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IMPROVING *SALVIA SCLAREA* L. SEED GERMINATION UNDER *IN VITRO* CONDITION

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ABSTRACT: It is easy to grow *Salvia sclarea* L. from seed on *in-vivo* condition and a cold period can improve seed germination but its germination on *in-vitro* condition is difficult, because of mucilage Production during surface sterilization of seeds that limit seed germination on *in-vitro* condition. This study was designed to optimize germination of sage seeds under *in-vitro* condition for tissue culture aims. The seeds were cultured in different media including Water Agar (WA), Wet Filter Paper (WFP), Murashige and Skoog (MS) medium, MS medium containing different Chitosan concentrations and MS medium with different pH. On the other hand, different methods of surface sterilization including several concentrations of Sodium hypochlorite in various time periods were tested. The results indicated significant differences among treatments. The highest percentage and rate of germination incubation, observed after 9 days in MS media containing chitosan, MS medium with a pH of 7-8 and seeds that sterilized with 1% sodium hypochlorite for 5 minutes. Also the Lowest percentage and rate of germination belonged to medium with a pH of 5-6, medium without Chitosan and in seeds that sterilized with 2.5% sodium hypochlorite for 10 minutes and 1% sodium hypochlorite for 12 and 15 minutes.

Key words: Clary Sage, Chitosan, Germination, Mucilage, Sterilization, Wet Filter.
Abbreviations: WA- Water Agar; WFP- Wet Filter Paper; MS- Murashige and Skoog.

INTRODUCTION

The genus *Salvia* belongs to the Lamiaceae family and covers about 900 species dispersed worldwide (Delamere et al., 2007; Mossi et al., 2011). Most of Salvia species are commonly utilized for their essential oils in the foods, medicines and perfumery industries (Goren et al., 2006; Ozcan et al., 2003; Ulubelen and Topcu, 1998).

Salvia sclarea L. (Clary Sage) has been known and used as a medicinal plant in phytotherapy since the antiquity. Sage in florescences has been proved to have a beneficial action in the treatment of cancer. The essential oils are also used in osteoarthritis treatment and rheumatic arthritis (Rusu et al., 1999). The main compounds in the oil were Linalyl acetate and Linalool (Dzamic et al., 2008).

Seed germination is a complex process various physical and biochemical cues such as water, light and phytohormones (Sen, 2010). Another factors such as temperature (Spechit and Keller, 1997; Iterranz et al., 1998; Iannucci et al., 2000; Eileen et al., 2001; Wuebker et al., 2001; Nyachiro et al., 2002), photoperiod (Anon, 2004), effected germination ability of plant genotypes. Numerous studies have been made of the factors that influence seed dormancy and elimination of seed dormancy in various plant species. Srivastara et al. (2011) observed that low temperature, induces *in vitro* seed germination in *Aconitum heterophyllum wall*. Soaking seeds of *Bobgannia malagascariensis* in hot water significantly improved seed germination percentage (Thokozani et al., 2011). Treatment seeds with gibberellic acid have been reported to overcome dormancy and ensure uniform germination (Al-Absi, 2010; Cetinbas and Koyunko, 2006).

Srinivasulu et al, (2011), documented that 0.1% mercuric chloride completely inhibits the tomato seed germination and optimum germination obtained with 4% sodium hypochlorite solution. Surface sterilization of mature seeds using 7.2% calcium hypochlorite stimulated the germination rate in Terrestrial Orchids (Vejsadova, 2006). Mechanical scarification increased average germination vigor of *Trifolium Pratense* (Onal Asci et al., 2011). Germination improved when seeds were smoke-treated or soaked in 70% (v/v) H_2SO_4 (Musarurwa et al., 2010). Jun-Feng, et al. in their studies on *Caragana korshinkii* (2010) showed that chitosan improves seed germination and at the same time shortens germination time. Chitosan generally improves growth of seedling, chlorophyll content, root length, dry and fresh-weight of roots. Treatment of wheat seeds with Chitosan induced resistance to certain disease and improved seed quality (Reddy et al., 1999). Seed priming with Chitosan significantly increased the shoot height and shoot dry weight and mean germination with the control (shao et al, 2005).

Germination of *Salvia sclarea* L. seeds is easy under *in-vivo* conditions. A cold period may improve germination, but this process difficult under *in-vitro* conditions, because seeds produce mucilage during the sterilization. The objective of this study was to improve seed germination of *Salvia sclarea* L. under *in vitro* conditions.

