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Micropropagation of Juniper (*Juniperus polycarpos*): Optimizing surface sterilization pretreatments can play key role in suppression of contamination and browning of shoot tip explants

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ABSTRACT

The juniper (Juniperus *polycarpos*) is enable to growth at the extremist of the geology, climatology and hydrology condition. The emptiness seed, embryo degeneration and seed dormancy are problems which necessitate the application of biotechnological technique to propagate mentioned juniper plant *in vitro*. Obtaining high efficiency of surface sterilization in order to prevent explants contamination and browning is necessary to success at initiate stage of juniper micropropagation. So, the effects of formulated of hypochlorite sodium, mercury chloride and ethanol as surface sterilization treatments on shoot tip explants of juniper were investigated. Optimized surface sterilization had a significantly effect on decrease contamination and browning in shoot tip explants ($P<0.01$). The results showed that surface sterilization treatments inhibited microbial or fungal contamination and browning of explants which can be minimized by optimizing mercuric chloride pretreatments. The formulated duration and concentration of hypochlorite sodium not only had no appreciable suppression effect on the contamination and browning in explants but also the browning extension in culture medium like same control was accelerated. It was found that mercuric chloride sterilization had distinguish or effective more than hypochlorite sodium on decrease contamination or browning. In contrast, lowest contamination or browning in culture medium and explants was displayed by formulated mercuric chloride. Therefore, the results suggested that in tissue plants if the aim is micropropagation of Juniperus *polycarpos*, elimination browning or having contaminated-free plants could be get in initiate state by using optimized mercuric chloride (Ethanol 70% at 1 min- sterile distilled water (SWD)- $HgCl_2$ 0.1% at 3 min-SDW) as surface sterilization.

INTRODUCTION

Junipers are evergreen shrubs or trees belonging to the family *Cupressacea*. The genus Juniperus consists of 67 species and 37 varieties. These plants grow in areas characterized by persistent drought and arid climate with high temperature ranges (El-Bana et al. 2010). In Iran, the geographic distribution of juniper includes the Alborz Mountains between Chaloos and Tehran, the Khorasan forest in the Hezar-masjed Mountains, as well as the forests of Bojnoord, Kalat, Bakhtiari, Kerman and Shahkooh of Baluchistan. Between the six junipers species presented in Iran, the most abundant are Juniperus *polycarpos* and J. excels (Ahani et al. 2013). The Juniperus *polycarpos* occurs in the southern part of Khorasan Razavi, usually at high altitudes, over 1000 m, which grown in areas characterized by sandy rocks soil, limestone soil with high pH and poor nutrition elements, persistent drought, extremely cold and arid climate with high temperature ranges. It has been reported that junipers are enable to growth at the extremist of the geology, climatology and hydrology condition (Earle 2013). However, it can be suitable option for preventing soil erosion, windy erosion, and desert

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greening in dry region of Iran country. Addition to, Juniperus species are used for many purposes including landscaping, wood and medicinal purposes to treat diarrhea (Qnais et al. 2005), to prepare leaves and berries mixtures that act as an oral hypoglycaemic agent (Amer et al. 1994), and to inhibit effects against bacteria and fungi by oils isolated from their leaves (Mazari et al. 2010).

Emptiness seed, not synchronize anther distribution and fertilize, high longevity of seed dormancy, lack of pollination, low pollen viability, and embryo degeneration are important causes for reducing seed production in Juniperus which led to sexual propagation has very difficult (Ortiz et al. 1998). Furthermore, it is reported for the genus Juniperus like J. *communis* and J. *sabina* L. that seed quality tends to decrease in drier and or colder regions and only very small percentage of the collected cones contain viable seeds (Wesche et al. 2005). J. *polycarpos*, similar to other Juniperus species, does not have a high rate of plant production through seed germination. furthermore, it is not efficiently propagated by traditional methods including cutting as vegetative propagation due to low successful and results are extremely inconsistent and not reproducible (Momeni et al. 2015). Also, conventional breeding of woody trees is a slow and difficult process due to elevated levels of heterozygosity and extended regeneration cycles which in Iran, the cycle of juniper seed production is usually four to eight years (Sriskanndarajah et al. 1994). These problems necessitate the application of biotechnological tools to propagate juniper plants in vitro.

