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Article *in* Journal of Plant Sciences (Science Publishing Group) · January 2019

DOI: 10.11648/j.jps.20190706.11

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Journal of Plant Sciences
2019; 7(6): 131-136
http://www.sciencepublishinggroup.com/j/jps
doi: 10.11648/j.jps.20190706.11
ISSN: 2331-0723 (Print); ISSN: 2331-0731 (Online)

Effect of Different Carbon Sources on *in vitro* Shoot Regeneration and Multiplication of *Cissampelos pareira* (L.) - An Important Medicinal Plant

Cingithalam Munaswamy Reddy Narendra Reddy, Palyam Venkatesh Chaithanya Lakshmi, Varagiri Narayanachetty Swetha Prasuna, Mustrahally Raja Gopal, Gunda Sudhasri Purna, Bathula Srinivas*

Department of Biotechnology, School of Herbal Studies and Naturo Sciences, Dravidian University, Kuppam, A. P. India

Email address:
bathulasrinivas71@gmail.com (B. Srinivas)
*Corresponding author

To cite this article:
Cingithalam Munaswamy Reddy Narendra Reddy, Palyam Venkatesh Chaithanya Lakshmi, Varagiri Narayanachetty Swetha Prasuna, Mustrahally Raja Gopal, Gunda Sudhasri Purna, Bathula Srinivas. Effect of Different Carbon Sources on *in vitro* Shoot Regeneration and Multiplication of *Cissampelos pareira* (L.) - An Important Medicinal Plant. *Journal of Plant Sciences.* Vol. 7, No. 6, 2019, pp. 131-136. doi: 10.11648/j.jps.20190706.11

Received: September 24, 2019; **Accepted**: October 17, 2019; **Published**: October 28, 2019

Abstract: An efficient *in vitro* protocol for shoot regeneration and production of multiple shoots of *Cissampelos pareira* (L) was developed. In the present study, the effect of different carbon sources, sucrose, fructose, maltose and glucose was investigated on *in vitro* shoot regeneration by using axillary bud of *Cissampelos pareira* (L). The frequency, growth and multiplication rate were highly influenced by the type and concentration of carbon sources used. The highest number of shoots (8.06±0.11) was observed on MS medium supplemented with 2% fructose and along with BAP 1.0mg/l and NAA 0.5mg/l and regeneration frequency 95%. The least number of shoots were obtained on MS medium supplemented with 4% maltose and (3.4±0.10) shoots and glucose (3.26±0.11). Among the different carbon sources used in the present study, fructose 2% proved to be a better choice for multiple shoot regeneration followed by Sucrose, maltose, and glucose from axillary bud explants of *Cissampelos pareira*. *In vitro* shoots were then excised from the shoot clumps and transferred to the rooting medium containing NAA, IBA (0.5-3.0mg/l). The well-rooted plantlets were then separated from the culture tubes and transferred into sterile soil and vermiculate (1:1) in the greenhouse. Finally, the hardened plants were transferred to the field environment for utmost survivability.

Keywords: Axillary Bud Explants, Carbon Sources, BAP-6-Benzyl Amino Purine, NAA-1-Napthalene Acetic Acid, Micro Propagation, *Cissampelos pareira*

1. Introduction

Cissampelos pareira (L.) belongs to the family Menispermaceae is a sub-erect or perennial climbing herb with small greenish-yellow flowers and also known as laghupath in Indian traditional medicine system. It is belongs to the genus *Cissampelos* of which 37 species are distributed in the tropical and subtropical world and India represents one among the species. This plant is common in orchards, parks, and gardens on moist soil distributed throughout tropical and sub-tropical in India. *Cissampelos pareira* is very widespread and locally common. It is used to locally to cure many diseases like gastro-intestinal, digestive problems, dysentery, menstrual problems, diarrhoea, infertility, and venereal diseases [1], uterine bleeding and threatening miscarriage [2]. There are several advantages of rhizome decoction or leaves of *Cissampelos pareira,* which are extensively used against jaundice, boils and childhood eczema, rheumatism, cough, and heart-related problems. [3-4]. In India Tribal, peoples are using this plant to prevent pregnancy [5-7]. *Cissampelos pareira* leaf extract used for several pharmacological effects such as anti-diabetics and antioxidant [8]. In *in vitro* culture of the plant cell, tissue and organ are largely determined by the composition of nutrient medium. Carbohydrates is one of

the most important components of the nutrient medium that strongly affects the growth and morphogenesis of *in vitro* cultured plants as it acts as a carbon source and osmoticum both [9].

