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Optimization of *in vitro* sterilization protocol for obtaining contamination-free cultures of *Tilia platyphyllos*

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Abstract. *Payamnour V, Ghasemi Bezdi K, Mehrdad M, Ahmadi A. 2014. Optimization of in vitro sterilization protocol for obtaining contamination free cultures of Tilia platyphyllos. Nusantara Bioscience 6: 7-12. Tilia platyphyllos* is one of threatened species of Caspian forests. Tissue culture techniques are applied for culture, regeneration and genetic resources preservation. Utilizing an accurate sterilization procedure is important to reduce the cost, time and energy. The aim of this present study was to provide optimization of *in vitro* sterilization protocol to obtain contamination-free cultures of *T. platyphyllos*. Explants were collected randomly from the best individuals of *T. platyphyllos*, which were located in Tooskestan forest of Gorgan, Iran. Results revealed that the optimum protocol for sterilization was when explants were exposed in pre-sterilizing solution of 600 mg L^{-1} ascorbic acid, 4 g L^{-1} captan fungicide and 5% commercial sodium hypochlorite (NaOCl) solution (5% Cl activated) for 20 minutes and then explants were exposed in sterilizing solution containing 600 mg L^{-1} ascorbic acid and 10% sodium hypochlorite.

Key words: Explants, pre-sterilization, sterilization, *Tilia platyphyllos*

INTRODUCTION

The Linden trees, from Tilia genus belong to the Salicaceae family and Malvales order (Sabeti 1965; Sadati 2002). Generally, Tilia genus includes woody plants (about 30-40 species) and 10 species of Linden are distributed in moderate areas of northern parts of the earth, 4 of which 4are found in Europe (Magherini and Nim 1993). *Tilia platyphyllos* is the only species of *Tilia* genus in Iran, which is one of the important species and has wide distribution (Mozafarian 1998). Natural site of *T. platyphyllos* is located in the northern forests of Iran (Sabeti 1965).

Tilia platyphyllos is hydro-phobe and hard on the land that prefers cold or moderate climate and also, distributes its root system (Kunneman and Albers 1991; Haller 1995). Its longevity is average (Moghadasi 2001) that in some species, reaches to 500-1000 years (Kunneman and Albers 1991; Haller 1995). Generally, the fruits are grayish, nut-shaped and oval-shaped capsule that matures in autumn to winter, but may also remain on the tree. Each includes a pod that contains a seed (Brinkman 1974; Pigott and Huntley 1981). External coating contains a layer with low fiber.

Linden can be included as a medical plant because its flower and leaves have medical usage (Moghadasi 2001). The honey produced by bees extracting its nectar is very famous in the world (Haller 1995). Its wood has the highest efficiency in the sculpture due to its beauty and softness. Usually, these trees are planted in many parks and roadsides in Europe, because they grow so fast and make the landscapes beautiful (Edlin 1976). *Tilia platyphyllos* can be planted with beech or hornbeam for rehabilitation of destroyed northern forests of Iran (Sadati et al. 2007).

Due to the many problems that exist in germination of *T. platyphyllos* seeds, techniques such as tissue culture are important for restoration and conservation of this genetic resource (Sadati 2002; Shahrzad 1997; Hartman et al. 1990; Bewley and Black 1985). The first study of tree tissue culture was conducted in the mid-1920s (Ghasemi Bezdi and Ahmadi 2009). Due to the presence of many microorganisms in the explants, contamination reveals in cultures despite the use of sterilization methods (Skirvin et al. 1999; Martinez and Wang 2009).

Tilia platyphyllos is valuable broad-leaved forest tree that much research should be done in tissue culture because the seed germination is negligible due to the hard seed coat. The first reports of the *T. platyphyllos* tissue culture was presented by Barker (1969). He proceeded through callus culture of *Tilia americana*. Chalupa (1984) was one of those who were very active in *T. platyphyllos* tissue culture and has done much research in this field. At first, he worked on *Tilia cordata*, and used the lateral buds to grow *in vitro* culture, in 1984.

