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Establishment of fast-growing *in vitro* root culture system in *Artemisia vulgaris*

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Artemisia vulgaris is a highly valuable medicinal plant belonging to the family Asteraceae. Adventitious roots were established from leaf and root explants cultured on Murashige and Skoog's medium containing various concentrations and combinations of Indole-3-acetic acid (IAA) (0.5-28.5 µM) and Indole-3-butyric acid (IBA) (0.5-24.6 µM). Maximum number of roots production was obtained on MS media supplemented with 11.4 µM IAA + 4.9 µM IBA. A maximum of 10.8 roots with 13.9 cms root length was achieved from root explants. Out of the different basal media and carbon sources tested, MS medium and sucrose was found to be more suitable for rhizogenesis. Full strength MS medium and 30 gl^{-1} sucrose was useful in biomass enhancement. This reproducible protocol can be used for the large-scale production of adventitious roots and for the subsequent production of root-specific secondary metabolites.

Key words: Adventitious roots, auxins, MS medium, sucrose, biomass

Introduction

Artemisia vulgaris L. is a tall aromatic threatened perennial herb belonging to the family Asteraceae. Mugwort is commonly used in traditional medicine as choleretic and for amenorrhoea and dysmenorrhoea (Teixiera da Silva, 2004).The plant is being widely used for the treatment of diabetes, liver disorders, epilepsy and in combination for psychoneurosis, depression, irritability, insomnia and anxiety stress (Walter *et al*., 2003; Gilani *et al*., 2005). Both the roots and aerial parts of *A.* v*ulgaris* are used as an herbal remedy (Kovacevic, 2004). They contain several medicinally active components including coumarins, sesquiterpene lactones, volatile oils and inulin (USDA-ARS-NGRL, 2004). Mugwort essential oil is used for its insecticidal, antimicrobial and antiparasitical properties (Judzentiene and Buzelyte, 2006). The essential oil of *Artemisia vulgaris* was reported to exhibit 90% repellency

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against *Aedes aegypti*, a mosquito that transmits yellow fever (Hwang *et al*., 1985). Repellant and fumigant activity of *Artemisia vulgaris* against *Musca domestica* and the stored-product insect pest *Tribolium castaneum* (Herbst) were also reported (Judzentiene and Buzelyte, 2006; Wang *et al*., 2006). The essential oil of mugwort exhibits many biological activities such as analgesic, antiseptic, antispasmodic, CNS-stimulant, larvicidal, insect repellant, insectifuge, antiasthmatic, antibacterial, Anti-inflammatory, sedative and are also used in flavor and perfumery industry (Teixiera da Silva, 2004).

The pharmacopoeias of traditional cultures throughout the world describe numerous uses of root extracts in the treatment of human diseases (Baker *et al*., 1995). Plant roots serve as a major source of natural products that include a bewildering diversity of secondary metabolites and bonafide proteins (Flores *et al*., 1999). Cell and tissue culture technology has facilitated development of methods for the production of useful biomass and secondary metabolites. *In vitro* root culture has become an alternative for the production of secondary metabolites (Verpoorte *et al*., 2002). The advantage of using root cultures is that they grow rapidly, are relatively easy to prepare and maintain, show a low level of variability and can be easily cloned to produce a large supply of experimental tissue. Root culture provides an efficient means of biomass production due to fast growth and stable metabolite productivity (Choi *et al*., 2000). Secondary metabolites from root and hairy root cultures have shown great promise for application in traditional medicine (Canto-Canche and Loyola-Vargas, 1999). The development of a fast growing root culture system would offer unique opportunities for producing root drugs in the laboratory without having to depend on field cultivation (Wadegaonkar *et al*., 2006). Thus, in the present study, pioneer experiments on adventitious root culture of *A. vulgaris* were conducted, where important factors affecting the induction of adventitious roots were evaluated.

Materials and methods

Explant collection

Seeds of *Artemisia vulgaris* L. were surface disinfected with 50% (v/v) ethanol for 30 sec, followed by rinsing three to five times in sterile distilled water. The seeds were then surface sterilized with 0.1% (w/v) aqueous mercuric chloride ($HgCl_2$) for 4-5 min and finally rinsed with autoclaved distilled water (three to five changes). Surface sterilized seeds were inoculated into culture flasks containing 25 ml MS basal medium and incubated in a dark chamber for 3-5 days at a temperature of 23 °C to facilitate germination. Later they were transferred to photoperiodic conditions (16/8 h light/dark) and maintained for

another 15 days for seedling growth. Leaf and root segments excised from 20 days old *in vitro* grown seedlings were used as the explant source for the present study.

