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Control of *In Vitro* Contamination in *Dionysia tapetodes* for Effective Micropropagation

Leila Samiei[1*], Maedeh Aghdaei[2] and Saeed Reza Vessal[3]
[1] Assistant Professor, Department of Ornamental Plants, Research Center for Plant Sciences, Ferdowsi University of Mashhad, Mashhad, Iran
[2] PhD Student, Department of Horticultural Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran
[3] Assistant Professor, Department of Legume Research, Research Center for Plant Sciences, Ferdowsi University of Mashhad, Mashhad Iran

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*Corresponding author's email: Samiei@um.ac.ir

Abstract

In vitro microbial contaminations are the most serious problems in plant tissue culture. Initial attempts for micropropagation of *Dionysia tapetodes*, a beautiful alpine cushion plant from north-east Iran, led to 100% explants contamination. Hence, the present study was conducted to develop an efficient surface sterilization protocol for *in vitro* culture of field collected *D. tapetodes* using combinations of different disinfection agents including sodium hypochlorite (NaOCl), ethanol and mercuric chloride ($HgCl_2$) in different concentrations and exposure times. The most effective sterilization treatment with 91.6% healthy explants was the application of 0.1% mercuric chloride for 4 min. However, mercuric chloride in higher concentration (0.2%) was toxic to explants tissues and resulted in 41.66% necrosis. Moreover, the results indicated that in spite of being a general disinfectant in tissue culture studies, NaOCl was not as effective as mercuric chloride in elimination of microbial contaminations and led to 75% total infection when used in concentration of 3% for 15 min. The current experiment represented a potential method for disinfection of *D. tapetodes* and provided aseptic and clean explants for future *in vitro* culture studies.

Keywords: Mercuric chloride, Microbial contamination, Sodium hypochlorite (NaOCl), Surface sterilization.

INTRODUCTION

The genus *Dionysia* belonging to Primulaceae family includes about 50 species in the world from which 27 are endemic to Iran (Lidén, 2007; Valant-Vetschera *et al*., 2010). *Dionysia* can be considered as a very economically important plant since they attract many tourists from all over the world to appreciate the natural outstanding colorful image they create on sloppy cliffs of the Zagros mountains in spring. *Dionysia tapetodes* is one of the only two species of *Dionysia* which are found in northeast Iran (Jamzad, 1999). It forms a large cushion covered with beautiful yellow flowers in early spring (Fig 1). It is widely distributed from Turkmenistan and north eastern Iran to the borders of Pakistan on shaded or semi-shaded limestone or dolomitic cliffs (Jamzad, 1999).

Many members of genus *Dionysia*, despite having a great ornamental value, have proven difficult to propagate either vegetatively or sexually. The technique of micropropagation has been used commercially to propagate many horticultural plant species. This has included species which could not have been propagated in the sufficient numbers by traditional propagation methods so far (Daniels, 1994). In spite of the successful micropropagation of many plant species, the technique still has some drawbacks including *in vitro* contamination control during propagation.

In vitro bacterial, fungi and yeast infection are the most serious challenges for commercial micropropagation laboratories. The difficulties in properly eliminating the contamination are one of the basic reasons for failures in commercial laboratories (Niedz and Bausher, 2002). Some surface sterilization agents include ethanol, sodium hypochlorite, and mercuric chloride that ave been used in various concentrations for contamination control of many plant species (Barampuram *et al.*, 2014; Patel *et al.*, 2014). For clonal propagation of *Primula veris*, 0.1% mercuric chloride has been used for *in vitro* seed disinfection (Morozowska and Wesolowska, 2004). For *Primula* spp., the optimum disinfection treatment for flower bud explants was reported to be with 70% ethanol for 30 s followed by 0.8% silver nitrate plus few drops of Tween 20 (Schween and Schwenkel, 2002). Mercuric chloride (0.1%) for 9 min followed by 1% sodium hypochlorite for 8 min was reported to be efficient in decontamination of *Cyclamen persicum* when applied after disinfection with 70% ethanol for 30 s (Naderi *et al.*, 2011).

During our preliminary attempts for *in vitro* propagation of *D. tapetodes* with general disinfection method using lower concentration of sodium hypochlorite (1%), a heavy contamination occurred, resulting in the 100% loss of explants collected from the field. To address this problem an experiment with various sterilization agents was designed to obtain contamination-free explants.