MATERIALS AND METHODS

Plant Material

This experiment was carried out in the Biotechnology Institute of Urmia University during 2011-2012. Seeds of Sage were obtained from medicinal plants garden of the Horticultural Sciences Department of Urmia University. Freshly harvested seeds from garden were tested for germination and viability.

Sterilization treatments

Two different methods of sterilization were tested in order to overcome seed dormancy. In the first experiment, the seeds were surface sterilized with 70% ethanol for 1 min. and sodium hypochlorite (2.5%, 1%) for 10 min. In second experiment, Surface sterilization with 70% ethanol for 1 min. and 1% sodium hypochlorite for different time intervals of 2, 5, 8, 10, 12 and 15 min. were studied (Figure 2A-F). Then seeds were rinsed three times with sterile distilled water.

Seed culture media

Sage seeds were cultured in different media including: Water-Agar (WA), Wet Filter Paper (WFP), MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and 0.8% agar without or with Chitosan at different concentrations (0, 40, 60, 80 ppm), MS medium with different pH values (5-6, 6-7, 7-8). The number of germinated seeds was counted every day and germination percentage and rate was calculated on the ninth day after seed culture.
All the cultures were kept in growth chamber at 24 ± 2°C under a 16/8 h (light/dark) photoperiod at a photon flux rate of 60 µmol m^{-2} s^{-1} provided by cool daylight fluorescent lamps.

Percentage and rate of germination

In determining the final density of plants per unit area, germination stage is important and this density is obtained when the seeds are planted with a good germination rate and percentage (Huang and Redman, 1995). At the end of the germination period, the germination percentage and germination speed, subjected to different treatments, were calculated using appendix equations (Gharineh et al., 2004):
Gp = 100 (NG / NT)
(Gp: germination percentage. NG: The number of germinated seeds. NT: The total number of seeds).
Germination rate (Rs) was calculated according to the following equation (Rajabi and Poostini, 2005):

$$Rs = \sum_{i}^{n} = \frac{Si}{Di}$$

(Rs: germination rate. Si: The number of germinated seeds per day. Di: Days to count the number).

Statistical analysis

Each treatment had 3 replications consisting of 100 seeds per replication. Analysis of variance (ANOVA) based on completely randomized design (CRD) was performed on the data with the General Linear Model procedure using SAS 9.1 software and the means were compared using Duncan's multiple range test (DMRT) at the 5% probability level.

RESULTS

Sterilization treatments

The results of this study showed that sterilization of seeds at different times and

different concentrations with sodium hypochlorite were effective on the germination of Clary sage seeds. Comparative data on concentrations of 1% and 2.5% for 10 minutes in preliminary experiment showed that the best treatment was 1% sodium hypochlorite (data not shown).

The results showed that different times of sterilization with 1% sodium hypochlorite had significant effects on the germination percentage at probability level of 5% (Figure 1). The highest germination percentage (68.36%) and germination rate (6.7) was obtained in 1% sodium hypochlorite for 5 min. The lowest percentage (5%) and rate (0.2) of germination were observed on 15 min. sodium hypochlorite. There was no significant difference between 12 and 15 min. treatments (Figure 2).

Seed culture media

Comparison of data revealed that there were significant differences between WA, WFP and MS media (P < o.o5). The maximum percentage (68. 36%) and germination rate (6.7) obtained in the MS medium and the minimum percentage (6.5) and germination rate (0.4) was observed in the wet filter paper (Table 1).

In this study, it was observed that germination was improved by adding chitosan into MS medium (Figure 3). Comparison of data showed that chitosan had significant effects (P < o.o5) in terms of speed and germination percentage of seeds (Figure 4). The highest percentage of germination (85.81%) was obtained in 80 ppm of chitosan and the highest germination rate (17.85) was observed on 40 ppm chitosan. In media containing chitosan, leaf color was darker than control plants and with increasing concentration of chitosan, seedling height was reduced (Figure 5).

Clary sage seeds grown in media with different pH levels showed different germination percentage and rate. With increasing pH levels greater than normal (pH =5-6) in MS basal medium, seed germination percentage and rate were raised. The highest percentage (86.66%) and germination rate (3.57) was obtained in pH 7-8 values and the lowest percentage (49.99%) and germination rate (1.6) was observed in pH= 5-6 (Table 2).