Culture conditions

Explant segments were cultured on Murashige and Skoog (1962) basal medium supplemented with 3% (w/v) sucrose (Himedia, Mumbai, India) and 0.7% agar (Himedia, Mumbai, India). The pH of the medium (supplemented with respective growth regulators) was adjusted to 5.7 with 1N NaOH or 1N HCl before solidification. In all the experiments the chemicals used were of analytical grade (Himedia, Mumbai, India, Sigma-Aldrich, USA and E. Merck, Germany). The medium was dispensed into culture vessels (Borosil, India) and autoclaved at 105 K Pa and 121°C for 15 min. The explants were implanted horizontally on the culture medium (test tubes (150X25 mm) containing 15 ml medium) and plugged tightly with non-absorbent cotton. All cultures were incubated at 25±1 °C under a 16/8 (light/dark) photoperiod of 45-50 μ mol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes (Philips, India) and with 55-60% relative humidity (RH). All the subsequent subcultures were carried out at 2 week intervals.

Induction of adventitious roots from explants on solid medium

Explants were cultured on MS medium containing 3% (w/v) sucrose and 0.7% (w/v) agar supplemented with various concentrations of IAA (Indole-acetic acid) (0.5 – 28.5 μM) and IBA (Indole-3-butyric acid) (0.5 – 24.6 μM) individually and in combination for root induction and proliferation. The effect of IAA / IBA on root induction was assessed after 30 days of culture. A control group (MS basal medium without hormone supplementation) was maintained to compare the frequency of response. Fifty explants were used per treatment and the experiments were repeated five times.

Effect of basal media

Three different media including MS (Murashige and Skoog, 1962); B_5 (Gamborg *et al*., 1968) and SH (Schenk and Hilderbrandt, 1972) were evaluated for their effects on root induction in *A. vulgaris*. All the three basal media were supplemented with 3% (w/v) sucrose, 0.7% (w/v) agar and 11.4 μM IAA + 4.9 μM IBA.

Effect of different carbohydrates on root induction

Explants were cultured on MS basal medium supplemented with 11.4 μM IAA + 4.9 μM IBA, 0.7% (w/v) agar and different carbohydrates, including 3% (w/v) of sucrose, fructose, maltose, glucose and mannitol to evaluate the adequate carbon source for root culture.

Establishment of suspension cultures

Roots were isolated carefully and rinsed in sterile distilled water *in vitro* to remove the adhering medium. Then they were cut into small segments (1.5 – 2.0 cms length) and transferred immediately to conical flasks containing fresh MS liquid medium containing 11.4 μM IAA + 4.9 μM IBA. The influence of various media strengths such as ¼ MS, ½ MS and full strength were tested for root proliferation. The cultures were maintained in a rotary shaker with gentle shaking at 90 rpm (Neolabs Technology, Ghaziabad, India). Growth was assessed after 4 weeks of culture.

Effect of sucrose on root growth in liquid media

Liquid cultures were obtained by incubating 1.5 – 2.0 cms long roots (fresh weight 2.0 gms) in 250 ml Erlenmeyer flask containing 50 ml of MS medium + 11.4 μM IAA and 4.9 μM IBA supplemented with sucrose at 10, 20, 30, 40 and 50 gl^{-1}. Roots were grown in the dark at 25 ± 1°C on a rotary shaker at about 90 rpm. Biomass accumulation was calculated after 4 weeks of culture.

Observations and statistical analysis

Experiments were setup in a randomized completely block design (RCBD), about fifty explants were used per treatment and each experiment was repeated five times. Observations were recorded on the frequency (number of cultures responding for root initiation and growth) and the number of roots per explant, root length and fresh weight, respectively. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means and the treatment means were compared using Duncan's Multiple Range Test (DMRT) at a 5% level of significance (Gomez and Gomez, 1976). The results were analyzed statistically using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA) for windows.

Results and discussions

Effect of explants and exogenous auxins on adventitious root induction

The effect of two different explants (leaf and root) and two exogenous auxins IAA (0.5 – 28.5 μM) and IBA (0.5 – 24.6 μM) (individual and combination treatments), were tested for root induction in MS solid medium. Root initiation was observed after 2 weeks of culture (Figs. 1a, 1c). They were further subcultured on the same medium for further growth and development.