MATERIALS AND METHODS

Dionysia tapetodes plants were collected in late April from sloppy cliffs of Kalat district

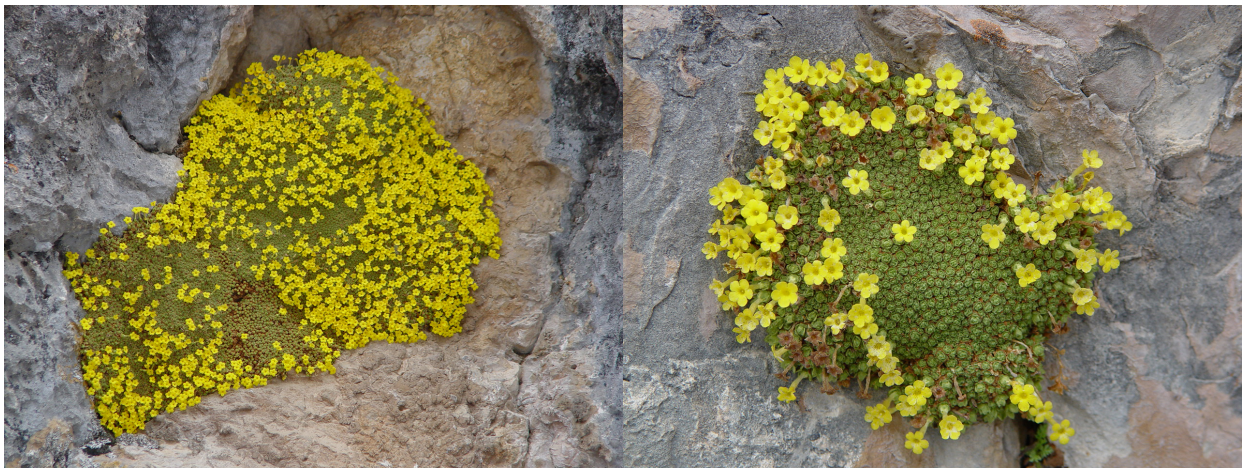

Fig. 1. Dionysia tapetodes plants in their habitat (Kalat district, North-East of Iran).

located in north east of Iran. About 2 cm shoot tips were excised from the stock plants and were rinsed under running tap water for one hour to remove soil and other superficial contaminations. A combination of the disinfectant agents including sodium hypochlorite (5% commercial NaOCl), ethanol (70%) and mercuric chloride with different exposure times composed 19 sterilization treat-

Table 1. The disinfection treatments applied for surface sterilization of Dionysia tapetodes explants.

Disinfection treatments	Ethanol (%)	Sodium hypochlorite		Mercuric chloride (%)
		Concentration (%)	Time (min.)	
T1	-	-	-	-
T2	-	1	10	-
T3	-	1	15	-
T4	-	2	10	¬-
T5	-	2	15	-
T6	-	3	10	-
T7	-	3	15	-
T8	70	0	10	-
T9	70	0	15	-
T10	70	1	10	-
T11	70	1	15	-
T12	70	2	10	-
T13	70	2	15	-
T14	70	3	10	-
T15	70	3	15	-
T16	-	-	-	0.1
T17	-	-	-	0.2
T18	70	-	-	0.1
T19	70	-	-	0.2

ments for *Dionysia* explants (Table 1). All treatments with 70% ethanol applied for 30 s while all treatments with mercuric chloride applied for 4 min. Explants treated with sterile distilled water were used as control.

After applying the treatments, the explants were washed three times with sterile double distilled water for 15 min to remove all traces of disinfectants. The explants were trimmed to 1 cm shoot length containing 4-6 apical leaves. MS basal medium supplemented with vitamins, sucrose (30 g/l), and myoinositol (100 mg/l) was used for culture initiation. The pH of the medium was adjusted to 5.8 with 1N NaOH and HCl before adding agar (8 g/l). The media were autoclaved at 121ºC and 15 kPa for 20 min. Cultures were incubated at 25 ± 2ºC under 16 h photoperiod regime with 50 µM m^{-2} s^{-1} photosynthetic photon flux density provided by cool white fluorescent lamps. The percentage of contamination was determined by visual evaluation of bacteria or fungi presence in the explants and recorded on a daily basis up to one month. Data were collected for percentage of bacterial infection, fungal infection, necrosis and explant survival within 4 weeks of the inoculation.

