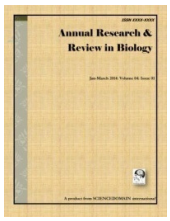

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Optimization of Somatic Embryogenesis in *Ferula assa-foetida L.* (a Rare Medicinal Plant)

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Experiments was carried out to examine the effect of abscisic acid (ABA), source of carbon and temperature on secondary somatic embryogenesis and somatic embryo germination of *Ferula assa-foetida L.* Cotyledonary somatic embryos were cultured on MS (Murashig and Skoog, 1962) medium supplemented with 0, 1.0, 1.5 or 2.0 mg/l ABA and on MS medium with 3% mannitol, sorbitol or sources. The highest number of secondary somatic embryos (35.94) was observed on MS medium devoid of ABA at 25℃, but the addition of abscisic acid, sorbitol or manitol to the MS basal medium significantly reduced the formation of secondary somatic embryos at 5℃. The concentration of abscisic acid had a significant effect on the rate of secondary somatic embryogenesis. Among the carbon source studied, mannitol significantly suppressed the induction of secondary somatic embryos. Abscisic acid had a positive effect on the percentage of somatic embryo germination. Higher germination was observed with1.0 or 1.5 mg/l ABA, but it was not significantly affected by sorbitol or manitol. Abscisic acid increased germination at 15℃ and 25℃ while sorbitol and manitol enhanced it at 5℃.

Keywords: Abscisic acid (ABA); germination; manitol; secondary somatic embryogenesis; sorbitol.

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1. INTRODUCTION

Ferula assa-foetida L. is a medicinal plant from the Apiaceae family [1]. It is native to Iran and Afghanistan [2]. It has medicinal and culinary properties including antispasmodic, aromatic, carminative, digestive, expectorant, laxative, sedative, nervine, analgesic, anthelmintic, aphrodisiac and antiseptic effects [3].

Secondary somatic embryogenesis involves initiating new somatic embryos from somatic embryos [4]. Secondary somatic embryogenesis leads to forming abnormal somatic embryos then it produces abnormal seedlings with single, three or more cotyledon structures. In *Ferula assa-foetida* L., abnormal secondary somatic embryos are also sporadically formed all over the surface of the primary embryos [1].

According to Daigny et al. [5] moderate concentrations of 2,4-D induces secondary somatic embryogenesis, though high auxin concentrations (NAA or 2,4-D) strongly inhibit secondary somatic embryogenesis and somatic embryo production. Also Karami et al. [4] showed the effect of concentration of 2,4-D on secondary embryos formed on primary embryos.

Structural abnormality of somatic embryos brings about low conversion into plants [6]. Abscisic acid (ABA) treatment promotes the conversion rate of somatic embryos to plantlets in some plants such as alfalfa, carrots and hybrid larch [6]. ABA can raise the expression of maturation genes in embryos, including the *Em*(Early methionine labeled) genes, which may be important for desiccation tolerance [7]. ABA pretreatment promoted accumulation of dry matter including proline, glycine-betaine, cyclitols and soluble carbohydrates and raised the concentration of K^+ and Na^+ ions in explants [8], thus leads to inducing desiccation tolerance and normal plant regeneration in somatic embryos [9]. Moreover, it describes that producing normal somatic embryos depends on the time of using ABA in *Daucuscarota*and *Aralia cordata* [10]. Exogenously supplied ABA is the important part of the maturation medium. In the absence of ABA, maturation results in poorly developed somatic embryos, which often shows abnormal morphology, asynchronous development and precocious germination [11].

In cyclic somatic embryogenesis, unlimited numbers of embryos are proliferated in a repetitive way from single culture of primary embryos [12]. ABA prevents initiating recurrent embryogenesis [13].

Mauri and Manzanera [13] reported that stratification can neutralize the effect of ABA as a germination inhibitor: ABA treatment for 1 week at different concentrations (1–40 µM), followed by stratification at 4°C for 1 month, significantly affected germination of immature somatic embryos. Fernandez-Guijarro et al. [14] reported that chilling slightly reduced secondary embryogenesis but gave a modest raise in germination. Stratification also promotes gibberellin action and somatic embryo germination in several plant species, including *Rosa* spp., *Juglans regia*, and *Quercus suber* [13].

Sugar added to the culture medium as a carbon source can also contribute to osmotic regulation of water stress. Adding an osmoticum raises the water stress during somatic embryo germination [15]. According to Tang et al. [16] secondary somatic embryo production depends on sucrose; manitol and sorbitol strongly suppress somatic embryo production compared with sucrose [16]. Many researchers have reported that various sources of carbon such as manitol etc., are important in germinating somatic embryos [15].

