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Optimization of Sterilization and Micropropagation of Water Lettuce (*Pistia stratiotes* L.)

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Abstract

Water lettuce (*Pistia stratiotes*) is an important medicinal aquatic plant that is also used for phytoremediation or as bioindicator. It is very difficult to multiply the plant using micropropagation techniques due to high contaminations. The study aimed to alleviate this problem for efficient multiplication of the plant subsequently. The study made use of 60% commercial bleach (5% NaOCl) or 60% H_2O_2 for surface sterilization for 10 min followed by adding 500 mg/l Duocid® (a wide spectrum antibiotic) in the culture medium. The results showed that commercial bleach was not proper sterliser as all explants bleached; whereas, 60% H_2O_2 based surface sterilization was most optimum for the purpose. The Surface sterilized plants were *in vitro* cultured on agar solidified or liquid MS medium containing 0.05-0.40 mg/l kinetin. Cent percent shoot regeneration frequency was on both agar solidified or liquid medium. However, all explants on agar solidified or liquid medium had tenancy to die turning all of them to albino. Therefore, multiplication under *ex vitro* conditions on liquid MS medium containing 0.05-0.40 mg/l kinetin or BAP using unsterilized shoot meristems without adding sucrose. Subsequently, the explants induced 2.0-40 plantlets per explant on 2.0-3.0 mg/l kinetin or BAP containing culture medium. The plants regenerated on both culture media were comparable with plants growing under natural conditions and did not show any sign of stress causing albinism or necrosis.

Key Words: *Ex vitro*, plant growth regulators, micropropagation, sterilization, water lettuce

INTRODUCTION

Water lettuce (*Pistia stratiotes* L. family Araceae) also known as water cabbage, Nile cabbage or shellflower is an important aquatic stoloniferous and floating plant of tropical and subtropical regions of Asia, Africa, and America [1]. Water lettuce is a perennial monocotyledonous plant with soft leaves that can grow to 14 cm in length and forms a rosette shape. It requires hot season for flowering and bear fruit after rainfall [2]. Water lettuce is one of the world's most productive freshwater aquatic plants and contain biologically active chemicals like alkaloids, glycosides, flavonoids, and steroids. The leaves and stem contains 92.9% moisture, 1.4% protein, 0.3% fat, 2.6% carbohydrate, 0.9% fibers, 0.2%, calcium, 0.06% phosphorus and 1.9% ash [3]. The ash is rich in potassium chloride and sulfate.

Leaves are rich in vitamin A, B, C, and are used in eczema, leprosy, ulcers, piles, and syphilis in traditional therapy systems of many countries. Leaf extracts boiled with coconut oil is used for chronic skin diseases [4]. Water lettuce has diuretic, antidiabetic, antidermatophytic, antifungal, and antimicrobial properties [5]. It also has antiseptic, antitubercular, and antidysentric activities. Besides that, water lettuce is an excellent plant for phytoremidiation of Cd [6], nitrate and ammonium [7], and other heavy metals [8]. Water lettuce also provides attractive foliage that inhibits growth of algae and help keep water clear. Due to its importance as medicinal and use for phytoremediation activities, there is need to work extensively on different aspects of the plant. In line with these objectives as initial step, the present study was designed to multiply water lettuce under *in vitro* or *ex vitro* conditions.

MATERIAL AND METHODS

Water Lettuce plants were obtained Department of Fishries and Aquaculture, Faculty of Agriculture, Ankara University, Ankara, Turkey. Plants with two leaves were selected carefully and washed under plenty of tap water for 5 minutes prior to sterilization. Therafter, these were subjected to surface sterilization with 1.0-3.0 % (v/v) NaOCl (commercial bleach) or diluted 8-24 % H_2O_2 for 12 min followed by 3x5 min rinsing with distilled water. Thereafter, they were cultured on MS medium [9] supplemented with 3.0 percent sucrose and solidified with 0.65 percent agar for two weeks. Thereafter, these plants were cultured on agar solidified or liquid MS medium without sucrose and supplemented with 0.05-0.40 mg/l kinetin in Magenta GA7 vessels. Culture medium was also provided with 500 mg/l Duocid (Broad Spectrum Antibiotic) to eradicate bacterial contamination. The experiments were run in triplicate with the pH adjusted to 5.8±0.1 before autoclaving at104 kPa atmospheric pressure, 120^{o}C for 21 min. For experiments under *ex vitro* conditions, the plants were not subjected to surface sterilization and directly cultured on tap water in

aquariums (40 x 30 x 15 cm) equipped with oxygen supply, using 0.05-0.40 mg/l for each of kinetin or BAP; without any concentration of sucrose or salts. Both *in vitro* and *ex vitro* experiments made use of explants cultured on growth regulator free medium as control. All cultures were incubated under 16 h light photoperiod (6000 lux). After 6 weeks of culture, *in vitro* regenerated shoots were isolated and the data about shoot regeneration frequency and number of plantlets per explants were scored. Each treatment contained 10 explants and was replicated 6 times. The experimental data was analyzed using One Way ANOVA of SPSS 17 computer statistical software and post hoc tests were performed using Duncans multiple range test. Data given in percentages were subjected to arcsine transformation [1] before statistical analysis.

