ISSN: 0976-3031

*Available Online at http://www.recentscientific.com*

*International Journal of Recent Scientific Research*
*Vol. 7, Issue, 7, pp. 12696-12700, July, 2016*

**International Journal of Recent Scientific Research**

**Research Article**

# EFFECT OF SILVER NITRATE AND VARIOUS SUGARS ON *IN VITRO* PLANT REGENERATION OF *Marsilea quadrifolia* (L.)

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**ARTICLE INFO**

***Article History:***

Received 17th April, 2016
Received in revised form 21st May, 2016
Accepted 05th June, 2016
Published online 28th July, 2016

***Key Words:***

*Marsilea quadrifolia* (L.); silver nitrate; carbon sources; *In vitro* plant regeneration

**ABSTRACT**

Optimization on *In vitro* production of multiple shoots of *Marsilea quadrifolia* (L.) was established by using various carbon sources and silver nitrate. In this study the carbon sources used are sucrose, fructose, maltose and glucose by using rhizome explants of *Marsilea quadrifolia* (L.).The data was collected with respective to growth rate and regeneration frequency. The multiple shoots are developed on MS media of various carbon sources along with 0.5µM concentration of NAA. After testing various carbon sources, Fructose has given more number of shoots (9.00 ± 1.00) with 88% of regeneration frequency and least number of shoots are found with maltose (1.66 ± 0.57) and glucose (1.33 ± 0.57). Compare to fructose, more number of shoots (17.00 ± 2.00) and 96% regeneration frequency was found on MS Media with silver nitrate (60µM).



## INTRODUCTION

*Marsilea quadrifolia* (L.) is an aquatic fern belongs to Genus *Marsilea*, Family Marsileaceae, Order Hydropteridales, and Class Filicopsida (CIOCARLAN V 2000). It is commonly known as European water clover. In eastern parts of India it is known as Sushni. The plant is widely distributed throughout India. Its roots are embedded in the soil, mud or in shallow pools. The plant prefers light (sandy) and medium (loamy) soils. It can grow in semi-shade (light woodland) or no shade and requires moist or wet soil and can also grow in water (Marwat *et al*., 2007). It forms more or less monospecies communities (Hulina 1998). It grows from the rhizome over the water level and the aerial leaf with long petioles that resembles a four-leaf clover. At the base of the petiole grows 1-3 sporocarps of kidney shape on a short peduncle (branched or not) attached 1-12 mm above the petiole base. Plant body of this fern is a sporophytic, resembles a four leaved clover plant shows differentiation into stem, leaves and roots. Rhizome is at the tip of each petiole; there are four leaflets of equal size, and hence commonly known as four leaf clover. It is also rooted in the bottom of the soil in submerged water. In Europe *M. quadrifolia* represents a rare species, included in Red list floras (Wraber and Scoberne, 1989).

*M. quadrifolia* is also eaten by various tribal communities such as Kadars, Pulaiyars, Malasars, Malaimalasars, Mudhuvars of Anamalais hills, Western Ghats, Coimbatore district Tamil Nadu, India as per seasonal availability (Ramachandran 2007).

This fern Propagates by using spores and produces sporocarps those need to be lightly abraded and then immersed in water. The sporocarps then swell and burst to release the spores which germinate immediately. The highly developed prothallus remains inside the large seed-like spores. The gametophyte generation is completed in 24 hours and the first roots and shoots appear in 2- 3 days. Mature plants bearing sporocarps can develop in as little as 3 months. Spore germination (gametophyte growth) and fertilization occur immediately (Huxley 1992). The leaves arise alternately in two rows from the upper surface of the creeping rhizome. Leaves, when young, show circinate venation. The petioles of submerged species are long, weak, cylindrical and flexible, with leaf-lets floating on the surface of water. However, the petiole of species growing on mud or ground, are short, cylindrical and upright. *M. quadrifolia* Linn. Has got profound antibacterial, cytotoxic and antioxidant effect and may have potential use in medicine (Ripa *et al*., 2009).