This study envisaged to investigate the effect of ABA, source of carbon (sorbitol or manitol compared with sucrose) and temperature on reduction of secondary somatic embryogenesis and increasing embryo germination of *Ferula assa-foetida* L.

2. MATERIALS AND METHODS

Experiments were carried out at Tissue Culture Lab of Agricultural Biotechnology Research Institute of Iran in 2011. In Experiment 1, different concentrations of ABA (0, 1.0, 1.5 or 2.0 mg/l) and different temperatures (5°C, 15°C and 25°C) were tested to assess the effect of ABA and temperature on the secondary somatic embryogenesis and germination of the embryos. In Experiment 2, effects of sorbitol (30 g/l), manitol (30 g/l) and sucrose (30 g/l) on secondary somatic embryogenesis and germination of embryos were examined at different temperatures.

2.1 Explant Preparation

Somatic embryos used in this experiment were obtained from preliminary work on direct somatic

embryogenesis of *Ferula assafoetida* [17]. In order to produce somatic embryos, the roots obtained from *in vitro* plants were cut into 0.5-1 cm pieces. The prepared explants were cultured on MS (Murashige and Skoog, 1962) medium containing 2,4-D (0.2 mg/L) + Kin (0.2 mg/L) to induce embryogenesis. After one month the explants were transferred to MS medium without any growth regulators for embryogenesis. Produced single somatic embryos were selected using a binocular microscope and used as explants for following experiments.

2.2 Experiment 1: ABA and Temperature Treatment

Somatic embryos were cultured on MS (Murashige and Skoog, 1962) basal medium supplemented with different concentrations of ABA (0, 1, 1.5 and 2 mg/l). Each treatment consisted of three Petri dishes and 20 single embryos were cultured in each Petri dish. Petri dishes were closed and sealed with household plastic foil and were randomly placed in growth chambers set at 5°C, 15°C or 25°C and 16/8 h (light/dark) photoperiod with a light intensity of 3000 lux for a period of one week. Then explants were transferred to MS medium without hormone and put back to the first same temperature again and incubated for three more weeks. After four weeks of stratification, all the Petri dishes were transferred to 25°C, 16/8 h (light/dark) photoperiod and 3000 lux for two more weeks.

2.3 Experiment 2: Carbon Sources and Temperature Treatment

Experiment 2, was carried out to assess the effect of source of carbon on secondary somatic embryogenesis and germination of embryos at different temperatures. Three kinds of carbon sources (sorbitol, manitol and sucrose) were tested. Standard MS (Murashige and Skoog, 1962) medium was prepared in three treatments with 30 g/l sorbitol, 30 g/l manitol and 30 g/l sucrose as mentioned above. Firstly, media were prepared; pH was adjusted to 5.8 and 6 g/l "plant agar" was added before autoclaving. Single somatic embryos were cultured in sterilized Petri dishes containing 30 ml of the above mentioned media. Each treatment consisted of three Petri dishes. 20 single somatic embryos were cultured in each Petri dish. The lids of Petri dishes were sealed with household plastic foil and were randomly placed in growth chambers set at 5°C, 15°C or 25°C, under 16/8 h (light/dark) photoperiod and a light intensity of 3000 lux for a period of four weeks. After four weeks Petri dishes were transferred to 25°C with the same photoperiod and light intensity for two more weeks.

2.4 Data Collection

Petri dishes were incubated for a period of 5 weeks. After that time, the number of produced secondary somatic embryogenesis was measured and recorded; after one more month, germinated embryos were measured. Germination was defined as evaluation of root and epicotyl emission [13].

2.5 Experimental Design and Statistical Analysis

Each experiment was conducted as a completely randomized factorial design with two factors and three replications. Data were analyzed using the SAS package (version 8). ANOVA used to find out the significant treatment effects (5 or 1%) based on the F-test, the Duncan's Multiple Range Test ($P \leq 0.05$) in order to compare the means.

3. RESULTS

3.1 Experiment 1: Effects of Abscisic Acid and Temperature Treatment

ABA showed highly significant effects on the number of secondary somatic embryos and the percentage of embryo germination (Table 1). The control showed the highest value for secondary somatic embryogenesis (Fig. 1, 2a). The minimum number of secondary somatic embryos and the highest percentage of germination and normal growth were observed in treatment of ABA 1.5 mg/l (Fig. 1, 2b).