Due to many difficulties in plant tissue culture, especially in forest trees, optimizing the sterilization procedure of plant material seems necessary (Osterc 2004). Analogous investigations were carried out about sterilizing forest trees explants and achieving standard method for sterilization (Osterc 2004; Meier-Dinkel 1986; Preil 1997; Langens-Gerrits 1998). In Linden, due to the difficulties in propagating even through laboratory procedures, some

researches have been done (Shahrzad 1997; Mehrdad et al. 2007, 2011). Shahrzad (1997) harvested the twigs of *T. platyphyllos* from three provinces of Tehran (botanic garden), Mazandaran (Waz forests) and Gilan (Shafaroud forest) in different seasons. She found that the best method for sterilization of this species was washing explants with 70% ethanol, with 1 and 2% sodium hypochlorite (NaOCl) and 1% $HgCl_2$ and the best time to harvest buds was from the fall to early winter. Also, she stated that suitable buds for culturing were apical buds or near the terminal end buds. Also, Mehrdad et al. (2011) studied the micropropagation of *Tilia begonifolia*, *in vitro*.

Therefore, this experiment was undertaken with an objective of finding out the optimized *in vitro* sterilization protocol to obtain contamination-free cultures of *T. platyphyllos*.

MATERIALS AND METHODS

In the present research, twigs of *Tilia platyphyllos* were used as a source plant. Twigs were harvested from Tooskestan forest of Gorgan, Iran, transferred to the laboratory and were cleaned. At first, water and soap water were used to eliminate surface pollution. In order to reduce pollution, pre-sterilizing was carried out, in which explants were set at a definite volume of sterilized water (dependent on explants volume) with 300 and 600 mg L^{-1} ascorbic acid, 2 and 4 g L^{-1} captan (1, 2, 3, 6-tetrahydro-N-trichloromethyl thiophthalmide) fungicide and 5% and 10% commercial sodium hypochlorite (NaOCl) and then were shacked in 10, 20 and 30 minutes (Table 1). Then, explants were exposed to running water for washing.

Table 1. Type and amount of used compound at pre-sterilizing solutions

Code of pre-sterilizing solution	Ascorbic acid (mg L^{-1})	Captan fungicide (g L^{-1})	Commercial sodium hypochlorite (%)
1	300	2	5
2	300	2	10
3	300	4	5
4	300	4	10
5	600	2	5
6	600	2	10
7	600	4	5
8	600	4	10

Then, to achieve suitable sterilizing of explants and successful culture, the effects of different concentrations of commercial sodium hypochlorite (0, 10, 20, 30, 40 and 50%) and time of sterilizing (5, 10 and 20 minutes) were investigated. Likewise, different concentrations (0, 300 and 600 mg L^{-1}) of ascorbic acid in the media were studied to eliminate the harmful effects of phenol compound that exist in explants at micro-propagation culture. The MS medium (Murashige and Skoog 1962) containing 30 g L^{-1} sucrose and 6.7 g L^{-1} agar was used for all experiments. The pH of the medium was adjusted to 5.7 before autoclaving, and ascorbic acid added to the medium after autoclaving.

The buds were used as explants for accessing best protocol to obtain contamination free-cultures of *T. platyphyllos*. Different treatments of time and material were used for pre-sterilizing and sterilizing of explants. Then, viability (in percent) of explants was investigated after sterilizing and transferring to a MS basal medium using a completely randomized design with three replications. Data obtained from this research were analyzed statistically using one-way analysis of variance (ANOVA) and the significant differences among means were assessed using Duncan's multiple range test with MSTATC software.

RESULTS AND DISCUSSION

Variance analysis results of different factors in order to pre-sterilizing of explants, ascorbic acid amounts at sterilizing solutions, necessary time for sterilizing treatments, sodium hypochlorite concentrations of sterilizing solutions and interaction effects of these factors on viability (in percent) of explants are shown in Table 2. According to the results, viability (percent) of explants under pre-sterilizing factors, ascorbic acid amount, sterilizing time, sodium hypochlorite concentration and their interaction effects was significant ($p \leq 0.01$).

