicate significant differences ($p<0.05$).

diameter and 15 cm in height). All cultures were incubated in a culture room at 24 ± 2° C under 16 h photoperiods that provided by cool white fluorescent light (63 µmol $m^{-2}s^{-1}$).

The experiments were set up in completely randomized design (CRD) and there were 10 replicates per treatment and each treatment was repeated in three sets. The data were analyzed by Analysis of Variance (ANOVA) using Duncan's multiple range test. Data analysis was carried out using SAS version 9.3.

Results

The multi-factorial analysis of variance demonstrated that the interaction between various concentrations of Sodium hypochlorite solution and immersion times of seeds did not have a significant effect on contamination percentage and seed germination. The results also suggest that different times of immersion and various concentrations of Sodium hypochlorite had significant effects on contamination percentage separately. Also, the results shown that various concentrations of Sodium hypochlorite had a significant effect on seed germination, but different times of immersion did not have any significant effect on seed germination (Table 1).

The lowest rate of contamination (0%) was obtained in the treatments containing 20% Sodium hypochlorite at 10 and 15 min immersion

and 25% Sodium hypochlorite at 5, 10, and 15 min immersion (Figs. I, II). Treatments including 10% Sodium hypochlorite at 5 and 10 min immersion resulted in the highest seed germination (63.33%), which was significantly higher than other treatments except for the treatment containing 10% Sodium hypochlorite at 15 min immersion (Table 1; Fig. III).

Discussion

Successful micro-propagation of all plants depends on the condition of plant material at the time of collection of the explant from the grown trees in the field and removal of exogenous and endogenous contaminating microorganisms. Fungi and bacteria are the most common microorganisms to be found on or in plant tissues (Pierik, 1987). Establishing a sterile *in vitro* culture is the first and most important step to success in commercial micro-propagation of plants (Arab et al., 2014).

A wide range of surface disinfectants, such as ethanol, hydrogen peroxide, bromine water, mercuric chloride, silver nitrate, and antibiotics are used for surface-sterilization; however, sodium hypochlorite (NaOCl) has been most widely used. NaOCl is highly effective against all kinds of bacteria, fungi, and viruses (Pierik, 1987). Moreover, NaOCl has a strong oxidizing property which makes it highly reactive with amino acids (Kantouch and Ardel-Fattah, 1971), nucleic acids (Hayatsu et al., 1971), amines, and amides (Sandford et al., 1971). The general reaction between amino acids and NaOCl produces the respective aldehyde, NH_4Cl and CO_2 (Kantouch and Ardel-Fattah, 1971).

In vitro seed germination, seedling growth, and the viability of the tissue were negatively affected by sodium hypochlorite (NaOCl) at high concentrations (Hsiao et al., 1984) while it was ineffective for sterilization of tissues at low concentrations. The negative effects of NaOCl concentration became more severe with increasing application period. Since regeneration capacity of the tissue is negatively affected by higher concentrations and longer application periods of disinfectants (Pierik, 1987), sterilization process under *in vitro* conditions should aim to use the lowest concentration of disinfectant for the shortest time.

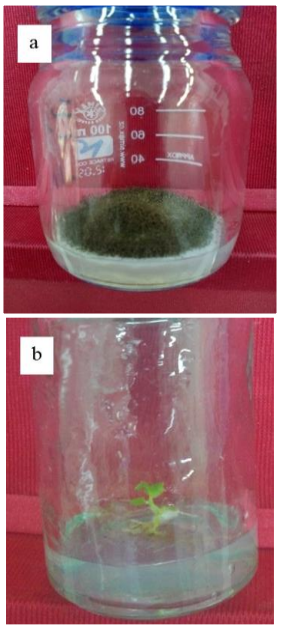

Fig. I. Effect of Sodium hypochlorite on *in vitro* contamination: (a) infected medium, (b) clean medium

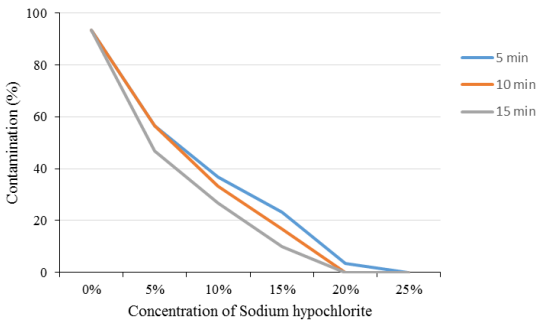

Fig. II. Effect of different levels of Sodium hypochlorite on *in vitro* contamination reduction of *F. religiosa*

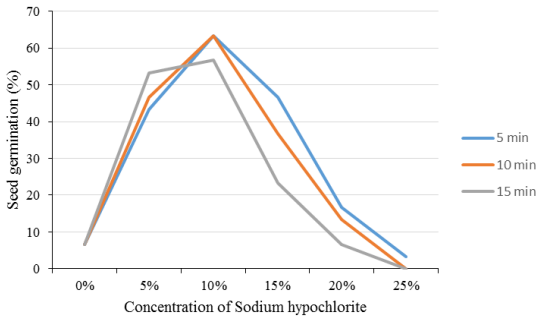

Fig. III. Effect of different levels of Sodium hypochlorite on *in vitro* seed germination of *F. religiosa*

Results showed that with increasing concentration of sodium hypochlorite and immersion time, the percentage of contamination is reduced (Table 1). Sodium hypochlorite has been reported to be very effective against many types of bacteria. Even micromolar concentrations are enough to significantly reduce bacterial populations (Nakagawara et al., 1998). It has also been reported that, when diluted with water, the hypochlorite salts used e.g., NaOCl, $Ca(OCl)_2$, LiOCl, and KOCl lead to the formation of HClO, the concentration of which is negatively correlated with contamination, perhaps in part due to lethal DNA damage (Wlodkowski and Rosenkranz, 1975; Dukan et al., 1999).

In this study, increase of sodium hypochlorite concentration and immersion time showed a negative effect on seeds, resulting in blackish color with lower germination rate (Table 1). The use of sodium hypochlorite for surface sterilization of plant explants from different sources has been widely reported (Badoni and Chauhan, 2010; Maina et al., 2010; Colgecen et al., 2011).

In the study aiming to evaluate the effects of NaOCl solutions used for sterilization on *in vitro* seed germination and seedling growth in *Lathyrus chrysanthus* Boiss., the best results were obtained from 3.75% NaOCl concentration and 15 min application period for all parameters examined (Telci et al., 2011). Telci et al. (2011) reported that seed-borne contamination increased gradually by decreasing concentrations and application periods of NaOCl below 3.75% and 15 min. Dramatic decreases were observed at 5.00% NaOCl concentration in all cases. At this concentration, NaOCl showed deleterious effects on the embryo of the seeds. Seed germination decreased to 65.18% when NaOCl concentration increased to 5.00% from 3.75% for 15 min application period. Seedling growth from seeds sterilized with 3.75% NaOCl concentration for 15 min were observed faster than that of the seeds sterilized with other concentrations and application periods of NaOCl.

There are a number of reports (Deshpande et al., 1998; Gill and siwach, 2009; Parasharami et al., 2014) for sterilization of *F. religiosa* explants with different concentrations of sodium hypochlorite. Parasharami et al. (2014) reported that the highest rate of sterilization (80%) for fruit of *F. religiosa* was obtained in the treatment containing 2% sodium hypochlorite for 30 min.

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