Sugar is the major source of carbohydrate which creates a suitable condition to grow plant tissue *in vitro*. The shoot regeneration frequency increases with increased concentration of sugars at optimum levels. Higher concentration of various sugars can also reduce the multiple shoots *in vitro*. The

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plantlets are growing in vitro requires carbohydrate as a energy source (De paiva Neto and Otoni *et al*., 2003). Sugars are working like a phytohormones to effect the photosynthesis and respiration (Rolland *et al*., 2002). Various types of carbon sources like sucrose, fructose, glucose, maltose, manitol and sorbitol are found to have positive effect on production of multiple shoots *in vitro* (Azar and Kazemiani 2011). Monosaccharides such as fructose and glucose have been widely used in plant tissue culture for the effective growth of plant cells and differentiation in to shoot and root (Faria *et al*., 2004; Aloni 1980).

Sucrose is highly preferred in organogenesis of plants due to its high solubility in water and transported throughout the plant. This sucrose has no inhibitory effect on various biochemical mechanisms existing in the plants (Smith *et al*., 1995). It is one of the major carbon sources found in the phloem sap of many plants (Lemos and Baker 1998). Higher concentrations of sucrose found to be more efficient in regeneration of plants in *Zea mays* L. (Lu *et al*., 1983). In some plants, sucrose is not a effective carbon source for somatic embryogenesis and plant regeneration (Thompson and Thorpe, 1987). Glucose is an effective stimulant of regeneration frequency in *Triticum aestivum* L. (Chu *et al*., 1990). Whereas Maltose enhances the somatic embryogenesis and regeneration frequency in *Medicago sativa* L. (Strickland *et al*., 1987). The present study focused on the effect of various carbon sources and silver nitrate to produce high efficiency regeneration of multiple shoots from the *Marsilea quadrifolia* L. by using Rhizome explants.

## MATERIALS AND METHODS

### *Collection of plant material and surface sterilization*

*Marsilea quadrifolia* plants have been collected from a small pond near herbal garden, Department of Biotechnology, Dravidian university, Kuppam, Andhra pradesh, India.

Rhizomes are selected from *Marsilea quadrifolia* and used as explants. These explants were washed under running tap water for 10 min followed by immersing in Tween- 20, 5% (v/v) for 15 min and washed with running tap water. The explants were surface sterilized with 0.4% (w/v) Bavistin (BASF, India Ltd) and with 70% (v/v) ethanol for 90 seconds. The explants were further sterilized with 0.1 % (w/v) $HgCl_2$ (Merck, India) for 1-3 minutes and thoroughly washed with sterile doubled distilled water for thrice to remove the traces of $HgCl_2$ before inoculation.

### *Culture medium and culture conditions*

MS medium (Murashige and Skoog 1962) with half strength is used with different concentrations of various carbon sources such as fructose, glucose, sucrose and maltose with concentrations ranging from 1-5% (w/v). The silver nitrate (5-1000µM) also used in half strength of MS medium. The pH of the medium was adjusted to 5.8 and added 0.8% (w/v) agar. The molten medium was dispensed approximately 15 ml into culture tubes (25x150mm) and closed with non-absorbent cotton plugs. The medium was autoclaved at 15 lbs/sq inch pressure and 121$^{o}$C for 20 min. MS medium supplemented with 0.5µM Concentration of auxin for culture initiation and multiplication. All the cultures were incubated in an *in vitro* culture room maintained at 26 ± 2$^{o}$c temperature and 55-60% relative humidity with a photoperiod of 16 hrs day light and 8 hrs dark with a light intensity of 3000 lux units provided by cool white fluorescent tubes (Philips, India Ltd.).

### *Data analysis*

The observations are made with respective to number of shoots, shoot length and frequency of regeneration.

### Statistical analysis

The experiments conducted in this study were with a minimum of 10 explants.