Table 2. Variance analysis of pre-sterilizing effect, ascorbic acid, time of pre-sterilizing, sodium hypochlorite concentration and their interaction effects on viability percent of *T. platyphyllos* explants

Source of variation (S.O.V)	Degree of freedom (df)	Mean of Squares (MS)
Pre-sterilizing (P)	7	0.220**
Ascorbic acid (S)	2	0.192**
P×S	14	0.030**
Time of sterilizing (T)	2	0.788**
P×T	14	0.038**
S×T	4	0.059**
P×S×T	28	0.008**
Sodium hypochlorite concentration (N)	5	2.111**
P×N	35	0.035**
S×N	10	0.024**
P×S×N	70	0.005**
T×N	10	0.057**
P×T×N	70	0.006**
S×T×N	20	0.008**
P×S×T×N	140	0.003**
Error	864	0.003
Total	1295	

Note: **): significantly different at the 1% level.

The presented data in Table 3 showed the results obtained from means comparisons of pre-sterilizing factor effect as ascorbic acid amount and their interaction with *T. platyphyllos* explants viability by Duncan's multiple range

test at the 5% level. As presented, factors were investigated at five different groups. No. 7 pre-sterilizing treatment was the best (first group) and showed 17.6% viability, which consisted of 600 mg L^{-1} Ascorbic acid, 4 g L^{-1} captan fungicide and 5% commercial sodium hypochlorite. No. 8 treatment showed 16.1% viability and placed in second group Duncan's multiple range test which consisted of 600 mg L^{-1} ascorbic acid, 4 g L^{-1} captan fungicide and 10% commercial sodium hypochlorite. Table 3 clearly indicated that these two groups revealed higher viability than other groups. In contrast, No. 1 pre-sterilizing treatment showed the minimum percentage of explants viability (6.7%) which contained at least concentration of ascorbic acid, captan fungicide and sodium hypochlorite.

The results of mean comparison of ascorbic acid amount effect on *T. platyphyllos* explants (Table 3) showed that under study factors set at two different groups with the highest percentage of explants viability (13.8%) were observed when higher amount of ascorbic acid (600 mg L^{-1}) were used. The results clearly indicated that, 0 and 300 mg L^{-1} ascorbic acid were non-significant and placed in the second group with 10.1 and 10.2% viability, respectively. From the mentioned results, it can be concluded that with the increase of ascorbic acid until 300 mg L^{-1}, viability increased a little, but the viability showed significant increase when ascorbic acid changed from 300 to 600 mg L^{-1}.

The interaction between pre-sterilizing factor and ascorbic acid in sterilizing solution on *T. platyphyllos* explants viability (Table 3) revealed that data were classified into 18 groups according to viability. The highest viability percentage (24.1) at No. 7 pre-sterilizing treatment was observed that contained 600 mg L^{-1} ascorbic acid, 4 g L^{-1} captan fungicide and 5% commercial sodium hypochlorite and sterilizing solution with 600 mg L^{-1} ascorbic acid showed significant increase viability than other treatments. On the other hand, No. 1 pre-sterilizing treatment containing 300 mg L^{-1} ascorbic acid, 2 g L^{-1} captan fungicide and 5% commercial sodium hypochlorite and sterilizing solution without ascorbic acid showed the minimum amount of viability.

