1. Introduction

Lemon balm (*Melissa officinalis* L.) is a medicinal perennial herb belong to the family *Lamiaceae* widely growing in Southern Europe, Middle East and southern parts of North America. Today, the extract of Lemon balm is used extensively in various fields, such as medicine, food industries, as well as perfume and cosmetics productions. Lemon balm has great significance as it is having medicinal properties like anti-cancer and anti-inflammatory. It is also used in gastrointestinal disorders and Alzheimer’s disease. It is known that most of these effects are related to its active ingredient, rosmarinic acid. Its essence includes compounds such as citronella, sitral, geranial, linalool, beta-caryophyllene and oxide, germacrone D, neural and flavonoids.

Hence, efficient seed sterilization and germination is a prerequisite for successful regeneration and transformation. Commonly used surface sterilization agents include ethanol, sodium hypochlorite, calcium hypochlorite and mercuric chloride, which have been used for surface sterilization of plant and seed material of various species. Surface sterilized seeds were inoculated on different culture media such as Murashige and Skoog (MS) basal, half strength MS and B5 media with different sucrose concentrations.
Therefore, the present study has been done to standardize the sterilization and germination protocols for seeds of *Melissa officinalis* L. for *in-vitro* propagation intended for its conservation using different types of media and sterilizing agents by varying their concentration and duration of exposure.

### 2. Materials and Methods

#### 2.1 Plant Material

The healthy seeds of *Melissa officinalis* L. (German origin seeds with 90% viability) procured from Pakan Bazr Co. Esfahan, Iran.

#### 2.2 Surface Sterilization of Seeds

All seeds stored under unknown conditions and duration. Before seed surface sterilization the seeds were hand sorted to select healthy seeds. For each treatment 50 seeds were used. The seeds were soaked in distilled water for 10 hours and treated with various concentrations of different seed surface sterilization for different intervals of time in the laminar air flow cabinet as mentioned below with occasional swirling: 1. Sterilized distilled water for 15 min (control). 2. Mercuric chloride (HgCl₂) 0.1%, 2% and 0.3% (W/V) for 3 and 6 min, 3. Ethanol 70% + 100 μL Tween-20 for 3 min, 4. Sodium hypochlorite (NaOCl) 2.5% and 4% (W/V) for 3 and 6 min and 5. Ethanol 70% (W/V) for 1 min + sodium hypochlorite 2.5% and 4% (W/V) for 3 min. The seeds subsequently were rinsed with 3-4 changes of sterile distilled water. Surface sterilized seeds were inoculated on ½ strength MS (Murashige and Skoog, 1962) medium supplemented with 1% sucrose and solidified with 0.8% agar (HI MEDIA). The cultures were incubated in culture room at 25 ± 2°C under 16-h day light with intensity of -40 μmol/ms provided by cool-white fluorescent tube lights.

#### 2.3 Seed Germination Medium

Surface-sterilized seeds with sodium hypochlorite 2.5% (W/V) for 3 min, followed by 3-4 rinses in sterile distilled water were inoculated aseptically on eight media namely, T₁ - Mineral water, T₂ - B5 medium, T₃ - MS medium free sucrose, T₄ - MS medium with 1% (W/V) sucrose, T₅ - MS medium with 2% (W/V) sucrose, T₆ - ½ strength MS medium with 1% (W/V) sucrose, T₇ - ½ strength MS medium with 2% (W/V) sucrose, T₈ - ¼ strength MS medium with 1% (W/V) sucrose. The Medium was gelled with 0.8% (W/V) agar-agar and adjusted pH to 5.7 in culture bottles by using 1N HCl or NaOH followed by autoclave at 15 Ψ (for 15-20 min) at 121°C. The cultures maintained at 25 ± 2°C under fluorescent light with a 16/8 light/dark photoperiod.