**Table 1** Effect of carbon source on number of shoots and shoot length in *Marsilea quadrifolia*

| Carbohydrate source | | Concen-tration (%) | Shoot regene-ration frequency (%) | Number of shoots /explant (mean±SD) | shoot length (cm) (mean ± SD) |
|---|---|---|---|---|---|
| **sucrose** | **s-1** | **1** | 79 | $2.66 \pm 0.57^{abc}$ | $5.33 \pm 1.52^{efg}$ |
| | **s-2** | **2** | 80 | $3.00 \pm 1.00^{abc}$ | $6.66 \pm 1.15^{gh}$ |
| | **s-3** | **3** | **90** | $5.66 \pm 1.52^{ef}$ | 7.33 ± 1.52h |
| | **s-4** | **4** | 85 | $4.00 \pm 1.00^{cde}$ | $5.00 \pm 1.00^{defg}$ |
| | **s-5** | **5** | 75 | $3.00 \pm 1.00^{abc}$ | $4.33 \pm 0.57^{cdef}$ |
| | **s-6** | **6** | 60 | $3.00 \pm 1.00^{abc}$ | $3.00 \pm 1.00^{abc}$ |
| **Fructose** | **f-7** | **1** | 75 | $4.00 \pm 1.00^{cde}$ | $5.33 \pm 1.52^{efg}$ |
| | **f-8** | **2** | **88** | $9.00 \pm 1.00^{h}$ | $6.33 \pm 1.52^{gh}$ |
| | **f-9** | **3** | 84 | $6.66 \pm 1.52^{fg}$ | $6.00 \pm 1.00^{fgh}$ |
| | **f-10** | **4** | 75 | $7.33 \pm 1.52^{g}$ | $5.00 \pm 1.00^{defg}$ |
| | **f-11** | **5** | 70 | $5.00 \pm 1.00^{de}$ | $3.00 \pm 1.00^{abc}$ |
| | **F12** | **6** | 65 | $2.33 \pm 0.57^{abc}$ | $2.33 \pm 0.57^{ab}$ |
| **Maltose** | **M13** | **1** | 60 | $1.33 \pm 0.57^{a}$ | $1.66 \pm 0.57^{a}$ |
| | **M14** | **2** | 75 | $2.33 \pm 0.57^{abc}$ | $2.66 \pm 0.57^{abc}$ |
| | **M15** | **3** | **85** | $3.00 \pm 1.00^{abc}$ | $3.00 \pm 1.00^{abc}$ |
| | **M16** | **4** | 82 | $4.00 \pm 1.00^{cde}$ | $3.33 \pm 0.57^{abcd}$ |
| | **M17** | **5** | 75 | $2.33 \pm 0.57^{abc}$ | $2.66 \pm 0.57^{abc}$ |
| | **M18** | **6** | 55 | $2.66 \pm 0.57^{abc}$ | $2.00 \pm 1.00^{ab}$ |
| **Glucose** | **G19** | **1** | 65 | $2.66 \pm 0.57^{abc}$ | $2.33 \pm 0.57^{ab}$ |
| | **G20** | **2** | 72 | $2.66 \pm 0.57^{abc}$ | $2.66 \pm 0.57^{abc}$ |
| | **G21** | **3** | 75 | $3.00 \pm 1.00^{abc}$ | $3.00 \pm 1.00^{abc}$ |
| | **G22** | **4** | **82** | $4.00 \pm 1.00^{cde}$ | $3.66 \pm 0.57^{bcde}$ |
| | **G23** | **5** | 76 | $3.33 \pm 0.57^{bcd}$ | $2.66 \pm 0.57^{abc}$ |
| | **G24** | **6** | 60 | $1.66 \pm 0.57^{ab}$ | $2.66 \pm 1.15^{ac}$ |

All experiments were repeated at least three times. The experimental data were statistically analyzed by one-way ANOVA using SPSS (11.5).