Table 3. The means comparison of explants viability (%) of *T. platyphyllos* in relation to pre-sterilizing factor effect, ascorbic acid amount and their interactions

Pre-sterilizing code	Ascorbic acid (mg L^{-1})			Mean of pre-sterilizing factor
	0	300	600	
1	4.4^{n}	6.4^{mn}	9.3^{i-l}	6.7^{e}
2	7.4^{lm}	10.1^{k-h}	11.0^{g-k}	9.5^{d}
3	9.9^{h-k}	7.5^{lm}	12.9^{d-g}	10.1^{d}
4	8.3^{klm}	11.5^{f-i}	14.8^{cd}	11.5^{c}
5	7.3^{lm}	8.4^{klm}	12.5^{efg}	9.4^{d}
6	9.4^{h-l}	9.0^{jkl}	11.8^{fgh}	10.1^{d}
7	15.4^{c}	13.5^{c-f}	24.1^{a}	17.6^{a}
8	18.9^{b}	15.3^{c}	14.3^{cde}	16.1^{b}
Mean of ascorbic acid	10.1^{b}	10.2^{b}	13.8^{a}	-

Note: Means separated by Duncan's multiple range test at alpha = 0.05. Means with the common letters were not significantly different (at the 5 % level) and they were in the same group.

The results obtained from a mean comparison of sterilizing time effect on *T. platyphyllos* explants viability are presented in Table 4. Time of 5 minutes for sterilizing with 15.4% viability placed in the first group, 10 minutes with 11.9% in the second group and 20 minutes with 6.9% viability in the third group. Therefore, it could be concluded that the viability percentage of explants decreased with the increasing time of sterilizing, and existing material at a sterilizing solution had a negative effect on explants viability. Thereupon, it is better to reduce the treating time with sterilizing solutions, if it is possible.

The data of the interaction between pre-sterilizing factors and time of exposing at sterilizing solution on *T. platyphyllos* explants viability are shown in Table 4, that indicated the highest value of free-contamination explants (24.8% and 24.3%, respectively) were obtained when explants were at a sterilizing solution for 5 minutes (No. 7 and No. 8 pre-sterilizing codes). It was evident that the pre-sterilizing solution had less effect than sterilizing time. On the other hand, with the increase of captan fungicide and ascorbic acid in pre-sterilizing solution, viability increased, but increasing the time more than optimum time, negative effects of solution increased and viability decreased extremely. As, with increasing the time from 5 to 10 minutes, viability decreased and with increasing the time to 20 minutes, viability decreased even more.

Table 4. The means comparison of explants viability (%) of *T. platyphyllos* in relation to pre-sterilizing factor effect, time of sterilizing and their interactions

Pre-sterilizing code	Time of sterilizing (minute)			Mean of pre-sterilizing factor
	5	10	20	
1	7.3^{ij}	6.9^{ij}	6.1^{jk}	6.7^{e}
2	10.6^{fgh}	11.4^{efg}	6.5^{j}	9.5^{d}
3	12.8^{def}	12.5^{fgh}	6.7^{j}	10.1^{d}
4	15.5^{c}	12.6^{def}	6.6^{j}	11.5^{c}
5	14.6^{cd}	9.4^{gh}	4.2^{k}	9.4^{d}
6	13.5^{cde}	10.2^{gh}	6.5^{j}	10.1^{d}
7	24.8^{a}	18.5^{b}	9.6^{gh}	17.6^{a}
8	24.3^{a}	15.2^{c}	9.0^{hi}	16.1^{b}
Mean	15.9^{a}	11.89^{b}	6.88^{c}	-

Note: Means separated by Duncan's multiple range test at alpha = 0.05. Means with the common letters were not significantly different (at 5% level) and they were in the same group.

The data in Table 5 showed the mean comparison of the sodium hypochlorite effect on viability of *T. platyphyllos* explants. The viability of explants at 10% and 20% sodium hypochlorite was 25.6% and 19.5% at sterilizing solution, respectively. Whereas, no explants were alive in sterilizing solution without sodium hypochlorite and 100% of them were contaminated. It is quite clear that increasing commercial sodium hypochlorite, explants viability decreased and too much of commercial sodium hypochlorite might cause the viability till zero. Thus, it can be concluded that sodium

hypochlorite is necessary, but should be used in minimum amount.