#### 2.4 Data Analysis

The percentage of contamination and germination were calculated and the root length and shoot length of plantlets were recorded 15 days after sterilization. For each treatment 50 seeds were treated with three replications. Mean Germination Time (MGT) was determined according the cited equation to measure the rate of germination:

\[
MGT = \frac{\sum D \times N}{n}
\]

Where; “D” is the number of certain day in each counting, “N” is the number of germinated seeds in the certain counting day and “n” is the total number of germinated seeds were used. In order to calculate Germination Percentage (GP) and Contamination Percentage (CP) seedlings grown in 15 days were used. The Germination Rate (GR) was computed by the following formula:

\[
GR = \frac{\text{No. of germinated seeds}}{\text{Days to first count}} + \cdots \frac{\text{No. of germinated seeds}}{\text{Days to final count}}
\]

Vigour index values were calculated adopting the following formula:

\[
V = GP \times T
\]

Where; “V” is Vigour index, GP is Germination Percentage and “T” is Total seedling length (cm). The Mean Germination Time and Germination Rate were calculated. For seed sterilization and germination, treatments of the experiment were conducted in IBM SPSS version 20 statistical software. All results were expressed as mean ± Standard Error (SE). Statistical analysis was performed by the Analysis of Variance (ANOVA) and means comparison analysis was made by Duncan's multiple range test (P ≤ 0.05).

### 3. Results and Discussion

#### 3.1 Seed Surface Sterilization

Surface sterilization of seeds is a compulsory pre-requisite technique for preservation and seedling production to
reduce the contamination and germinate dormant seeds. Various concentrations of different surface sterilization agent and time of treatment were presented in Table 1. The results of ANOVA indicated the effect of seed sterilization treatment were significant (p< 0.01) on Germination Rate and Contamination and Germination Percentage (GR, CP and GP), Mean Germination Time (MGT) and also on Number of Days to First Germination (NDFG). The maximum percentage of germination (81.33%) was observed at 2.5% Sodium Hypochlorite for 3 min and a minimum (32.66%) at 0.2% Mercuric Chloride for 6 min, while control showed 22.00% of Germination Percentage (Figure 1). Among different treatments, the highest Contamination Percentage (66.66%) was recorded for Sterilized Distilled Water for 15 min and the lowest (4.00%) for 0.3% Mercuric Chloride for 3 min and 4% Sodium Hypochlorite for 6 min treatment (Figure 2). 2.5% Sodium Hypochlorite for 6 min evolved the optimum Growth Rate (GR). The correlations between MGT-GP and GR-MGT-GP were significant and positive, whereas the correlations were negatively significant between MGT, GR, GP and NDFG (Table 2). The correlation between Germination Percentage and growth rate is strong, linear and positive at the 0.01 level (Figure 3). Bleaching activity of Mercuric Chloride (HgCl₂) due to chloride atoms as well as ions that can attach toughly with proteins and causing the death of organisms 15. Sodium hypochlorite significantly reduced the contamination and also affected the germination of seeds.

### Table 1. Effect of different surface sterilization methods on measured characteristics (Mean values ± Std. Error) in *M. officinalis*