MS medium containing BAP was found to be most favourable for regeneration of multiple shoots in nodal segments of *C.pareira* [10]. The whole plant regeneration from *Cissampelos pareira* has been established by using axillary buds [11]. In-plant tissue culture, sugar serves as a major source of carbohydrate, which provides a suitable condition to grow plant regeneration *in vitro.* The shoot regeneration frequency increases with increased concentration of sugars at optimum levels. A higher concentration of various sugars can also reduce the multiple shoots *in vitro.* The plantlets are growing *in vitro* requires carbohydrates as an energy source [12]. Sugars are working like phytohormones to affect the photosynthesis and respiration [13]. Different types of carbon sources like those that sucrose, glucose, fructose, maltose, mannitol, and sorbitol are found be a positive effect on the production of multiple shoots *in vitro* [14]. Monosaccharides such as fructose and glucose have been used in the plant tissue culture for effective plant cell growth and differentiation in to shoot and root [15-16]. Glucose and fructose are also known to hold up for the growth of some tissues [17]. Sucrose is most preferred in organogenesis of plants due to its high solubility in water and transported throughout the plant.

In some cases, sucrose is partially [18] or totally replaced by other carbon sources such as mannose, galactose, lactose and melibiose with different levels of success for supporting growth in culture [19]. This sucrose has no inhibitory effect on various bio-chemical mechanisms existing in the plants [20]. It is a highest source of carbon found in the phloem sap of several plants [21]. A higher concentration of sucrose found to be more efficient in the regeneration of plants in *Zea mays* L. [22]. In some plants, sucrose is not a proper carbon source for plant regeneration and somatic embryogenesis [23]. The effective stimulant such as glucose is highly involved in the regeneration frequency of *Triticum aestivum* L. [24]. Similarly, Maltose also enhances regeneration frequency and somatic embryogenesis in *Mediacago sativa* L. [25]. The current research work is focused on the effect of various carbon sources to produce high- efficiency regeneration of multiple shoots from the *Cissampelos pareira* L. by using axillary bud explants.

2. Materials and Methods

Cissampelos pareira plants were collected from the Herbal Garden, Department of Biotechnology, Dravidian University, Kuppam, Andhra Pradesh, India.

2.1. Preparation and Sterilization of Plant Material

Axillary buds of *Cissampelos pareira* were collected from the young sprouts of the stock plants were selected as explants. Axillary buds were washed thoroughly in running tap water for 10-15 min, then followed by immersing in 5% (v/v) Tween-20, a liquid detergent for 20 minutes. Followed by continuous washing in distilled water until all the traces of detergents were removed. Then the explants were soaked 0.4% (w/v) bavistine treatment, a systematic fungicide (BASF India Ltd) for about 15 to 20 minutes. After sterilizing fungicide, the explants were surface sterilized with 70% (v/v) ethanol for 90 seconds. The de-contamination of the explants was treated by the passing through a solution of 0.1%Mercuric Chloride (w/v) for 1-3 min and thoroughly washed with sterile double distilled water for thrice to eliminate the traces of mercuric chloride before inoculation. The cut end of the explants were further trimmed and axillary buds (1.0-1.5cm) were prepared. Then the explants were blotted on sterile filter paper discs to absorb the excess of water before planting them vertically on agar gelled MS media in culture vessels.

2.2. Culture Medium and Culture Conditions

MS medium [26] (Murashige and Skoog 1962) with full strength is used with different concentration of various carbon sources such as fructose, glucose, sucrose, and maltose with different concentration ranging from 1-6% (w/v), along with different types of plant growth regulators such as BAP, NAA and $AgNo_3$. The PH of the medium was adjusted to 5.8 with 0.1N HCl and 0.1N NaOH and it was made to a known volume. Before dispending the media into the containers (15ml for 25×150mm test tubes) 0.8% (w/v) agar was added to the media and melted. The medium was autoclaved 15 lbs/sq inch pressure and 121°C for 20 minutes. After the completion of sterilization, the tubes were removed from the autoclave and placed in a slanting position to get more surface area to inoculate the explants. All cultures of *Cissampelos pareira* were incubated in an *in vitro* culture room maintained at 26±2°C temperature and 55-65%relevant humidity with a photoperiod of 16 hrs daylight and 8 hrs dark with a light intensity of 3000-lux units provided by cool white fluorescent tubes (Philips, India Ltd). All sub-sequent sub-cultures were carried out at four weeks regular intervals.