Plant tissue culture technique had developed as a biotechnological tool to improve the propagation of many plants. Therefore, to enhance vegetative propagation in junipers, choosing a tissue culture system based on micropropagation technique as alternative mean can be best option to conserve unique species. Through micropropagation, rare genotypes could be cloned; thereby, the risk of rare populations of juniper germplasm being lost due to problems mentioned above could be eliminated (Momeni et al. 2015). The success of the initial stage of micropropagation is influenced by different parameters. The critical opinions for the effective in vitro culture establishment from juniper, similar to other woody plant are connected with the efficacy of sterilization of collected explants and the prevention of phenol-induced browning of explants which these dependent on choice of explant, the sterilization method, the in vitro physical and chemical conditions (Pérez-Tornero and Burgos 2007; Ríoz Leal et al. 2007). During Stage 1 of micropropagation explants are transferred to in vitro culture, which means that they should be surface sterilized so that they can survive and grow under artificial conditions. To prosperous initiation of an aseptic in vitro culture, stock plants should be selected and these or their parts often have to be pretreated. Physical or chemical pretreatments as surface sterilization can be necessary for decreasing the contamination of the explants and for improving the growth of explants in subsequent in vitro conditions. (George and Debergh 2008). Some studies has been investigated associated to micropropagation of different species of junipers as well as the rare researches has been reported dedicated to optimizing different surface sterilization methods of juniper explants and often it as treatments have not demonstrated, thus, it has not occurred to statistical analysis (Momeni et al. 2015). The main objective of this paper was to develop an optimized surface sterilization protocol for adult J. *polycarpos*. We tested various physical and chemical pretreatment on shoot tip explants derived from material collected from 5-8 years-old junipers trees. Emphasis was put on attaining the greatest satisfactory response from optimized surface sterilization on the shoot tip explants of J. *polycarpos* to decrease contamination rate and to reduce browning in order to achieve to more success in initiate state of juniper micropropagation.

MATERIALS AND METHODS

Plant material and explant preparation

Shoots with buds (20-30 cm) were obtained from four individuals of Juniperus *polycarpos* trees about seven years old grown in natural habitat located in Shirvan, Khorasan Razavi, Iran. To provide plant materials for micropropagation and following organogenesis experiments, shoot tip explants were collected during two different periods, that is, December 2014 (winter season) and March 2015 (spring season). To prepare explants, plant materials chosen were first exposed to running tap water for 2 h and then for remove effect of explant size on contamination rate, browning phenomenon, and also to eliminate their overlap effect companied with surface sterilization, all of the explants were desiccated in same size (4 cm from shoot tip branches) containing 3-5 lateral buds.

Surface sterilization treatments

The surface sterilization of explants were performed with optimized formulate protocol (duration, kind and concentration of chemical antiseptic) from hypochlorite sodium (NaOCl), mercuric chloride ($HgCl_2$) and ethanol (C_2H_6O) which described in Table 2. The shoot tip explants were exposed to surface sterilization treatments as followed in Table 2. The write down for assaying of contamination and browning was carried out after 33 day.

Basal medium and explant culture

To culture of shoot tip explants, the MS medium (Murashige and Skoog 1962) supplemented by 1.5 ml naphthalene acetic acid (NAA), 0.5 ml kinetin, 30 gl^{-1} sucrose and 8 gL^{-1} agar was applied. The shoot tip explants (microshoots) cultured in tube tests containing 20 ml MS medium. Explants maintained in a medium without antioxidants such as ascorbic acid or citric acid and activated charcoal. All media were autoclaved for 15 min at 121°C and 1.1 kg cm^{-2}. The pH of the medium was adjusted to 5.8 before autoclaving. Cultures were grown at 21°C with 16 h photoperiods provided by white fluorescent lamps with an intensity of 5000 lux.

Contamination assay and browning measurement in medium and explants

In order to check the browning due to phenol oxidation in medium and shoot tip explants, they were exposed to formulated protocol from chemical substances as sterilization treatments. The browning intensity of medium scored from 1 to 5 numbers by observations of researcher and also the browning intensity of shoot tip explants were evaluated and they were expressed as percentage. Consequently, the contamination rate of explants including bacterial and fungi were checked by researcher during cultivation and scored from 0 to 4 numbers.