Temperature showed a highly significant effect on the number of secondary somatic embryos, and the percentage of embryo germination (Table 1). The maximum rate of secondary somatic embryogenesis was observed at 25°C, though the minimum value of secondary somatic embryogenesis was observed at 5°C (Table 2). At 15°C and 25°C, embryo germination percent was higher than at 5°C (Table 2).

The interaction effect of ABA and temperature on the number of secondary somatic embryos

and percentage of embryo germination was highly significant (Table 1). At 0 mg/l of ABA, the temperature effects on secondary somatic embryogenesis were much higher than ABA was supplied; resulting in a high level for the combination of no ABA concentration and 25℃, but at 1.5 mg/l ABA and 5℃, there seemed to be an optimum temperature for reduction of secondary somatic embryogenesis (Table 3). In contrast, ABA (1 mg/l) at 5℃ and ABA (1.5 mg/l) at 15℃ and 25℃ caused the highest percentage of embryo germination (Table 3).

Table 1. Analysis of variance of the effect of abscisic acid (ABA) and temperature on the number of secondary somatic embryos (SSE), and the percentage of germinated embryos

Source of variation	DF	SSE	Germination (%)
ABA	3	1097.79 **	413.66 *
Temperature	2	2126.62 **	2029.86 **
ABA × Temperature	6	620.41 **	398.38 **
Error	24	15.39	112.50

*ns: non significant (p>0.05), * significant at p≤0.05, ** significant at p≤0.01*

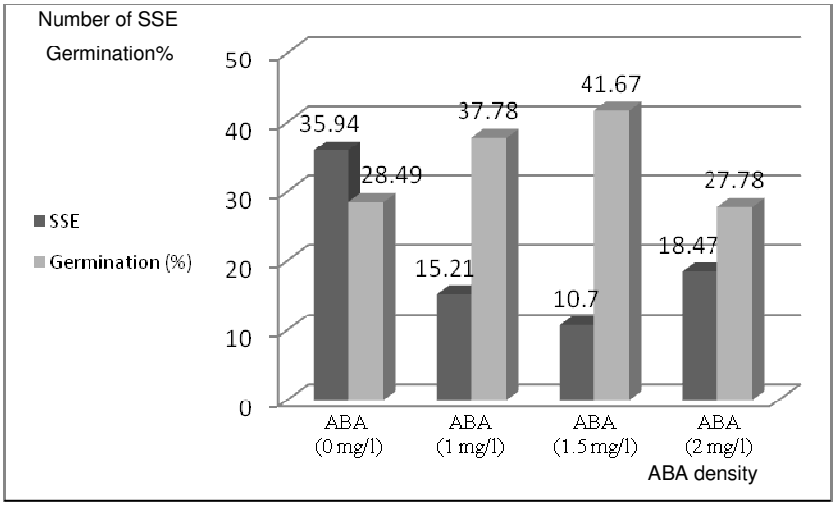

Fig. 1. The effect of different concentrations of abscisic acid (ABA) on the number of secondary somatic embryos (SSE) produced, and the percentage of germinated embryos

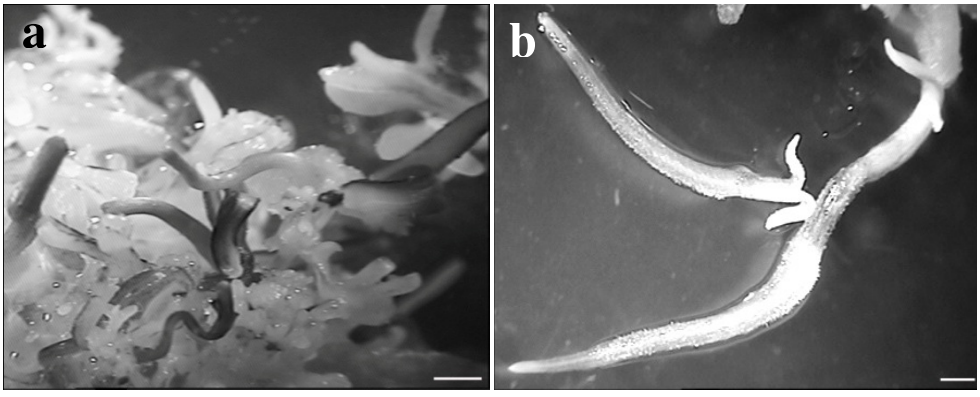

Fig. 2. (a) Secondary somatic embryogenesis on medium without ABA (0 mg/l); bar=2mm, (b) germination and normal growth of somatic embryos without secondary somatic embryogenesis in medium containing ABA (1.5 mg/l); bar=3mm