Data analysis

All *in vitro* experiments were done in a completely randomized design. The experiment had 6 replications with 12 explants for each treatment. Percentage data were arcsine transformed to validate the one way analysis of variance (ANOVA) assumption of normality of distribution. Duncan's multiple range test (DMRT) was used for separating mean value using SPSS (IBM, SPSS statistics 19 R, USA) and significant level was set at $P< 0.050$.

RESULTS

Our preliminary experiments revealed that the *Dionysia* explants collected from the field were heavily infected with microbial contaminations. Therefore, the current experiment was carried out to optimize the sterilization protocol for fast multiplication of *D. tapetodes*. The results showed that the disinfection treatments had a significant effect on bacterial infection, total infection, the percentage of necrosis, and the explant survival rate (Table 2). Bacterial infection was affected by disinfectant agent. Treatments containing mercuric chloride were successful in complete elimination of the bacterial infection. In fact, no bacterial contamination was observed when mercuric chloride was applied in either 0.1 or 0.2% concentrations. Sodium hypochlorite was not as effective

Fig. 2. Left: Healthy explants from the treatment of 0.1% mercuric chloride for 4 min, Right: Infected explants of control.

as mercuric chloride in controlling bacterial infection even in higher concentrations and longer exposure times. Also, among the disinfection treatments, 70% ethanol plus 3% sodium hypochlorite for 15 min resulted in the least bacterial infection with 41.6% contamination. With an exception of mercuric chloride, treatments with ethanol and sodium hypochlorite led to 41.6 to 100% bacterial infections.

The appearance of fungal colonies normally occurred after 4-6 days while the bacterial contamination was observed 7-10 days after culture initiation. The results indicated that the rate of bacterial infection was much higher than that of fungal contamination in our experiments. It means that the average bacterial infection was 61.4% whilst the rate of fungal contamination was 7.8%. No distinct differences were observed among chemical disinfectants in fungal contamination. However, it was completely controlled by applying any concentrations of mercuric chloride in sterilization treatments.

The application of mercuric chloride in surface sterilization of *D. tapetodes* led to the com-

Table 2. The effect of different disinfectant treatments on surface sterilization of *Dionysia tapetodes*.

Disinfection treatments	Bacterial infection (%)	Fungal infection (%)	Total infection (%)	Necrosis (%)	Surviva (%)
T1	100.00 ^{c*}	0.00 ^{a}	100.00 ^{c}	0 ^{b}	0 ^{d}
T2	83.33 ^{bc}	16.67 ^{a}	100.00 ^{c}	0 ^{b}	0 ^{d}
T3	100.00 ^{c}	0.00 ^{a}	100.00 ^{c}	0 ^{b}	0 ^{d}
T4	66.66 ^{bc}	16.67 ^{a}	83.33 ^{bc}	0 ^{b}	0 ^{d}
T5	100.00 ^{c}	0.00 ^{a}	100.00 ^{c}	0 ^{b}	0 ^{d}
T6	75.00 ^{bc}	16.67 ^{a}	91.66 ^{c}	0 ^{b}	0 ^{d}
T7	75.00^{bc}	0.00 ^{a}	75.00 ^{bc}	8.33 ^{b}	16.66 ^{cd}
T8	100.00 ^{c}	0.00 ^{a}	100.00 ^{c}	0 ^{b}	0 ^{d}
T9	83.3333 ^{c}	16.67 ^{a}	100.00 ^{c}	0 ^{b}	0 ^{d}
T10	75.00 ^{bc}	25.00 ^{a}	100.00 ^{c}	0 ^{b}	0 ^{d}
T11	83.33 ^{c}	16.67 ^{a}	100.00 ^{c}	0 ^{b}	0 ^{d}
T12	58.33 ^{bc}	0.00 ^{a}	58.33 ^{b}	0 ^{b}	41.66 ^{bc}
T13	66.66 ^{bc}	8.33 ^{a}	75 ^{bc}	8.33 ^{b}	16.66 ^{cd}
T14	41.66 ^{b}	16.67 ^{a}	58.33 ^{b}	0 ^{b}	41.66 ^{bc}
T15	58.33 ^{bc}	16.67 ^{a}	75.00 ^{bc}	16.66 ^{b}	8.33 ^{d}
T16	0 ^{a}	0.00 ^{a}	0 ^{a}	8.33 ^{b}	91.66 ^{a}
T17	0 ^{a}	0.00 ^{a}	0 ^{a}	8.33 ^{b}	91.66 ^{a}
T18	0 ^{a}	0.00 ^{a}	0 ^{a}	16.66 ^{b}	83.33 ^{a}
T19	0 ^{a}	0.00 ^{a}	0 ^{a}	41.66 ^{a}	58.33 ^{ab}
Average	61.40	7.89	69.30	5.70	25.00