Statistical analysis

The experimental data were analyzed according to factorial design on the basis of Completely Randomized Design (CRD) with ten treatments with three replications (containing one explant per tube which has existed totally 4 tubes tests). Values presented were means of three replicates. Data were subjected to analysis of variance (ANOVA) using MSTAT-C software. The means were compared by least significant difference (LSD) test to analyze the difference between treatments and intervals at 99% confidence level of each variable.

RESULTS AND DISCUSSION

The survival of explants depends on their rate of microbial contamination and of explant browning, which pertain not only to the explants used for culture initiation but also to the physiological stage of mother plants and to the season when explants are collected (Dobránszki et al. 2000). However, Even though in vitro cultures can be established at any time of the year, success depends on the season for collection of explants. Contamination of explants was found to be less if they were collected during spring or summer compared to explants collected in autumn or winter (Modgil et al. 1999). In our research, to counteract impact of spring collected explants on decrease contamination rate, at first the effect of explants collected season was investigated. The results revealed that the contamination and browning of explants were less and growth more rapid if they were collected in spring (March 2015) as compared to explants collected in winter on December 2014 (data not shown). Similar observations were recorded earlier (Webster and Jones 1991; Mahna and Motallebi-Azar 2007). Furthermore, the collected explants in winter were subjected to sterilization treatments.

The surface sterilization can be play an important role in the production of healthy, browning or contaminated-free plants. The effects of formulated surface sterilization treatments on contamination rate and browning of shoot tip explants was studied. The impacts of formulated surface sterilization (kind, duration, and concentration of chemical substances) were very important not only on the effectively decrease microbial contamination, but also on the prevention of browning appears in shoot tip explants ($P<0.01$; Table 1). The results of mean comparison from LSD test determined that furthest contamination rate of explants (4 score) were observed in control (A treatment) in which treated by ethanol 70% at 1 min-sterile distilled water followed by B (3.75 score) and H (3.5 score) treatments, respectively. In contrast, the lowest contamination rate was recorded in M (1 score) and L treatments (2 score), respectively, in which sterilized with duration and concentration of mercuric chloride (Figure 1). Two types contamination include microbial and fungal were recorded in shoot tip explants during cultivation. The contamination rate for shoot tip explants are shown in (Figure 2). The figure 2 obviously shows that both microbial and fungal appears and surrounded explant or dispersed on medium surface in control whereas neither microbial nor fungal were observed in M treatment which subjected to formulated mercuric chloride surface sterilization. However, there was no significant difference amid the application duration and concentration of hypochlorite sodium as chemical pretreatment ($P<0.01$). Thus, the duration and concentration of hypochlorite sodium had no appreciable suppression effect on the contamination rate in explants. It was found that mercuric chloride sterilization had distinguish or effective more than hypochlorite sodium on decrease contamination. At the 1% concentration in 1 min treatment of mercuric chloride, it had same behavior alike other formulated hypochlorite sodium sterilization, but with increasing sterilization duration from 1 to 3 min, both high suppression effect on contamination and prevent of browning appears in explants or medium was displayed (Table 2). Therefore, this positive effect

of duration sterilization of mercuric chloride imply on very vital of optimizing chemical surface sterilization to attaint to success in initiation state of micropropagation of any woody plants. Over all, it proved that contamination rate (microbial or fungal) of shoot tip explants depend on the type of chemicals pretreatments used as surface sterilization and the duration of their application.

Table 1. The variance analysis of kind, duration, and concentration of chemical substances including hypochlorite sodium, mercuric chloride and ethanol as surface sterilization treatments of shoot tip explants from *Juniperus polycarpos*.

Source of variation	df	Sum of squares	Average of squares
Sterilization treatments of explants	9	36.525	4.058**
Error experimental	30	14.25	0.475
Total	39	50.775	4.553

Coefficient variation is 23.56 %, ** Significant at P<0.01

Table 2. Effects of optimized surface sterilization treatments with hypochlorite sodium, mercuric chloride and ethanol on browning intensity of growth medium and shoot tip explants tissues demonstrated by means.