DISCUSSION

Effect of different methods of sterilization on seed germination was statistically significant. Results of seed sterilization with 1% sodium hypochlorite at different times showed that with increasing time of sterilization, percentage and rate of germination were decreased. With increasing time of sterilization, the amount of mucilage around seed was increased (Figure 6). Therefore uptake of oxygen was restricted and then seed germination rate was reduced. Orphanos (1983) stated that in Caper (*Capparis spinosa* L.) seed coat mucilage limited penetrate of oxygen to the embryo, therefore, caused main obstacles in the seed germination. It seems that in our study with increasing time of disinfection, sodium hypochlorite influenced the embryo and caused more damage to the embryo and therefore germination percentage and rate were decreased. Tomita (1998) reached the conclusion that with any longer time of *Calypso bulbosa* seeds treatment with sodium hypochlorite, seeds were damaged and the germination rate was decreased.

In this research, in WA and WFP media, germination percentage and germination rate was decreased compared to MS media. On the other hand, seedling vigor was reduced due to lack of nutrients.

Chitosan is an abundant and comparatively cheap organic compound. With adding chitosan to culture medium, significant difference in germination rate and germination percentage was obtained. All concentrations of chitosan significantly increased the GP when compared with controls in Clary sage seeds tested. Zhou et al. (2002) reported that seed soaked with chitosan increased the energy of germination, germination percentage, lipase activity, and gibberellic acid (GA3) and Indole acetic acid (IAA) levels in peanut. It seems that increase of Lipase and GA_3 activity in Clary sage seed has caused better germination. Chitosan increases the β-1,3-glucanases (β-Glu) enzyme, it is proposed that β-Glu not only helps defend seeds against pathogens, but is also a key factor in regulating coat-imposed dormancy and germination of seeds in response to environmental and hormonal cues (Gerhard, 2003). In this research with addition of chitosan to seed germination media, color of leaf due to more photosynthesis and chlorophyll content was darker than that of control plants. In accordance with our results, Ait Barka et al. (2004) reported that photosynthesis and related parameters were stimulated in chitogel treated *Vitis vinifera* L. plantlets.

In this research, the effects of pH on seed germination was studied, and observed that in

pH=7-8 seed germination was best. The results of this study were in accordance with the results in Begonia (*Begonia* sp.), Impatiens (*Impatiens wallerana)*, Allyssum (*Lobularia maritima)*, Petunia (*Petunia* sp.) and Salvia (*Salvia splendens)* that did not germinate at or below pH= 5.0. Germination was very low for Ageratum (*Ageratum houstonianum)* and Marigold (*Tagetes patula*) in this range; in these plants the exact pH range increased germination varied, 4.5 to 5.5 for Geranium and 5.0 to 6.5 for Salvia (Shoemaker and Carlson, 1990).

CONCLUSION

Seed germination in plants under different conditions is varied. In this study it was observed that MS medium is better for seed germination than WA and WFP media. With increase pH (7-8), both germination rate and percentage, improved. Add chitosan to the medium has caused better germination and also reduced time of seed surface sterilization with sodium hypochlorite, increased germination rate and percentage.

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Table 1. Seeds germination of Sage on different medium culture MS, WA and WFP.

medium	Germination percentage	Germination rate
MS	68.36 a	6.7a
WA	13.3 b	1.33 b
WFP	6.5 c	0.4 c

*Means in each column followed by the same letters are not significantly different at 5% level using DMRT.

Table 2. Effect of different pH levels on germination percentage and germination rate of *Salvia Sclarea* L. seeds.

Medium with different pH	Germination percentage	Germination rate
pH=5-6	49.99 c	1.6 c
pH=6-7	70.55 b	2.9 b
pH=7-8	86.66 a	3.57 a

*Means in each column followed by the same letters are not significantly different at 5% level using DMRT.

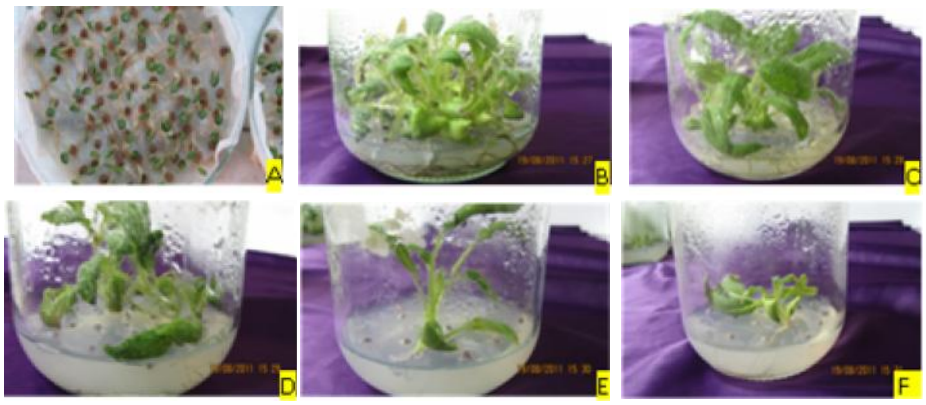

Figure 1. Effect of different time sterilization in *Salvia Sclarea* L. seed germination. A, Control seed . B, Seed sterilization at 5 min. C, Seed sterilization at 8 min. D, Seed sterilization at 10 min. E, seed sterilization at 12 min. F, Seed sterilization at 15 min.