Concerning the interaction between the pre-sterilizing factors and the sodium hypochlorite amount on explants viability (Table 5), the data indicated that the highest viability (40.9%) was related to pre-sterilizing No. 7 and 10% sodium hypochlorite. Viability difference between the first and second groups showed the effect of commercial sodium hypochlorite in pre-sterilizing solution that demonstrated the minimum necessity of using commercial sodium hypochlorite at pre-sterilizing solution. To study the negative effects of commercial sodium hypochlorite we increased up to 50% at sterilizing solution and it caused a remarkable reduction of explants viability. In all pre-sterilizing method, without using sodium hypochlorite in sterilizing solution, there was not any viability observed.

Table 5. The means comparison of explant viability (%) of *T. platyphyllos* in relation to pre-sterilizing factor effect, sodium hypochlorite and their interactions

Pre-sterilizing code	Sodium hypochlorite (%)						Mean of pre-sterilizing factor
	0	10	20	30	40	50	
1	0^{t}	15.9^{ni}	12.41^{jkl}	5.5^{o-r}	4.2^{p-s}	2^{st}	6.7 ^{e}
2	0^{t}	21.8^{ef}	14.4^{hij}	9.63^{lmn}	7.96^{mno}	4.14^{rst}	9.5 ^{d}
3	0^{t}	2^{fg}	17.2^{gh}	11.1^{klm}	8.3^{mno}	3.7^{grs}	10.1 ^{d}
4	0^{t}	26.7^{d}	20.2^{fg}	3.3^{ijk}	6.8^{n-q}	2.2^{rst}	11.5 ^{c}
5	0^{t}	2^{fg}	15.7^{hi}	10.9^{klm}	7^{nop}	2.8^{rst}	9.4 ^{d}
6	0^{t}	23.5^{e}	17.6^{gh}	11^{klm}	6.7^{n-q}	1.7^{st}	10.1 ^{d}
7	0^{t}	41^{a}	30.4^{c}	2^{fg}	12^{jkl}	2.6^{rst}	17.6 ^{a}
8	0^{t}	36^{b}	28^{cd}	2^{fg}	11^{klm}	2.4^{rst}	16.1 ^{b}
Mean of sodium hypo-chlorite	0^{f}	25.6^{a}	19.5^{b}	12.7^{c}	8^{d}	2.6^{e}	-

Note: Means separated by Duncan's multiple range test at alpha = 0.05. Means with the common letters were not significantly different (at the 5% level) and they were in the same group.

As for the interaction effect between ascorbic acid treatment and time of exposing explants to sterilizing solution on explants viability (Table 6), the data indicated that an increase of ascorbic acid in sterilizing solution had a direct effect (increasing viability) and time of exposing explants to sterilizing solution had the reverse effect (reducing viability) on explants viability. The highest viability (19.6%) was observed in 5 minutes-exposure to the sterilizing solution containing 600 mg L^{-1} ascorbic acid. Also, the minimum viability (5.7%) was observed in 20 minutes-exposure to the sterilizing solution containing 300 mg L^{-1} ascorbic acid.

The data obtained from the interaction between ascorbic acid amount and sodium hypochlorite at sterilizing solution on explant viability (Table 7) pointed out that the highest viability (31.1%) was related to 600 mg L^{-1} ascorbic acid and 10% sodium hypochlorite. It can be concluded that the minimum usage of commercial sodium hypochlorite and the maximum amount of ascorbic acid (600 mg L^{-1}) was necessary to increase explant viability. The explants in sterilizing solution without using sodium hypochlorite did not show any viability.

Table 6. The means comparison of explant viability (%) of *T. platyphyllos* in relation to ascorbic acid, time of sterilizing and their interactions

Time of sterilizing (minute)					Mean of pre-sterilizing factor
		5	10	20	
Ascorbic acid (mg L^{-1})	0	11.8^{c}	11.2^{cd}	7.4^{e}	10.1 ^{b}
	300	14.7^{b}	10.1^{d}	5.7^{f}	10.2 ^{b}
	600	19.6^{a}	14.3^{b}	7.5^{e}	13.8 ^{a}
Mean		19.8^{a}	11.9^{b}	6.9^{c}	

Note: Means separated by Duncan's multiple range test at alpha = 0.05. Means with the common letters were not significantly different (at 5% level) and they were in the same group.