Both the explants responded well for root initiation. In control (MS basal) medium, leaf explants did not respond for root induction whereas root explants exerted 20.6% root induction with a maximum of 1.5 roots production and 2.1 cms root length. Out of the different concentrations of IAA tested, 11.4 μM IAA was found to exert the maximum response with 96.3% and 92.3% root induction frequency and 5.7 and 7.8 roots with 8.2 and 10.2 cms root lengths in leaf and root explants, respectively (Table 1). The maximum root length of 11.9 cms was recorded from root explants cultured on 5.7 μM IAA. When the different concentrations of IBA were tested, 4.9 μM IBA was found to respond maximum with 95.9% (leaf) and 89.8% (root) for root induction. The maximum number of roots achieved was 3.5 and 6.3 and the maximum root length recorded was 5.7 cms and 8.9 cms respectively from leaf and root explants (Table 1). Thus IAA was found to respond maximum for root induction than IBA on individual treatments. Different concentrations of IAA and IBA were augmented together to test the synergistic effect in root promotion. Out of the different combination treatments evaluated, 11.4 μM IAA + 4.9 μM IBA were found to be most suitable for root culture (Table 1). A maximum of 98.2% root induction with 8.9 roots and 12.2 cms root length was observed in leaf explants.

Whereas 96.9% root induction with 10.8 roots and 13.9 cms root length was recorded from root explants (Table 1). On the whole, combination treatment was found to be more suitable for the present experiment. Though root induction frequency was maximum in leaf explants, higher biomass production was achieved in root explants (Table 1). Root explants were reported to be suitable for adventitious root culture in several plants (Raggio and Raggio, 1956; Kevers *et al*., 1999; Betsui *et al*., 2004). Similarly leaf explants were also used to induce adventitious roots in several plants (Panichayupakaranant and Tewtrakul, 2002; Wadegaonkar *et al*., 2006; Nandagopal and Ranjitha Kumari, 2007). IBA was reported to be the potent auxin for root induction in *Albizzia kalkora* (Park *et al*., 2006) and *Rapanus sativus* (Betsui *et al*., 2004). In agreement with our findings IAA and IBA combinations was found to respond maximum for root culture in *Withania*

somnifera (Wadegaonkar *et al*., 2006). Thus all further experiments were conducted in IAA (11.4 μM) + IBA (4.9 μM) combination.

Effect of different basal media on root induction

Of the three different basal media viz., B5, MS and SH evaluated, MS medium was found to yield the maximum result with 98.2% and 96.9% root induction frequency and 8.9 and 10.8 roots per leaf and root explants, respectively (Table 2). A similar report was stated earlier (Wadegaonkar *et al*., 2006). Explants cultured on B_5 medium induced a maximum of 82.7% (leaf) and 82.1% (root) root induction whereas SH medium exerted a maximum of 71.3% and 69.7% rhizogenesis (Table 2). The maximum number of roots were 6.8 and 7.4 in B_5 medium and 5.9 and 8.9 in SH medium from leaf and root explants (Table 2). Thus MS medium was found to yield the maximum response. A similar result was reported (Betsui *et al*., 2004). In contrary B_5 medium was found to respond better in *Plumbago rosea* (Panichayupakaranant and Tewtrakul, 2002) whereas SH medium was found to be suitable in *Brugmansia candida* (Nino *et al*., 2003). The most prominent difference between MS and other media is the lower NH_4^+ / NO_3^- ratio which might have influenced rhizogenesis (Russowski *et al*., 2006).

Effect of carbohydrates on root induction

The importance of carbon source in plant tissue culture has long been recognized. Carbohydrates are one of the five classes of essential substances needed for growth and development. Sugars have important signaling functions throughout all stages of the plant life cycle from germination and vegetative growth to reproductive development and seed formation. Carbohydrates can signal alterations in gene expression similar to the concepts developed for hormones. Sugar sensing can be defined as the interaction between a sugar molecule and a sensor protein in such a way that a signal is generated. This signal then initiates signal transduction cascades that result in cellular responses such as altered gene expression and enzymatic activities (Smeekens, 2000). In cultured plant tissues, a continuous supply of carbohydrates from the medium is necessary, since the photosynthetic activity of *in vitro* tissues is reduced because of low light intensity, limited gas exchange and high relative humidity (Kozai, 1991).

In the present study, different carbohydrate sources (each 3% (w/v)) were tested for their effect on rhizogenesis. Out of the five different carbohydrates experimented, sucrose exerted the maximum response (98.2% , 96.9%) in both the explants followed by fructose (91.6%, 90.5%), glucose (85.4%, 88.9%),

maltose (79.4%, 72.6%) and mannitol (71.2%, 67.3%) (Fig. 2). Sucrose is the most commonly used carbohydrate source for plant tissue cultures and most culture media have it as the sole carbohydrate source (Nin *et al*., 1997; Nandagopal and Ranjitha Kumari, 2007). However, other sugars have been reported as suitable carbon sources than sucrose (Scott and Lyne, 1994). It was found that different patterns of morphogenesis were attributable to the type of carbohydrate and its concentration (Thorpe, 1980; Romano *et al*., 1995).