Sterilization Treatments	Formulating protocol from kind, duration, and concentration of chemical substances	Browning intensity of medium (1-5 scores)**	Browning intensity of explants tissues (%)**
A	Ethanol 70% at 1 min-SDW	5cd	87d
B	Ethanol 70% at 1 min- SDW- NaOCl (5%) at 5 min-SDW	5cd	81cd
C	Ethanol 70% at 1 min- SDW- NaOCl (10%) at 5 min- SDW	4.5bc	75c
D	Ethanol 70% at 1 min- SDW- NaOCl (5%) at 15 min- SDW	4.5bc	77c
E	Ethanol 70% at 1 min- SDW- NaOCl (10%) at 15 min- SDW	4.25bc	76c
F	Ethanol 70% at 1 min- SDW- NaOCl (5%) at 30 min- SDW	4b	75c
G	Ethanol 70% at 1 min- SDW- NaOCl (10%) at 30 min- SDW	4b	76c
H	Ethanol 70% at 1 min- SDW-HgCl2 (0.1%) at 1 min-SDW	3.75b	85d
L	Ethanol 70% at 1 min- SDW- HgCl2 (0.1%) at 2 min-SDW	2a	46b
M	Ethanol 70% at 1 min- SDW- HgCl2 (0.1%) at 3 min-SDW	1.5a	13a

Mean values with different letters in the same column are significantly different at $P < 0.01$. SDW: sterile distilled water.

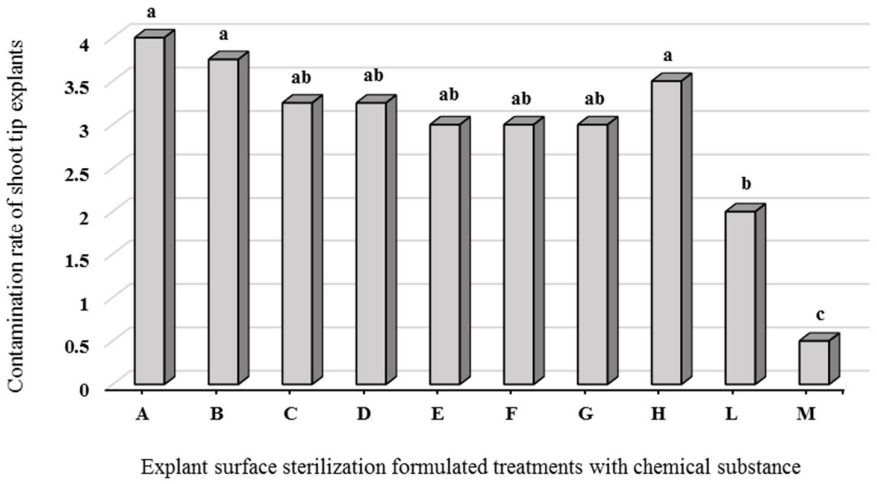

Figure 1. Main comparison of microbial contamination rate of shoot tip explants from *Juniperus polycarpos* under optimized surface sterilization treatments with hypochlorite sodium, mercury chloride and ethanol.