RESULTS AND DISCUSSION

Sterilization of explants is the major step for successful *in vitro* micropropagation and can be done by using various agents like sodium hypochlorite, calcium hypochlorite, ethanol, mercuric chloride, hydrogen peroxide and silver nitrate [11]. Successful tissue culture of all plant species depends on the removal of exogenous and endogenous contaminating microorganisms [12,13]. Sterilization of aquatic plants is the prerequisite for micropropagation [14], as whole plant or plant parts are directly subjected to sterilization and used as explants. Therefore, obtaining sterilized explants without any substantial damage is very important and selection of sterilizing agent is of utmost importance. Unsuccessful sterilization hinders the micropropagation and obtaining reduced or contamination free explants [15]. However, selection of sterilizing agent depends on the plant part depending on the morphological characteristics like hardness/softness of the tissue [14].

For aquatic plants, H_2O_2 is the most commonly used agent for sterilization as it is less damaging to plants or plant parts and has been successfully used for aquatic plants like Water Hyssop [16], Dwarf Hygro [17] and Coontail [18]. For sterilization of water lettuce plants, 1.0-3.0 percent diluted commercial bleach or 8-24 percent diluted H_2O_2 were used. All plants died with the usage of commercial bleach at all concentrations used. Whereas, various concentrations of H_2O_2 for 10 min resulted in variable results and 25.0-35.0 % (Table 1) sterilized plants were recorded but they also died within one week. However, 0.0-50.0 % plants survived with free of contamination after one week of culture. Farooq et al. [19] reported 50% sterilized plants with H_2O_2. Similar results are also obtained by Karatas et al. [17] using H_2O_2. However, after two weeks of culture, bacterial contamination was recorded on explants. To overcome the problem, broad spectrum antibiotic (500 mg/l Duocid) was added to both agar solidified or liquid medium that was effective to halt bacterial contamination. Application of antibiotic has been used to control bacterial contamination by Karatas et al. [17], who found that broad spectrum antibiotic in the culture medium was very effective to control bacterial contamination in dwarf hygro cultures. After four weeks of culture, degradation of chlorophyll was observed on leaves that turned albino with green meristematic zones that were used as explant for *in vitro* regeneration experiments.

For *in vitro* shoot regeneration, shoot meristem explants were cultured on MS medium supplemented with 3.0 % sucrose, 0.05-0.40 mg/l kinetin, 500 mg/l Duocid and solidified with 0.65 % agar. After two weeks of culture, direct plantlets development started but all they died within four weeks of culture. Contrarily, Yong et al. [20] reported successful *in vitro* shoot regeneration of water lettuce on agar solidified medium. However, they obtained first calli on MS medium containing 26 umol/L of 2,4-D and 0.88 umol/L of BA followed by culture of calli on shoot regeneration medium supplemented with 4.44 µmol/L BA and 0.54 µmol/L NAA. In order to obtain shoot regeneration under *in vitro* conditions, explants were also cultured on liquid mediums supplemented with 0.05-0.40 mg/l kinetin and 500 mg/l Duocid. In liquid mediums, sucrose and MS salts were not added. Liquid medium for propagation of aquatic plants has been reported in *Ipomoea aquatica* [21], *Trapa japonica* Flerov [22], *Spartina alterniflora* [23], *Lemna gibba* var. *Hurfeish* and *Spirodela punctata* [24], *Ludwigia repens* [25], *Bacopa monnieri* [26], dwarf hygro [17] and *C. demersum* [18]. After 6 weeks of culture, shoot regeneration frequency and number of plantlets were scored (Table 2).

Analysis of variance showed insignificant (<0.05) effects of kinetin concentrations on shoot regeneration frequency while, number of shoots per explant was found significant (<0.05). Results illustrated cent percent regeneration irrespective of concentration of kinetin in the growth mediums. Karatas et al. [17] reported 0.0-50.0 % shoot regeneration frequency of dwarf hygro using kinetin singly on agar solidified medium. Whereas, kinetin concentrations had clear effects on mean number of regenerated plantlets per explant and ranged 2.0-3.27. Maximum of 3.27 plantlets per explant were recorded on liquid medium with 0.10 mg/l kinetin. Whereas, further increase upto 0.40 mg/l kinetin resulted in 3.00 plantlets per explant. Minimum of 2.0 plantlets per explants were recorded on liquid medium without kinetin or supplemented with 0.05 mg/l kinetin. Yenice [27], obtained 57.82 shoots per explant of *Lemna minor* on liquid MS medium supplemented with 0.05 mg/l Kin. Thereafter, bleaching on leaves of regenerated plantlets was observed and the leaves turned in to albino without affecting meristematic zones. It is assumed that sterilization with H_2O_2 resulted in chlorophyll bleaching that affected cellular membranes and chloroplasts which failed to recover and resulted in reduced biosynthetic enzyme activity in the plant cells metabolism that resulted in changes and degradation of phospholipids causing albinism and hormonal treatments failed to recover negative effects due to H_2O_2 treatment. Similarly, Şumlu [28] reported death of shoot meristem, 1st and 2nd nodal meristem explants, 1st and2nd internodal segments of *Rotala macrandra* on MS liquid medium containing 0.25 mg/L BAP -0.50 mg/L NAA and 0.50 mg/L BAP - 0.50 mg/L NAA, after 4 weeks of culture.