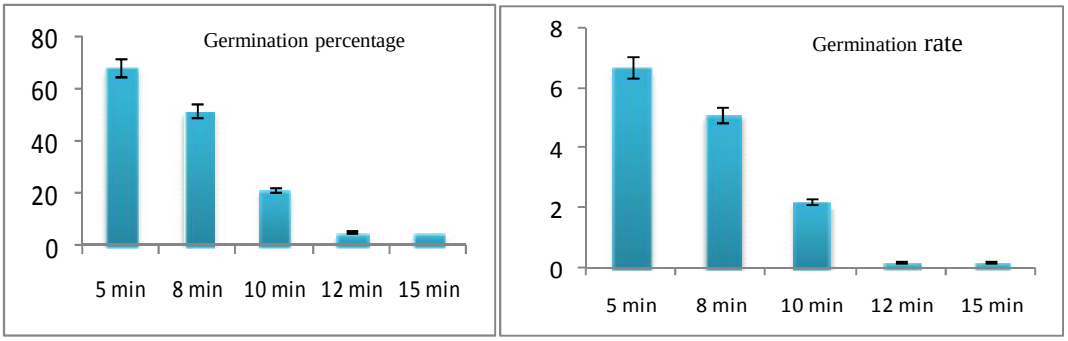

Figure 2. Effect of different time sterilization treatments on rate and percentage germination in *Salvia* sclarea L.

Figure 3. Seeds germination and plants growth in medium containing chitosan

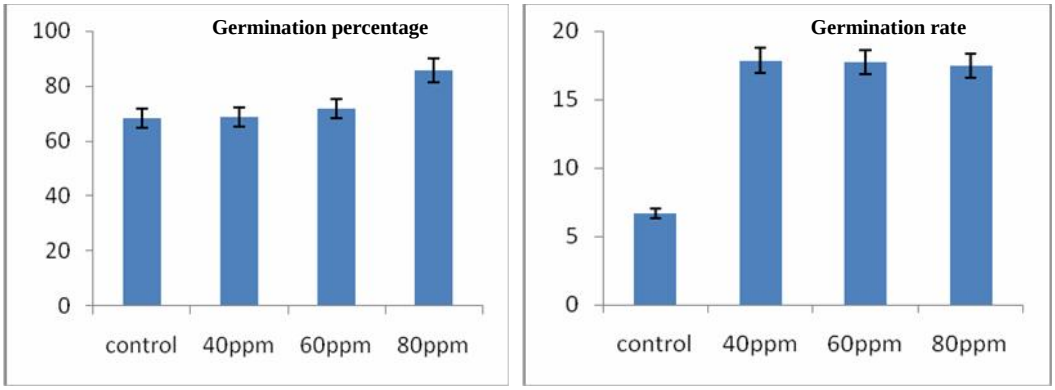

Figure 4. Seeds germination in mediums containing different concentrations of Chitosan

Figure 5. Effects of Chitosan concentrations on leaf color and seedling size. Treatments from left to right are: control, 40, 60 and 80 ppm Chitosan

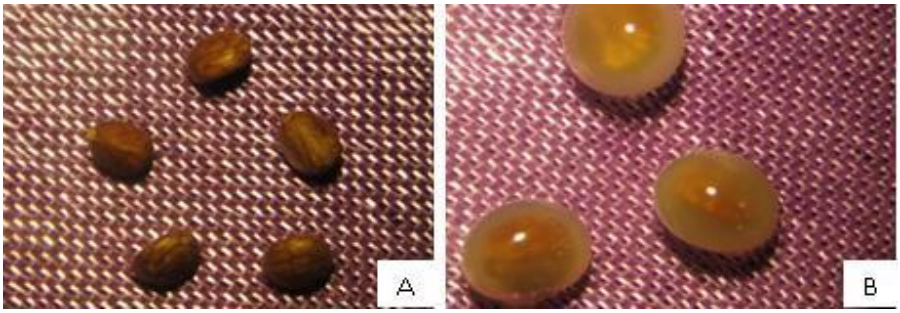

Figure 6. Seeds before sterilization (A). Seeds after sterilization (B).

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