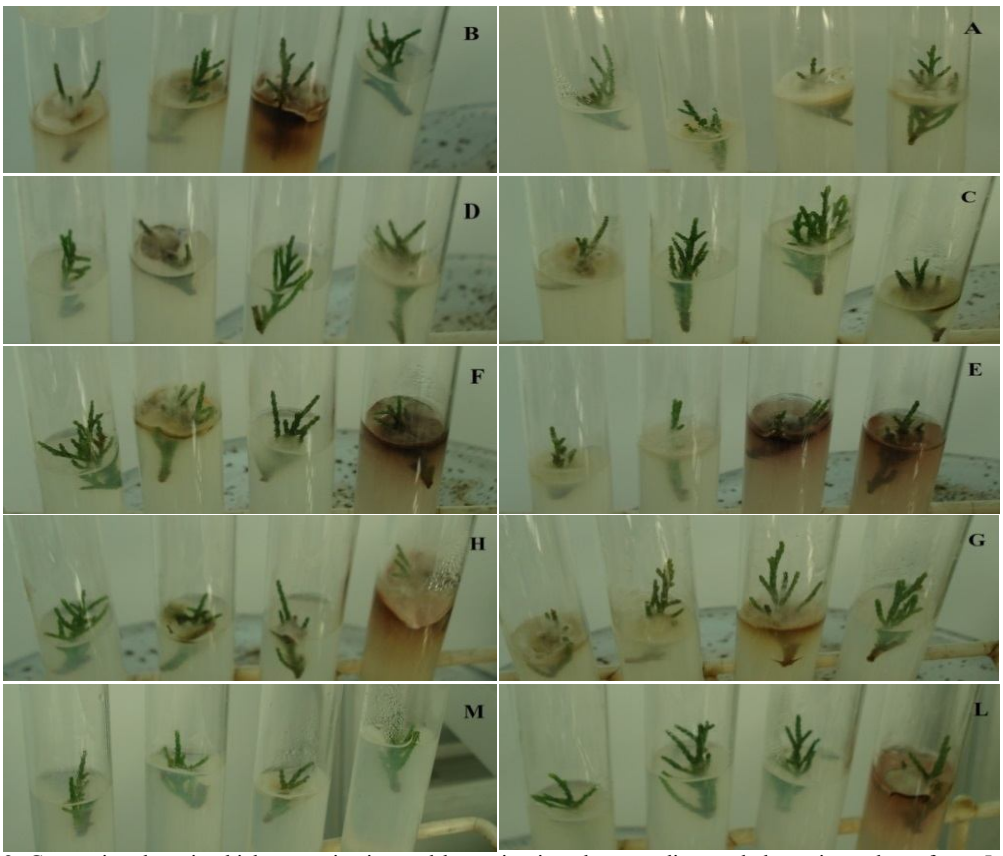

Figure 2. Comparing the microbial contamination and browning in culture medium and shoot tip explants from *Juniperus polycarpos* results in effects of optimized sterilization treatments with hypochlorite sodium, mercury chloride and ethanol

Explant browning due to oxidation of phenols and contamination were the two main problems associated with perennial woody trees in vitro culture (Mahna and Motallebi-Azar 2007). It occurs through the action of polyphenol oxidase (PPO) and peroxidases (POX) by triggering defense reactions induced by wounding (Dobránszki and da Silva 2010). PPO catalyzes the reaction between different phenolic compounds and molecular oxygen producing quinones which are highly reactive and non-specifically polymerize proteins and produce dark pigments, melanin (Leng et al. 2009). The variance analysis confirmed that significantly reduction ($P<0.01$) in browning content in medium culture or tissue explant was related to formulate surface sterilization treatments (Table 2). The results demonstrated that the browning intensity of the explants was controlled too much extent when they were subjected to formulated surface sterilization of mercuric chloride (Table 2). In the present work, microbial or fungal contamination and browning influenced by both concentration and duration of mercuric chloride treatments (Figure 2). The highest browning of medium (5 score) and tissue explants (87%) was observed in control while the lowest browning of medium (1.5 score) and tissue explants (13%) displayed by M treatment (formulated by mercuric chloride). However, the formulated sterilization of hypochlorite sodium cannot enable to prevent from explants brewing occurrence and it extension to growth medium, but mercuric chloride application suppressed the release phenolic compound, enhanced the color, texture of explants and guaranties or promoted their survival percentage (Figure 2). Thus, considering the freshness or health explants tissue and growth medium free of microbial or fungal contamination and browning, the best result was achieved using the M surface sterilization treatment (Ethanol 70% at 1 min- sterile distilled water (SDW)- $HgCl_2$ (0.1%) at 3 min-SDW). So, the optimized procedure of mercuric chloride (M treatment) used in our experiment was efficient for surface sterilization of explants from wild-grown juniper (Juniperus *polycarpos*) even when they were collected from intensely contaminated mother plants in winter season.

CONCLUSION

Different methods have been described for surface sterilization of explants (mainly shoot tips) in vitro culture of different species of junipers. It has been reported that different genotypes do not respond in the same way to surface sterilization chemical treatments, such as hypochlorite sodium or mercuric chloride (Momeni et al. 2015). Hence, a surface sterilization procedure developed for a juniper genotype could not always be extrapolated with the same success for another genotype. Surface sterilization protocol difference are commonly found between genotype which mainly in rate of browning due to synthesized phenolic compound presented in their organs followed by releasing it into growth medium and prevalence contamination in explants. To date, in vitro propagation of Juniperus *polycarpos* has not been reported and thus, in another our work, we reported first micropropagation of Juniperus *polycarpos* (Kazemzadeh et al. 2017; unpublished). Therefore, to obtain to more success in micropropagation of Juniperus polycarpos, the optimizing of surface sterilization is more vital. We can conclude that using optimized surface sterilization of mercuric (Ethanol 70% at 1 min- SDW- HgCl2 0.1% at 3 min-SDW) is the most effective method to suppress microbial contamination of explants and browning occurrence in culture medium and in vitro plant tissues of Juniperus *polycarpos*.

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