## RESULTS AND DISCUSSION

**Effect of Silver nitrate and different sugars on *in vitro* plant regeneration from Rhizome explants of *Marsilea quadrifolia*.**

In this study, different carbohydrate sources such as sucrose, fructose, maltose and glucose at different concentrations were tested for their efficiency on shoot regeneration. To find out the best concentration of carbohydrate source to get multiple shoots, sucrose was substituted with fructose, maltose and glucose. After comparing the data obtained with respective to various carbohydrates, we found that fructose is highly suitable carbohydrate source for obtaining more number of shoots.

The maximum number of shoots (9.00 ± 1.00), (Figure1B) were found in the medium containing 0.5µM of NAA and supplemented with 2 % fructose (w/v) and decreased shoot length (1.33 ± 0.57 cm) was observed at 1% maltose.

The next best carbon source for more number of shoots (5.66 ± 1.52) was found with 3 % sucrose (w/v) (Figure 1A and table 1). The maximum shoot length was obtained in 3 % sucrose (w/v) (7.33 ± 1.52cm). The least number of shoots (4.00 ± 1.00) were observed on MS medium (half strength) supplemented with 4% maltose (w/v) (Figure1C) and glucose (w/v) (Figure1D). Throughout the experiment, MS medium (half strength) with 3% sucrose (w/v) was used as a positive control (Figure 1A). MS medium supplemented with 2% fructose and various concentrations of silver nitrate was analyzed. Among the various concentrations, 60µM of silver nitrate has given more number of shoots (17.00 ± 2.00) and the maximum length of shoots (7.66 ± 1.52cm) at 20µM (Table 2, Figure1E).


**Fig 1** The effect of various carbon sources on the production of multiple shoots in *Marsilea quadrifolia.* Sucrose 3% is used and obtained 7 explants(A), Fructose 2% is used and obtained 10 explants(B), Maltose 3% is used and obtained 4 explants(C), Glucose 4% is used and obtained 5 explants(D), Multiple Shoot production in the presence of Silver nitrate 60µM (E), Acclimatization and hardening of plantlet(F).

Without silver nitrate and with 2% of fructose has given shoots (9.00 ± 1.00) and shoot length was found to be (6.33 ± 1.52cm).

**Table 2** Effect of silver nitrate on number of shoots and shoot length in *Marsalea quadrifolia*

| Silver nitrate (µm) | With silver nitrate and fructose (2%) | | | | Without silver nitrate and fructose | | | Fruct-ose (%) |
|---|---|---|---|---|---|---|---|---|
| | Regene- ration frequency (%) | No.of shoots (mean±SD) | Shoot length (cm) (mean ± SD) | | Regene-ration frequency (%) | No.of shoots (mean±SD) | Shoot length (cm) (mean ± SD) | |
| *control | - | - | - | | - | - | - | - |
| 5 | 79 | Ag1 | 8.33 ± 2.09$^{a}$ | 5.00 ± 1.00$^{ab}$ | 75 | 4.00 ± 1.00$^{ab}$ | 5.33 ± 1.52$^{c}$ | 1 |
| 10 | 85 | Ag2 | 10.00 ± 2.00$^{a}$ | 7.33 ± 1.15$^{bc}$ | 88 | 9.00 ± 1.00$^{e}$ | 6.33 ± 1.52$^{c}$ | 2 |
| 20 | 93 | Ag3 | 10.66 ± 2.51$^{a}$ | 7.66 ± 1.52$^{c}$ | 84 | 6.66 ± 1.52$^{cd}$ | 6.00 ± 1.00$^{c}$ | 3 |
| 40 | 92 | Ag4 | 10.66 ± 2.08$^{a}$ | 6.66 ± 1.52$^{bc}$ | 75 | 7.33 ± 1.52$^{de}$ | 5.00 ± 1.00$^{ac}$ | 4 |
| 60 | 96 | Ag5 | 17.00 ± 2.00$^{b}$ | 6.66 ± 1.15$^{bc}$ | 70 | 5.00 ± 1.00$^{bc}$ | 3.00 ± 1.00$^{ab}$ | 5 |
| 80 | 89 | Ag6 | 10.66 ± 1.52$^{a}$ | 5.33 ± 1.52$^{abc}$ | 65 | 2.33 ± 0.57$^{a}$ | 2.33 ± 0.57$^{a}$ | 6 |
| 100 | 85 | Ag7 | 8.66 ± 2.08$^{a}$ | 5.66 ± 1.52$^{abc}$ | | | | |
| 1000 | 80 | Ag8 | 8.33 ± 2.51$^{a}$ | 4.00 ± 1.00$^{a}$ | | | | |