Table 7. The means comparison of explant viability (%) of *T. platyphyllos* in relation to ascorbic acid, sodium hypochlorite and their interactions

Sodium hypochlorite (%)								Mean of pre-sterilizing factor
		0	10	20	30	40	50	
Ascorbic acid (mg L-1)	0	O^{h}	22.9^{b}	17.3^{c}	11.5^{e}	6.9^{f}	2.2^{g}	10.1 ^{b}
	300	0^{h}	22.8^{b}	17.6^{c}	11.1^{e}	7.2^{f}	2.4^{g}	10.2 ^{b}
	600	0^{h}	31.1^{a}	23.5^{b}	15.4^{d}	7.8^{e}	3^{g}	13.8 ^{a}
Mean		0^{f}	25.6^{a}	19.5^{b}	12.7^{c}	8^{d}	2.6^{e}	

Note: Means separated by Duncan's multiple range test at alpha = 0.05. Means with the common letters were not significantly different (at the 5% level) and they were in the same group.

Table 8. The means comparison of explant viability (%) of *T. platyphyllos* in relation to time of sterilizing (minute), sodium hypochlorite and their interactions

Sodium hypochlorite (%)								Mean of pre-sterilizing factor
		0	10	20	30	40	50	
Time of sterilizing (minute)	5	0^{h}	31.6^{a}	24.5^{b}	17.8^{d}	14^{e}	4.4^{g}	15.8 ^{a}
	10	0^{h}	25.8^{b}	21.8^{c}	13.96^{e}	7.2^{f}	2.6^{g}	11.9 ^{b}
	20	0^{h}	19.4^{d}	12.1^{e}	6.25^{f}	2.7^{g}	0.8^{h}	6.9 ^{c}
Mean		0^{f}	25.6^{a}	19.5^{b}	12.7^{c}	8^{d}	2.6^{e}	

Note: Means separated by Duncan's multiple range test at alpha = 0.05. Means with the common letters were not significantly different (at the 5% level) and they were in the same group.

The results showed in Table 8 revealed that the highest percentage of viability (31.6%) was related to treatment with 10% sodium hypochlorite for 5 minutes. Other treatments pointed out that the time of sterilizing, increased the negative effects of sodium hypochlorite in sterilizing solution and with the increasing time and sodium hypochlorite, viability decreased (severely).

Also, the results of the mean comparison of interaction effects between pre-sterilizing factors, ascorbic acid amounts, time of sterilizing and sodium hypochlorite amounts in sterilizing solution (the data were not shown)

implied that No. 7 pre-sterilizing with 600 mg L^{-1} ascorbic acid in sterilizing solution containing 10% sodium hypochlorite for 10 minutes and 5 minutes showed 68.3% and 65% viability, respectively and they were the optimum treatments for sterilization of *T. platyphyllos* explants. Meanwhile, Viability was not observed in treatments without sodium hypochlorite and also with high concentration of sodium hypochlorite (over 50%).

Discussion

Tilia platyphyllos is one of the valuable and high longevity (sometimes until 1000 years) plant species that exists individually under ecological and economical value of beech and oak trees (Sabeti 1965). These typical trees are planted in parks and streets in Europe and America. The leaves of this tree which are processed in tea and the honey produced by bees which extract nectar from their flowers are popular in the world, as well (Sadati et al. 2007). This species have been distributed from 100 to 1600 meters and even, sometimes, to 2400 meters above sea level (Sheikh Al-Eslami and Namiranian 2002). Because of the wide range of altitude distribution and genetic variability preservation of this species and efforts in preserving the natural structure of Iran forests, the importance of this species is clear for everyone. Many studies demonstrated that producing *T. platyphyllos* seedlings have encountered serious problems such as weak germination in nurseries until now. It was an interesting fact that although mature seeds are healthy, their germination is low (Tabari et al. 2007). Although there are problems, using biotechnology tools such as tissue culture might be a proper solution. Tissue culture and regeneration of *T. platyphyllos* are difficult and the success of regeneration is very low. Hence, regeneration may also be an effective technique to multiply stock plants of this species.