2.3. Data Collection and Statistical Analysis

The observations were documented with respective to the number of shoots and shoot length per explants. Similarly, it is also analysed for the length and number of roots per explants. In each and every experiments, 20 replicates were taken in each treatment and each experiment repeated three times and the cultures were analysed in regular intervals. The qualitative data were subjected to statistical analysis by using one-way ANOVA and were obtained as the average ± standard error (SE) using SPSS 16.0 Software.

3. Result and Discussion

In this study, Four different types of carbon sources such as Sucrose, Fructose, Maltose and Glucose with six different concentrations (1-6%) were used for shoot regeneration and multiplication in MS Medium supplemented with 1mg/l BAP

+ 0.5 mg /lNAA. To find out the best concentration of carbohydrate source to get multiple shoots, sucrose was substituted with fructose, maltose, and glucose. The data were recorded after 4 weeks of inoculation. Among all carbon sources with different concentrations, fructose is highly suitable followed by sucrose, maltose, and glucose for obtaining more number of shoots. The maximum number of shoots were recorded in the medium containing BAP and NAA augmented with 2% fructose (w/v) (8.06±0.11) (Figure 1B) with shoot length (7.56±0.20) and the regeneration frequency is about 95%. The next best carbon source for more number of the shoot (5.2±0.25) was found with 3% sucrose (w/v) (Figure 1A, Table 1&Figure 2 A, B) and the maximum shoot length (6.53±0.05). In the maltose, the maximum number of shoots was obtained at 4% (3.4±0.10) (Figure 1C) and the shoot length at 3% (3.7±0.05). The shoots and shoot length obtained in maltose carbon source contain very stunted appearance. The regeneration frequency obtained 85%. Where as in glucose at 4% the least mean number of shoots (3.26±0.11), (Figure 1D) shoot length (3.13±0.05) are obtained, and the frequency of regeneration dropped to 80%. From the above results, it is clear that the lesser concentration of carbohydrates favour for the shoot regeneration and proliferation whereas at high concentration it reduces the shoot organogenesis. In all the carbon sources, the frequency of shoot regeneration, mean number of shoots and mean number of shoot length was increased profusely up to 1-3% and this was contrary in the concentration of 4-6%, where the frequency of regeneration, shoot number and shoot length was decreased.

In-plant tissue culture carbohydrates continuous supply is essential since the photosynthetic activity of *in vitro* plant tissue culture is reduced due to low light intensity, high humidity and exchanges of gaseous is limited [27].

Efficient shoot regeneration is pre-requisite before producing any transgenic plant for improved qualities. The effective growth and multiplication of shoots in vitro is depending on many factors. One of such factors is the concentration and type of exogenous carbohydrate source, which was, supplemented in the MS medium [9].

In several 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 carbon dioxide *in vitro,* an effective carbon source is needed to promote efficient shoot regeneration [15].

Nevertheless, in the current investigation fructose along with BAP and NAA was performed well to produce a maximum length of shoots and number of shoots. These results are similar to earlier reports on *Stevia rebiudina* [28-29] and *Sphaeranthus indicus* [30]. Along with in recent *Marsilea quadrifolia* [31] and *Oxalis corniculata* [32] shows similarity results. There are many reports to support that sucrose was the best carbohydrate source for plant regeneration [33-34]. It is well known that the need for carbohydrate depends on the period of culture and also based on species [23]. However, in contrast to our results obtained, replacement of maltose instead of sucrose was found to be the best carbon source to get highest number of shoots in *C.asiatica* [35]. Sucrose is degraded during autoclaving and changed to glucose and fructose by the action of *invertase* [36]. Then the order of utilization is from glucose followed by fructose. There are more number of reports on additional carbon sources such as sorbitol, mannitol that plays a major role in the *in-vitro* culture of *Zea mays* (L.) [18]. The results from our experimental work are in accordance with results produced for the regeneration of *Lillium* [37]. 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 the regeneration of shoots, it is due to metabolized at a low level but they are important in osmotic regulation. The positive effect of glucose also reported in *Prumus mume koehne* [38]. This may be due to uptake at a low level and low activity of the responsible enzyme. In the present investigation, four different carbon sources were used (Sucrose, Fructose, Maltose, and Glucose). In our study, Fructose showed maximum shoot regeneration performance compared to sucrose, maltose, and glucose.