Table 2. The effect of temperature on the number of secondary somatic embryos (SSE) produced, and the percentage of germinated embryos in experiment 1

Temperature treatments	SSE	Germination (%)
5°C	5.8[c]	19.2[b]
15°C	22.4[b]	43.3[a]
25°C	32.1[a]	39.6[a]

Values followed by a similar letter are not statistically significantly different

3.2 Experiment 2: Effect of Carbon Sources and Temperature Treatment

The effect of carbon sources on the number of secondary somatic embryos was highly significant but had no effects on the percentage of embryo germination (Table 4). The presence of sorbitol and manitol in the culture medium reduced the number of secondary somatic embryos. The minimum number of secondary somatic embryos was observed in manitol (Fig. 3).

A highly significant effect of temperature on the number of secondary somatic embryos and the percentage of embryo germination was also observed (Table 4). Minimum of secondary somatic embryogenesis and maximum of embryo germination were found at 5℃ (Fig. 4).

The interaction effect of carbon sources and temperature on secondary somatic embryogenesis was highly significant but, there was no significant interaction observed on the percentage of embryo germination (Table 4). Having sucrose as carbon source the number of secondary somatic embryos was produced as the highest at 25℃, however, in presence of manitol, there was a steady rise in secondary somatic embryogenesis when temperature rose from 5℃ and 15℃ to 25℃ (Fig. 5). At 5℃ using Sorbitol resulted in a minimum of secondary embryogenesis.

To hardening normal plantlets were cultured in pots containing coco peat and perlite (50:50). 80% of the regenerated plants were developed and acclimatized into young plants (Fig. 6).

Table 3. Interaction effects of abscisic acid (ABA) and temperature on the number of secondary somatic embryos (SSE) produced, and the percentage of germinated embryos

Hormonal treatments	temperature	SSE	Germination (%)
ABA (0) (control)	5℃	10.1[hi]	28.3[def]
ABA (0) (control)	15℃	25.5[bc]	25.0[efg]
ABA (0) (control)	25℃	72.3[a]	33.3[de]
ABA (1 mg/l)	5℃	5.6[j]	15.0[ghi]
ABA (1 mg/l)	15℃	21.8[cde]	61.7[a]
ABA (1 mg/l)	25℃	18.3[efg]	36.7[d]
ABA (1.5 mg/l)	5℃	1.9[jk]	20.0[fgh]
ABA (1.5 mg/l)	15℃	19.5[def]	53.3[ab]
ABA (1.5 mg/l)	25℃	10.7[h]	51.7[abc]
ABA (2 mg/l)	5℃	5.4[jk]	13.3[hi]
ABA (2 mg/l)	15℃	22.9[cd]	33.3[de]
ABA (2 mg/l)	25℃	27.1[b]	36.7[d]

Values followed by a similar letter are not statistically significantly different

Table 4. Analysis of variance of the effect of carbon source and temperature on the number of secondary somatic embryos (SSE) produced, and the percentage of germinated embryos

Source of variation	DF	SSE	Germination (%)
Carbon source	2	3406.83[**]	67.00[ns]
Temperature	2	1201.88[**]	901.44[**]
Carbon source ×Temperature	4	985.10[**]	348.11[ns]
Error	18	7.83	153.29

*ns: non-significant (p>0.05), * significant at p≤0.05, ** significant at p≤0.01*

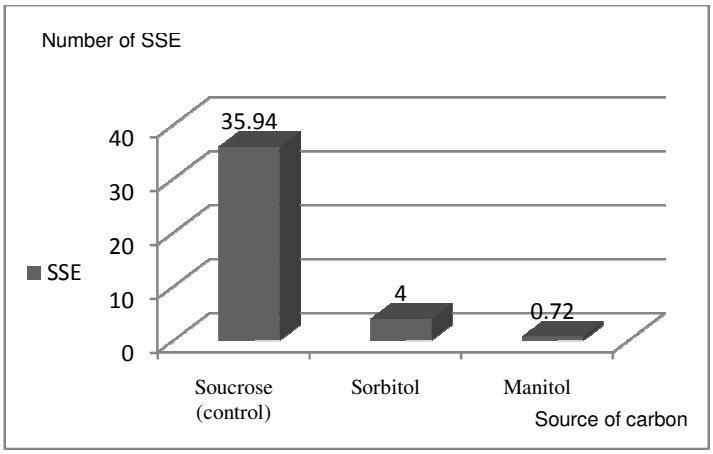

Fig. 3. The effect of carbon source on the number of secondary somatic embryos (SSE) produced