Table 1. Effect of different H_2O_2 treatment on sterilization of Water lettuce

H_2O_2 Concentration in percent (%)	Percentage of contaminated plants with death	Percentage of Sterilized plants with death	Percentage of living sterilized plants
20.0	65.00a	35.00a	0.00d
30.0	60.00a	35.00a	5.00c
40.0	60.00a	35.00a	5.00c
50.0	60.00a	25.00b	15.00b
60.0	25.00b	25.00b	50.00a

Means followed by different small letters within columns are significantly differentusing Duncan test at $p<0.05$

Table 2. *In vitro* plantlet regeneration of water lettuce on liquid medium containing different concentrations of Kinetin

Kinetin (mg/l)	Frequency of regeneration (%)	Number of plantlets per explant
0.05	100.0ns	2.00c
0.10	100.0	3.27a
0.20	100.0	3.00b
0.40	100.0	3.00b
Control	100.0	2.00c

Means followed by different small letters within columns are significantly different using Duncan test at $p<0.05$

Table 3. *Ex vitro* plantlet regeneration of water lettuce on liquid medium containing different concentrations of Kinetin

Kinetin (mg/l)	Frequency of regeneration (%)	Number of plantlets per explant
0.05	100.0ns	2.00d
0.10	100.0	3.00c
0.20	100.0	3.67b
0.40	100.0	4.00a
Control	100.0	2.00d

Means followed by different small letters within columns are significantly different using Duncan test at $p<0.05$

Table 4. *Ex vitro* plantlet regeneration of water lettuce on liquid medium containing different concentrations of BAP

BAP (mg/l)	Frequency of regeneration (%)	Number of plantlets per explant
0.05	100.0ns	2.33ab
0.10	100.0	3.00a
0.20	100.0	2.67ab
0.40	100.0	3.00a
Control	100.0	2.00b

Means followed by different small letters within columns are significantly different using Duncan test at $p<0.05$

Direct plantlet regeneration or shoot regeneration under *ex vitro* conditions is an alternative way to regenerate plants. In these experiments, the explants were not subjected to sterilization and cultured in liquid medium containing 0.05-0.40 mg/l kinetin (Table 3) or BAP (Table 4) provided with continuous oxygen supply under ambient conditions of light in the culture room with no sucrose and any basal media. Cent percent shoot regeneration frequency was noted on both kinetin (Table 3) and BAP (Table 4) containing media. However, number of plantlets per explant (<0.05) were affected by type and concentration of growth variant used in the experiments. Number of plantlets per explant on kinetin containing medium ranged 2.0-4.0 with minimum plantlets obtained on 0.05 mg/l kinetin. Increase of kinetin concentration resulted in increased number of plantlets per explant with maximum of 4.0 plantlets per explant on liquid medium containing 0.40 mg/l kinetin (Table 3). On the other hand, different concentrations of BAP showed variable regeneration on regeneration per explant that ranged 2.0-3.0 in number. Maximum of 3.0 plantlets per explantlet was reorded on 0.10 mg/l and 0.40 mg/l BAP (Table 4). A review of literature shows that micropropagation of aquatic plants under *ex vitro* conditions was used for rapid propagation of *Microsorium pteropus* [29] by culturing sterilized leaf explant on liquid MS medium without sucrose that resulted in 6.5 shoots per explant. In another experiment, leaf explants were pulse treated with 250 mg/l BAP and 250 mg/l IBA and cultured in aquariums that resulted in regeneration of 14 shoots per explant [29].

The results of present study shows that newly developed plantlets in both experiments remained green and did not show any sign of stress, chlorosis or necrosis due to external agents in the culture medium. Furthermore, continuous provision of oxygen also helped to obtain live and healthy plantlets under *ex vitro* conditions. It is supposed that this plant can be propagated more efficiently by temporary immersion system where oxygen can also be provided. The study presents problems in optimization of sterilization and micropropagation of water lettuce under *in vitro* and *ex vitro* conditions. There is need for more work for development of efficient and repeatable *in vitro* propagation protocols to alleviate the problems of aquatic plants under *in vitro* conditions.

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