Table 1. *Effect of different carbon sources using MS Medium supplemented with BAP 1.0mg/l and NAA 0.5mg/l on multiple shoot induction by using axillary bud of Cissampelos pareira (L).*

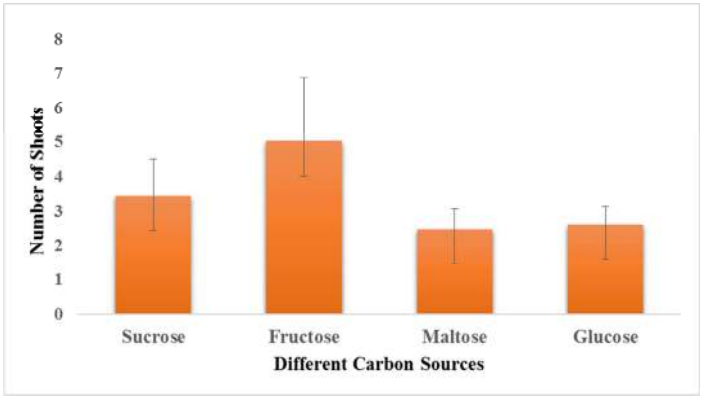
CarbohydrateSource	Concentration	RegenerationFrequency	Mean Number ofshoots	Mean Number ofshoot Length
Sucrose	1	98	2.63 ± 0.05^{de}	5.26 ± 0.11^{i}
	2	80	3.03 ± 0.05^{f}	6.06 ± 0.11^{j}
	3	90	5.2 ± 0.25^{i}	6.53 ± 0.05^{k}
	4	85	4.43 ± 0.11^{h}	5.23 ± 0.25^{i}
	5	75	3.23 ± 0.25^{fg}	4.36 ± 0.11^{h}
	6	60	2.16 ± 0.28^{bc}	3.16 ± 0.15^{f}
Fructose	1	75	4.26 ± 0.25^{h}	6.46 ± 0.05^{k}
	2	95	8.06 ± 0.11^{k}	7.56 ± 0.20^{l}
	3	85	6.33 ± 0.28^{j}	6.3 ± 0.26^{jk}
	4	80	5.16 ± 0.28^{i}	5.33 ± 0.15^{i}
	5	75	4.20 ± 0.17^{h}	3.16 ± 0.28^{f}
	6	70	2.06 ± 0.11^{b}	2.16 ± 0.15^{bcd}
Maltose	1	60	1.53 ± 0.05^{a}	1.6 ± 0.11^{a}
	2	65	2.23 ± 0.057^{bc}	2.66 ± 0.11^{e}
	3	75	3.06 ± 0.11^{f}	3.7 ± 0.05^{g}
	4	85	3.4 ± 0.10^{g}	3.06 ± 0.11^{f}

CarbohydrateSource	Concentration	RegenerationFrequency	Mean Number ofshoots	Mean Number ofshoot Length
Glucose	5	72	2.4 ± 0.17^{cd}	2.33 ± 0.23^{cd}
	6	65	2.23 ± 0.05^{bc}	2.03 ± 0.05^{b}
	1	60	2.56 ± 0.05^{de}	2.26 ± 0.05^{bcd}
	2	70	2.63 ± 011^{de}	2.66 ± 0.11^{e}
	3	75	3.16 ± 0.28^{fg}	3.16 ± 0.11^{f}
	4	80	3.26 ± 0.11^{fg}	3.13 ± 0.05^{f}
	5	76	2.73 ± 0.11^{e}	2.43 ± 0.20^{de}
	6	70	1.6 ± 0.17^{a}	2.13 ± 0.11^{bc}