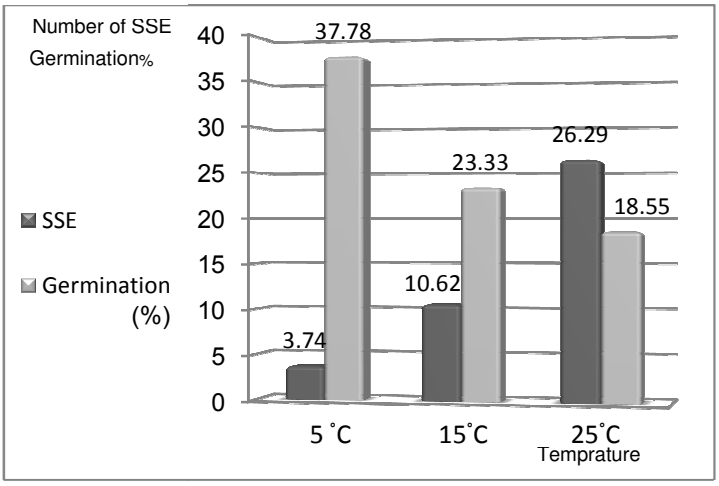

Fig. 4. The effect of temperature on the number of secondary somatic embryos (SSE) produced, and the percentage of germinated embryos (in experiment 2)

4. DISCUSSION

4.1 Effects of Abscisic acid and Temperature Treatment

The control treatment showed the highest value for secondary somatic embryogenesis. Similar results were also reported by [18]. While treatment ABA with 1.5 mg/l of concentration showed the minimum number of secondary somatic embryos. The same results were also reported by [13]. The latter researchers also reported that ABA significantly reduced unwanted recurrent embryogenesis in mature somatic embryos and stated that the frequency of secondary somatic embryos was reduced by ABA compared with the control. ABA applied to embryogenic cell clumps of caraway prevented secondary somatic embryogenesis [19] but secondary somatic embryogenesis occurred in celery cell cultures [20] and also in carrot cell cultures [21] with ABA application. In our study, decrease in somatic embryo induction was observed in presence of ABA with concentration of 1.5 mg/l, so the concentration of ABA was very important for secondary somatic embryogenesis. Torres et al. [22] reported that ABA has the most effect on embryo formation at low concentration. When embryos were treated with 0.2 mg/l ABA they showed profuse secondary somatic embryogenesis [10]. Haddadi et al. [9] found that the optimum concentration of ABA was different among the species of

Brassica. Increasing the concentration of ABA from 0.1 to 7 µM inhibited secondary somatic embryogenesis; however at the concentration of 10 µM, secondary embryogenesis was arrested [23]. The same results were also confirmed by Finkelstein [24].