*Different letters within a indicate significant differences ($P < 0.05$)

plete removal of all contamination (Fig. 2), whereas 58-100% total infection was observed in the remaining treatments. Sodium hypochlorite was not effective in controlling the infection rate as it only decreased the total contamination by 42% when applied in higher concentrations of 2 to 3%. Mercuric chloride in the concentration of 0.2% along with 70% ethanol brought about 41% losses of explants due to necrosis. However, the rate of damage to explants was distinctly decreased to 16% when ethanol was removed from surface sterilization procedure.

DISCUSSION

The control of contamination is one of the main factors of the success of *in vitro* plant propagation. The best results for controlling *in vitro* contamination of *Dionysia tapetodes* explants with the highest survival rate were obtained with the treatments containing mercuric chloride due to high efficiency, low necrosis, and lower handling requirement in this study. The use of mercuric chloride as an effective surface sterilizing agent has been reported for *in vitro* decontamination of the species belonging to *Primulaceae* family (Jalali *et al.*, 2010; Kocak *et al*., 2014; Morozowska and Wesolowska, 2004; Naderi *et al.*, 2011). In the present study, the use of ethanol in sterilization procedure along with the higher concentration of mercuric chloride caused nearly 41% loss of survival rate of the explants. This may be attributed to the dehydration of explants tissue exposed to alcohol. Ethanol is a strong surface disinfectant agent, but at the same time it is highly toxic to plant tissues (Tiwari *et al.*, 2012).

The use of alcohol along with mercuric chloride has been effective in surface sterilization of some plant species (Islam *et al.*, 2010; Kumari *et al.,* 2011). However, the application of only mercuric chloride was more efficient in our experiment as it has been confirmed by other studies too (Joseph *et al*., 2003; Saxena, 1990; Yadav *et al.*, 2012). Mercuric chloride has been very effective when used for surface sterilization of different explants in several important plant species (Goyal and Bhadauria, 2006). NaOCl, despite being a widespread chemical *in vitro* disinfectant, was not effective in eliminating the contamination of *Dionysia* explant even in higher concentrations and exposure times. It is well known that NaOCl has a broad-spectrum of antimicrobial activity and it is able to rapidly kill vegetative spores, bacteria, fungi, protozoa, and viruses (Dychdala, 1991). However, the results showed that it was not as effective as mercuric chloride in the current experiment. It can be interpreted by the conditions in which the plants have been grown up as the *Dionysia* explants have been collected from the stock plants living in their native habitat in the sloppy cliffs where they had been exposed to extensive contamination. So, it may not be surprising that a more vigorous sterilizing agent like mercuric chloride is needed to eliminate contaminations.

The rate of bacterial infection was 8.5 folds higher than fungal infection in the whole experiment. It means that the bacterial infection is a greater threat to *in vitro* establishment of *Dionysia* explants. However, mercuric chloride was effective in controlling both pathogens. In contrast to our study, Khan *et al.* (2002) reported that the main contamination was due to fungal growth in their experiment with olive explants. Furthermore, they observed the appearance of contamination after seven days in olive explants (Khan *et al.*, 2002), whereas in our experiment fungi growth were observed after four days.

CONCLUSION

In conclusion, for achieving a successful micropropagation of *Dionysia tapetodes*, the application of mercuric chloride as the main disinfecting agent may be crucial for sterilizing the plants collected from the field. Sodium hypochlorite as the main disinfectant failed to eliminate the microbial contamination effectively. Currently, we are using this optimized protocol for surface sterilization of *D. tapetodes* explants which allows us to perform substantial experiments to develop an efficient micropropagation protocol for *D. tapetodes*.

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