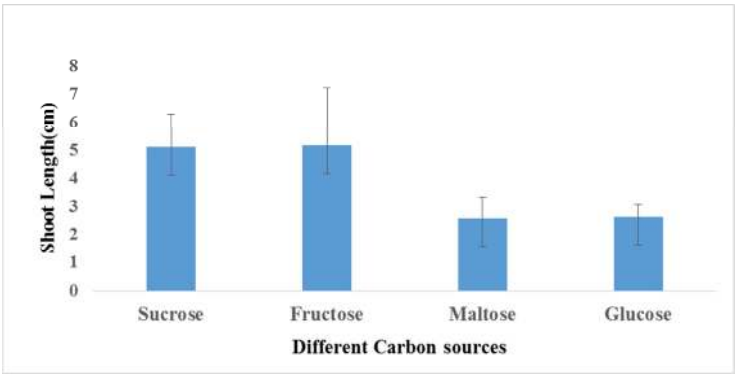
Data shown treatment means ± SE followed by a different letter (s) within a columnindicate significant differences according to ANOVA and DMRT test ($P<0.05$).

A. MS+3% Sucrose B. MS+2% Fructose C. MS+4% Maltose D. MS+4% Glucose.

Figure 1. *Effect of different carbon sources on in vitro shoot proliferation of Cissampelos pareira L. on MS + 1.0mg/l BAP + 0.5mg/l NAA Supplemented with different concentrations of carbon source.*



A



B

Figure 2. *A. Effect of different carbon sources on shoot number of Cissampelos pareira (L.) B. Effect of different carbon sources on shoot length of Cissampelos pareira (L.).*

Rooting and Acclimatization of Plants

Induction of rooting is an important step in the propagation of plant species. Healthy shoots that were formed *in vitro* were taken out from the medium, and it was shifted to fresh MS Medium improved with various concentration of auxins such as NAA and IBA. The comparison of all concentration, the best rooting was obtained in IBA. The highest number roots (12.5+0.15), root length (6.46+0.05) (Table 2) and maximum regeneration frequency (95%) was recorded at 1 mg/l IBA (Figure 3A&B). The plantlets of *in vitro* developed grown *Cissampelos pareira* with well-developed roots and shoots were shifted to polybags containing autoclaved vermiculate and soil in 1:1 ratio for hardening (Figure 3C). Finally, the hardened plantlets were transferred to pots and acclimatized to the natural environment (Figure 3D). All the plantlets were morphologically indistinguishable from the parent plants.

Table 2. *Effect of different concentration of auxins on rooting of invitro derived micro shoots of C.pareira on MS medium after 20 days.*

Plant growthregulators (mg/l)		Root regeneration frequency (%)	Mean no. ofRoots	Mean no. of rootLength (cm)
NAA	IBA			
0.5		82	9.4 ± 0.17^{c}	5.36 ± 0.15^{c}
1.0		90	10.4 ± 0.25^{d}	6.43 ± 0.20^{d}
2.0		85	8.36 ± 0.15^{b}	4.46 ± 0.35^{b}
3.0		80	7.3 ± 0.15^{a}	3.16 ± 0.28^{a}
	0.5	78	10.4 ± 0.25^{d}	5.50 ± 0.4^{c}
	1.0	95	12.5 ± 0.15^{f}	6.46 ± 0.05^{d}
	2.0	80	11.36 ± 0.15^{e}	4.40 ± 0.17^{b}
	3.0	75	9.7 ± 0.87^{c}	4.26 ± 0.20^{b}

Data represent treatment means ± SE followed by a different letter (s) within a columnindicate significant differences according to ANOVA and DMRT test ($P< 0.05$).

Figure 3. *A. Rooting of in vitro regenerated shoots on MS Medium supplemented with IBA at 1.0mg/l& NAA 1.0mg/l; B. In vitro regenerated micro shoots with roots ready for hardening; C. Plantlets kept in poly bags for hardening in greenhouse conditions; D. Hardened Plantlets transferred to earthen pots having soil and vermiculite in 1:1 ratio.*

4. Conclusion

From the present study, it can be concluded that more number of shoots and shoot length was obtained with fructose by using axillary bud explant of *Cissampelos pareira.* This efficient method of plant regeneration is highly useful in genetic transformation and manipulation for improving medicinal values of *Cissampelos pareira.*

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