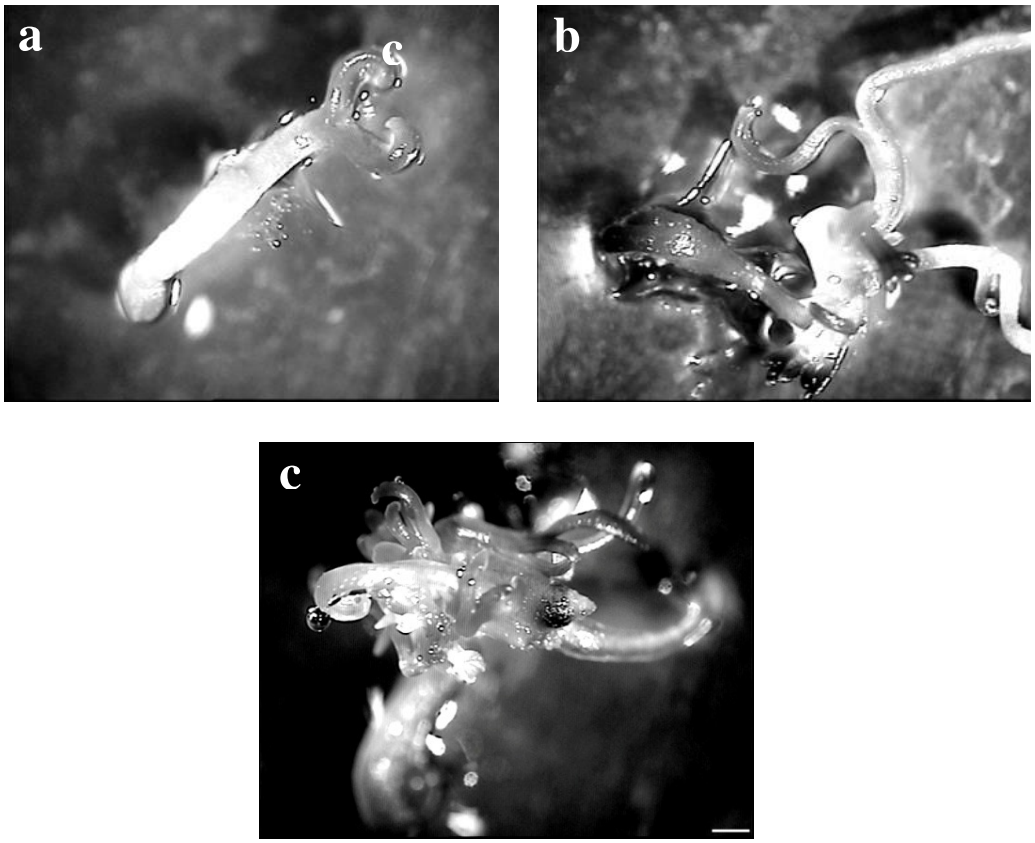

Fig. 5. Effect of manitol and temperature on secondary somatic embryogenesis. (a: minimum of secondary somatic embryogenesis at 5℃, b: secondary embryogenesis at 15℃, c: increased secondary embryogenesis at 25℃). bar=1mm

Fig. 6. Acclimatization of normal plantlets

Medium containing ABA (1.5 mg/l) also showed the highest percentage of germination (Table 2). Nickle and Yeung [25] reported that ABA improved conversion of carrot somatic embryos. Liu et al. [26] also confirmed the same. According to Brown et al. [27] desiccation was needed for higher rate of embryo germination in many plant species and also increased the embryos germination.

At 5℃ the minimum of secondary somatic embryogenesis was observed. Fernandez-Guijarro [14] reported that chilling treatment for 30 days reduced adventitious embryogenesis. At 15℃ and 25℃ temperature, the germination was observed as higher. Gingas and Lineberger [28] indicated that low temperature for maturing somatic embryos to induce their germination has sometimes been unsuccessful.

4.2 Effect of Carbon Sources and Temperature Treatment

Presence of sorbitol and manitol reduced secondary somatic embryogenesis. The minimum number of secondary somatic embryos was observed in manitol into all temperature treatments. Karami et al. [4] and Nadel et al. [20] reported that, in celery, singular embryos can be controlled by manitol. Torres et al. [22] confirmed the same results as well. Due to osmotic potential of mannitol, used as the only carbon source, no secondary somatic embryos were formed [4]. These results are supported by the findings of Ikeda-Iwai et al. [29] and Kamada et al. [30]. Shibli et al. [31] stated that sucrose at 0.2 M promoted higher embryogenesis, while sorbitol and mannitol reduced the embryogenesis significantly and inhibited at 0.4 M concentration. Presence of sucrose stimulated secondary somatic embryogenesis. Karami et al. [4] and Agrawal et al. [23] reported that sucrose in culture medium enhanced the secondary embryogenesis. Daigny et al. [5] observed that in the presence of sucrose, secondary embryogenesis was significantly higher than sorbitol; sorbitol was found to be inefficient in stimulating secondary embryogenesis.

Minimum secondary somatic embryogenesis and maximum germination were obtained at 5℃. Mauri and Manzanera [13] reported that a stratification treatment at 4℃ significantly improved germinating somatic embryos. Yang et al. [32] reported that temperature strongly affected induction frequency of secondary embryogenesis. They stated that high temperature (30℃) is effective in induction of secondary somatic embryos, and low temperature (20℃) is more suitable for further embryo development and plantlet conversion.

5. CONCLUSION

1- ABA concentration has a large effect on secondary somatic embryogenesis. The minimum number of secondary somatic embryos and the highest percentage of germination are obtained from treatment ofABA 1.5 mg/l
2- Sorbitol and manitol reduce secondary somatic embryogenesis while sucrose stimulates it. The minimum number of secondary somatic embryos is observed in manitol.
3- Low temperature (5℃) can be used to reduce secondary somatic embryogenesis. The maximum rate of secondary somatic embryogenesis is obtained at 25℃.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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