| Sterilant and Treatment Duration (min) | NDFG        | GP          | MGT         | GR          | CP          |
|---------------------------------------|-------------|-------------|-------------|-------------|-------------|
| Sterilized Distilled Water, 15 min (control) | 2.33 ± 0.33** | 22.00 ± 3.05* | 1.37 ± 0.30* | 2.36 ± 0.38* | 66.66 ± 1.76* |
| 0.1% Mercuric Chloride, 3 min         | 2.00 ± 0.00** | 50.66 ± 1.76* | 2.38 ± 0.16** | 7.41 ± 0.32c | 6.66 ± 0.66b |
| 0.1% Mercuric Chloride, 6 min         | 2.66 ± 0.33ab | 41.33 ± 1.76e | 2.82 ± 0.16ab | 3.92 ± 0.33bc | 5.33 ± 0.66a |
| 0.2% Mercuric Chloride, 3 min         | 2.66 ± 0.33** | 38.66 ± 1.76d | 1.94 ± 0.18** | 4.33 ± 0.10d | 6.00 ± 1.15** |
| 0.2% Mercuric Chloride, 6 min         | 3.33 ± 0.33** | 32.66 ± 2.40f | 1.77 ± 0.10c | 3.39 ± 0.36** | 5.33 ± 0.66** |
| 0.3% Mercuric Chloride, 3 min         | 3.00 ± 0.00** | 34.66 ± 3.33** | 2.04 ± 0.23** | 3.51 ± 0.28** | 4.00 ± 1.15c |
| 0.3% Mercuric Chloride, 6 min         | 1.66 ± 0.33** | 39.33 ± 2.40** | 2.22 ± 0.10** | 4.65 ± 0.45** | 5.33 ± 0.66** |
| 2.5% Sodium Hypochlorite, 3 min       | 1.33 ± 0.33** | 81.33 ± 1.76c | 4.03 ± 0.40** | 10.82 ± 0.51bc | 8.00 ± 1.15b |
| 2.5% Sodium Hypochlorite, 6 min       | 1.00 ± 0.00** | 78.66 ± 2.40** | 4.00 ± 0.40** | 12.04 ± 0.94c | 6.66 ± 0.66a |
| 4% Sodium Hypochlorite, 3 min         | 1.66 ± 0.33** | 73.33 ± 2.90bc | 3.46 ± 0.12** | 10.57 ± 0.91bc | 6.66 ± 0.66a |
| 4% Sodium Hypochlorite, 6 min         | 1.66 ± 0.33** | 68.00 ± 3.05bc | 2.94 ± 0.12** | 9.76 ± 0.77b | 4.00 ± 1.15** |
| 70% Ethanol + 2.5% Sodium Hypochlorite, 3 min | 2.00 ± 0.00** | 54.66 ± 2.90d | 2.67 ± 0.26** | 6.89 ± 0.59c | 6.00 ± 1.15bc |
| 70% Ethanol + 4% Sodium Hypochlorite, 3 min | 2.00 ± 0.00** | 50.66 ± 0.66d | 2.04 ± 0.07** | 8.10 ± 0.51c | 5.33 ± 0.66** |

Table 2. Phenotypic correlation coefficient of measured traits under various sterilization treatments in *M. officinalis*

|         | NDFG | GP  | MGT  | GR    | CP   |
|---------|------|-----|------|-------|------|
| NDFG    | 1    | -0.701 ** 1 |
| GP      | -0.620 ** 0.909 ** 1 |
| MGT     | -0.366 ** 0.903 ** 0.803 ** 1 |
| GR      | -0.766 ** 0.951 ** 0.803 ** 1 |
| CP      | 0.059 -0.426 -0.389 -0.359 1 |

**Significant correlation at 0.01 level (2-tailed) and* Significant correlation at 0.05 level (2-tailed).
Studies on the Effect of Various Seed Surface Sterilization and Growing Media on the In-vitro Germination of Lemon Balm (Melissa officinalis L.)

3.2 Effect of Different Germination Media Composition on Seeds

The data on the effectiveness of various media to improve better germination media for ideal seedling production in respect of Germination Percentage (GP), Germination Rate (GR), shoot and root length were given in Table 3 (Figure 4). The highest rate of seed germination (GR) and Germination Percentage (GP) were obtained on ½ strength MS medium with 1% (W/V) sucrose and the lowest were observed on control and B5 medium (Figure 5). Our findings showed ½ strength MS medium with 1% (W/V) sucrose has better response in terms of shoot and root length (Figure 6). Maximum value of vigour index (703.72) was achieved by ½ strength MS medium with 1% (W/V) sucrose. As it is described in Table 4, analysis of data indicated that the effect of media was significant (p<0.01) for GP, GR, shoot length and root length as well as mean germination time. Subsequently, the correlation between GP, GR, shoot length and root length were significant and positive. Sucrose is a source of carbon and energy in higher plants that works as an important signalling molecule to regulate genes involved in photosynthesis, respiration and metabolism processes. Effect of sucrose in culture media on seed germination and seedling development were reported in different plants. In this study, we indicated greater concentration of sucrose and MS medium strength, caused to delay of seed germination and also decrease the percentage of seeds germination, length of shoot and length of root.