*** No energy source**

Efficient shoot regeneration is prerequisite before producing any transgenic plant for improved qualities. The effective growth and regeneration of shoots is depending on many factors. One of such factors is the concentration and type of carbohydrate source which was supplemented in the MS medium (Lipavska and Konradova 2004). In many reports after comparing various carbohydrate sources, it is found that sucrose had a maximum positive effect. Plant cells depend on carbon source for the energy and osmotic regulation. Due to insufficient levels of carbondioxide *in vitro,* an effective carbon source is needed to promote efficient shoot regeneration (Faria *et al*., 2004). But in the present study fructose along with… hormone was performed well to produce maximum length of shoots and number of shoots. These results are similar to earlier reports on *Stevia rebiudina* (Preethi and Naidu 2011). There are many reports to support that sucrose was the best carbohydrate source for plant regeneration (Sujana and Naidu 2011; Sridhar and Naidu 2011). It is well known that need of carbohydrate is depends on the period of culture and also based on species (Thompson and Thorpe 1987). But in contrast to our results obtained, replacement of maltose instead of sucrose was found to be the best carbon source to get maximum number of shoots in *C. asiatica* (Anwar Hussain *et al*., 2005). Sucrose is broken down during autoclaving and converted to glucose and fructose by the action of invertase (Pierik 1987). Then the order of utilization is from glucose followed by fructose. There are more number of reports on other carbon sources such as mannitol, sorbitol which plays a major role in *In Vitro* culture of *Zea mays* (L.) (Gauchan 2012). The results from our experimental work are in accordance with results produced for the regeneration of *Lillium* (Shin *et al*., 2002). The degradation of sucrose results in the formation of glucose and fructose which are very important in breaking dormancy. Glucose and maltose have given least effect on regeneration of shoots, it is due to metabolized at low level but they are important in osmotic regulation. The positive effect of glucose also reported in *Prumus mume koehne* (Hisashi and yasuhiro 1996). This may be due to uptake at low level and low activity of the responsible enzyme.

***Acclimatization and hardening***

The rooted plants were carefully removed from culture tubes and washed with water to remove the residual amounts of agar. These healthy rooted shoots were transferred to tray containing soil and vermiculite in 1:1 ratio for acclimatization (Saritha *et al*., 2002; Preethi *et al*., 2008). These plants were transferred to polythene bag (Figure1F) for one week.

After this period, plants were transferred to pots and maintained under green house for hardening.

## CONCLUSION

From the present study, it was concluded that more number of shoots and shoot length was obtained with fructose and silver nitrate by using rhizome explants of *Marsilea quadrifolia.* This efficient method of plant regeneration is highly useful in genetic transformation and manipulation for improving medicinal values of *Marsilea quadrifolia*.

**Acknowledgements**

The authors are thankful to the UGC BSR (Non-SAP), New Delhi, India for providing infrastructure facilities for research work.

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**How to cite this article:**

Sudhasri purna G *et al*.2016, Effect of Silver Nitrate And Various Sugars on *In Vitro* Plant Regeneration of *Marsilea quadrifolia* (L.). *Int J Recent Sci Res.* 7(7), pp. 12696-12700.