One of important problems in case of *T. platyphyllos* or every plant, which their explants collected from *in vivo* special forest areas, is the control of contamination at *in vitro* condition. It is necessary to note, that pre-sterilizing is preliminary stage that can reduce contamination of explants *in vitro*. Although, this stage was conducted in laminar airflow, which is not sterile, but this contamination could not be included in the data in the independent stage for calculations. According to this point and achieved experiments in this study, it could not eliminate pre-sterilizing effect from calculations and considered it as one of dependent on sterilizing stage. Therefore, with used material at pre-sterilizing, eight treatments of pre-sterilizing were adopted and included in this investigation. The present study demonstrated that the best sterilization method was using 4 g L^{-1} captan and 600 mg L^{-1} ascorbic acid and 5% v/v commercial sodium hypochlorite. In general, increasing chlorox concentration increased percentage of contamination-free explants that presented results were coordinated with those of Abou Dahab et al. (2005). Sharifkhani et al. (2011) demonstrated that the most suitable concentration of sodium hypochlorite for sterilization is 5%, which agrees with these results.

A wide range of contamination has been indentified in plant tissue culture that causes considerable economical losses (George 1993; Altan et al. 2010). Fungi are from contaminations in plant tissue culture that may arrive with explants, airborne or enter a culture (Babao et al. 2001; Altan et al. 2010). In the present study, a fungicide was used to reduce fungal contaminations observed during the research. Observed fungi were identified and were reported in the other research that consisted of *Penicillium* sp., *Fusarium* sp. and *Alternaria* sp. (Mehrdad et al. 2010). Shahrzad (1997) reported that using fungicide reduced remarkably fungal contaminations, which agrees with these results.

The effectiveness of sterilization may depend on the type, concentration and time of treatment with the sterilant (Roxas et al. 1996; Tomaszeska-Sowa and Figas 2014). The best sterilization method was found for a solution containing 600 mg L^{-1} ascorbic acid and subsequently, 10% v/v commercial sodium hypochlorite while the optimal time was 5 minutes. The explant viability decreased with the increment of sterilization time and materials in sterilizer solution that have a negative effect on explant viability. Researchers working on sodium hypochlorite concentration and percentage of the surviving explants (Sharifkhani et al. 2011) agree with mentioned results. Also, Researchers working with NaOCl reported that using sodium hypochlorite for 5-10 minutes was considered a suitable time (Miller and Lipschutz 1984; Naik and Chandra 1993; Gopal et al. 1998; Villafranca 1998; Badoni and Chauhan 2010).

Due to poisonous effects resulted from high concentration of sodium hypochlorite, all explants except complete seeds that contained embryo preservative membrane turned yellow and dried after several days. Preservative membrane causes material transferring, slowly. Sodium hypochlorite is necessary, but it should be used at least amount. Increment of sodium hypochlorite concentration will decrease the viability of *T. platyphyllos* explants. Similar results were recorded by Shahrzad (1997) using sodium hypochlorite and sterilizing time, but in that project $HgCl_2$ was used. However, Shahrzad (1997) reported that the optimum concentration of sodium hypochlorite 1% and 2%, but in this study, the best concentration of this sterilizer solution was 0.5%.

CONCLUSION

In sterilization of *T. platyphyllos*, it was concluded that superabundant of phenolic compound was reduced by using ascorbic acid. The optimum protocol was using pre-sterilizing solution of 600 mg L^{-1} ascorbic acid, 4 g L^{-1} captan fungicide and 5% commercial sodium hypochlorite (NaOCl) solution for 20 minutes. The results of present research will be applicable for propagation in large scale, conservation, breeding and further genetic improvement programs.

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