Table 3. Effect of different media with various concentrations of sucrose on measured characteristics (Mean values ± Std. Error) in M. officinalis

| Composition of Media                  | NDFG  | GP       | GR       | Mean Shoot Length (cm) | Mean Root Length (cm) | Vigour Index |
|--------------------------------------|-------|----------|----------|------------------------|-----------------------|--------------|
| Mineral water                        | 2.00 ± 0.57a | 43.33± 3.71c | 5.76 ± 0.49d | 1.92 ± 0.17b | 1.18 ± 0.14d | 133.41 ± 5.19f |
| B5 medium                            | 2.33 ± 0.33c | 54.00± 2.30d | 6.20 ± 0.48d | 2.12 ± 0.07c | 1.71 ± 0.12bc | 206.91 ± 9.79c |
| MS medium free sucrose               | 2.00 ± 0.00a | 55.33± 0.66d | 6.82 ± 0.19d | 2.78 ± 0.08b | 2.01 ± 0.14b | 265.75 ± 11.44c |
| MS medium with 1% (W/V) sucrose      | 2.33 ± 0.33c | 62.00± 1.15e | 7.52 ± 0.63e | 3.44 ± 0.01c | 2.06 ± 0.12b | 341.67 ± 13.50c |
| MS medium with 2% (W/V) sucrose      | 2.33 ± 0.33c | 57.33± 1.76e | 6.58 ± 0.26d | 2.98 ± 0.06c | 1.75 ± 0.02bc | 271.72 ± 8.52d |
| ½ strength MS medium with 1% (W/V) sucrose | 1.33 ± 0.33a | 81.33± 1.76e | 10.82 ± 0.51d | 5.16 ± 0.02b | 3.48 ± 0.15c | 703.72 ± 27.75c |
| ½ strength MS medium with 2% (W/V) sucrose | 2.00 ± 0.00a | 72.66± 4.37b | 9.33 ± 0.43b | 3.87 ± 0.02b | 1.74 ± 0.06bc | 408.11 ± 22.97b |
| ¼ strength MS medium with 1% (W/V) sucrose | 2.33 ± 0.33c | 62.66± 2.66d | 7.14 ± 0.64ad | 3.18 ± 0.11d | 1.50 ± 0.11ad | 293.55 ± 8.87d |
Table 4. Phenotypic correlation coefficient of measured traits under different types of media in *M. officinalis*

|        | GP   | MGT  | GR   | Shoot Length | Root Length |
|--------|------|------|------|--------------|-------------|
| GP     | 1    |      |      |              |             |
| MGT    | 0.919*** | 1    |      |              |             |
| GR     | 0.865*** | 0.652*** | 1    |              |             |
| Shoot Length | 0.907*** | 0.967*** | 0.885*** | 1            |
| Root Length | 0.695*** | 0.591*** | 0.711*** | 0.817***     | 1           |

***Significant correlation is significant at the 0.01 level (2-tailed).

Figure 4. *In vitro* seed germination of *M. officinalis*. a. seeds of *M. officinalis*; b and c. *In vitro* germination of seedlings; d. *In vitro* germination on different types of media: T₁ - Mineral water, T₂ - B5 medium, T₃ - MS medium free sucrose, T₄ - MS medium with 1% (W/V) sucrose, T₅ - MS medium with 2% (W/V) sucrose, T₆ - ½ strength MS medium with 1% (W/V) sucrose, T₇ - ½ strength MS medium with 2% (W/V) sucrose, T₈ - ¼ strength MS medium with 1% (W/V) sucrose.

Figure 5. Effect of different types of media on growth rate in *M. officinalis*.

Figure 6. Comparison of mean shoot length and root length by using different type of media in *M. officinalis*.

4. Conclusion

The different system for seed surface sterilization and growth media composition not only has effect on the uniform growth of seedlings, but also affects the Germination Percentage. Hence, in our study, using 2.5% Sodium Hypochlorite for 3 min was more suitable surface sterilization agent and uniformity growth of seedlings in lemon balm. In addition, ½ strength MS medium with 1% (W/V) sucrose proved to be the best medium for germination and seedling development on in-vitro conditions. More concentration of sucrose and MS medium strength, caused to delay of seed germination and also decrease the percentage of seeds germination, length of shoot and length of root. The analysis of data indicated the correlation between GP, GR, shoot length and root length were significant and positive.

5. References

1. Meftahizade H, Moradkhani H, Naseri B, Lofti M, Naseri A. Improved in vitro culture and micropropagation of different *Melissa officinalis* L. genotypes. Journal of Medicinal Plants Research. 2010; 4(3):240–4.
2. Adinee J, Piri K, Karami O. Essential oil component in flower of lemon balm (*Melissa officinalis*). American Journal of Biochemistry and Biotechnology. 2008; 4(3):277–8.
3. Park SU, Uddin R, Xu H, Kim YK, Lee SY. Biotechnological applications for rosmarinic acid production in plant. African Journal of Biotechnology. 2008; 7(23):4959–65.
4. Aharizad S, Rahimi MH, Toorchi M, Mohebalipour N. Assessment of relationship between effective traits on yield and citral content of lemon balm (*Melissa officinalis* L.) populations using path analysis. Indian Journal of Science and Technology. 2013; 6(5):4448–50.

5. Blumenthal M, Goldberg A, Brinckmann J. Herbal Medicine. First ed. USA: American Botanical Council; 2000. p. 230–1.

6. Evans WC. Trease and Evans' pharmacognosy. 15th ed. UK: W. B. Saunders and Company; 2002. p. 221–53.

7. Zhang BH, Liu F, Yao CB. Plant regeneration via somatic embryogenesis in cotton. Plant Cell, Tissue and Organ Culture. 2000; 60(2):89–94.

8. Talei D, Valdiani A, Abdullah MP, Hassan SA. A rapid and effective method for dormancy breakage and germination of King of Bitters (*Andrographis paniculata* Nees.) seeds. Maydica. 2012; 57(2):98–105.

9. Daud NH, Jayaraman S, Mohamed R. An improved surface sterilization technique for introducing leaf, nodal and seed explants of *Aquilaria malaccensis* from field sources into tissue culture. Asian Pacific Journal of Molecular Biology and Biotechnology. 2012; 20(2):55–8.

10. Gamborg OL, Miller R, Ojima K. Nutrient requirements of suspension cultures of soybean root cells. Experimental Cell Research. 1968; 50(1):151–8.

11. Ellis RH, Roberts EH. The quantification of ageing and survival in orthodox seeds. Seed Science and Technology (Netherlands). 1981; 9(2):373–409.

12. Van De Venter HA, Grobbelaar N. Influence of sub-optimal imbibition temperatures on seed vigour and respiration in maize (*Zea mays* L.). South African Journal of Plant and Soil. 1985; 2(4):203–6.

13. Tajbakhsh M. Relationships between electrical conductivity of imbibed seeds leachate and subsequent seedling growth (Viability and vigour) in Omid wheat. Journal of Agricultural Set Technology. 2000; 2(3):67–71.

14. Baki AA, Anderson JD. Vigour determination in Soybean seed by multiple criteria. Crop Science. 1973; 13(6):630–2.

15. Pauling L. College chemistry. San Francisco: WH Freeman and Company; 1955. p. 578.

16. Baiyeri KP, Mbah BN. Surface sterilization and duration of seed storage influenced emergence and seedling quality of African breadfruit (*Treculia africana Deene*). African Journal of Biotechnology. 2006; 5(15):1393–6.

17. Newman PO, Krishnaraj S, Saxena PK. Regeneration of tomato (*Lycopersicon esculentum Mill.*): Somatic embryogenesis and shoot organogenesis from hypocotyl explants induced with 6-benzyladenine. International Journal of Plant Sciences. 1996; 157(7):554–60.

18. Moghaieb RE, Saneoka H, Fujita K. Shoot regeneration from GUS-transformed tomato (*Lycopersicon esculentum*) hairy root. Cellular and Molecular Biology Letters. 2004; 9(1):439–49.

19. Xu F, Tan X, Wang Z. Effects of sucrose on germination and seedling development of Brassica napus. International Journal of Biology. 2010; 2(1):150–1.

20. Gibson SI. Control of plant development and gene expression by sugar signaling. Current Opinion in Plant Biology. 2005; 8(1):93–102.

21. Finkelstein RR, Gibson SI. ABA and sugar interactions regulating development: Cross-talk or voices in a crowd? Current Opinion in Plant Biology. 2002; 5(1):26–32.

22. Younesikelaki FS, Ebrahimzadeh MH, Desfardi MK, Banala M, Marka R, Nanna RS. Optimization of seed surface sterilization method and in vitro Seed Germination in *Althaea officinalis* (L) - An important medicinal herb. Indian Journal of Science and Technology. 2016; 9(28):1–5.