Influence of MS media strength on root culture in liquid medium

Roots were cultured on ¼, ½ and full strength MS liquid medium to evaluate root proliferation. It was found that full strength MS medium was required for continuous production of roots. The maximum proliferation rate observed was 99.8% (Fig. 3; Figs. 1b, 1d). Whereas ¼ MS exerted a maximum proliferation rate of 67.4% and ½ MS exerted a maximum of 75.2% (Fig. 3). Half-strength medium was found to respond well for root cultures (Nino *et al*., 2003; Park *et al*., 2006). But in the present investigation, maximum response was achieved only in full strength MS medium. This clearly depicts that the supply of adequate concentrations of nutrients is essential for excised organ culture.

Impact of different concentrations of sucrose on root growth

During 4 weeks of culture, the fresh weight increased markedly with higher sucrose concentrations after which it declined (Fig. 4; Figs. 1b, 1d). The biomass increase appeared to be high compared to previous reports (Panichayupakaranant and Tewtrakul, 2002). Sucrose at 30 g/l was found to yield the maximum biomass of 8.2 gms fresh weight than other concentrations tested (Fig. 4). This was due to the intense profusion of lateral branches. A similar result was reported (Nin *et al*., 1997). In contrast, root growth inhibition and root morphological modifications when the sucrose concentration exceeded 30 g/l. In this condition, roots appeared callused and without branching, probably due to osmotic stress.

Thus the present study describes a simple, efficient protocol for adventitious root induction from the two different explants and the various parameters to be considered for enhanced root induction. This reproducible protocol can be used for the large-scale production of adventitious roots and for the subsequent production of root-specific secondary metabolites, thus conserving the wild. To-date this is the first report on adventitious root induction in *A. vulgaris*.

Table 1. Effect of different auxins on adventitious root induction from leaf and root explants of *A. vulgaris*

Concentration of auxins (μm)	Root induction frequency (%)		Number of roots		Root length (cms)	
	Leaf	Root	Leaf	Root	Leaf	Root
Control	-	20.6 g	-	1.5 f	-	2.1 f
IAA						
0.5	77.1 d	78.2 cd	2.8 d	3.4 d	4.9 d	7.8 d
2.8	80.2 cd	80.9 c	3.0 cd	3.8 c	5.3 c	9.6 bc
5.7	87.6 b	86.5 b	3.2 c	4.5 bc	6.6 b	11.9 a
11.4	96.3 a	92.3 a	5.7 a	7.8 a	8.2 a	10.2 b
17.1	81.0 c	74.9 d	4.1 b	5.1 b	6.1 bc	8.9 c
22.8	73.4 de	69.1 e	3.5 bc	3.9 c	4.8 d	7.8 d
28.5	67.3 e	60.7 f	2.3 de	2.7 e	3.2 e	6.4 e
IBA						
0.5	78.6 cd	69.1 cd	2.1 c	3.2 de	3.7 d	6.6 c
2.4	86.4 b	79.3 b	2.7 b	4.8 bc	4.1 c	7.4 b
4.9	95.9 a	89.8 a	3.5 a	6.3 a	5.7 a	8.9 a
9.8	81.5 c	79.8 b	3.1 ab	5.4 b	5.2 ab	7.2 bc
14.7	76.1 d	73.7 c	2.5 bc	4.3 c	4.8 b	6.7 c
19.6	63.2 e	64.8 d	2.0 c	3.7 d	4.1 c	5.8 d
24.6	52.7 f	51.9 e	1.7 d	2.9 e	3.6 d	4.9 e
IAA + IBA						
11.4 + 2.4	91.1 c	89.5 b	5.8 bc	7.6 c	8.5 cd	10.4 d
11.4 + 4.9	98.2 a	96.9 a	8.9 a	10.8 a	12.2 a	13.9 a
11.4 + 9.8	92.3 b	89.4 b	6.4 b	8.4 b	10.5 b	12.4 b
5.7 + 4.9	90.6 cd	88.8 c	4.0 c	7.3 cd	11.2 ab	11.3 c
17.1 + 4.9	85.4 d	82.7 d	3.2 d	6.5 d	9.1 c	9.5 de

- = nil response, * Data recorded after 30 days of culture, *Treatment means followed by different letters are significantly different from each other ($p<0.05$) according to Duncan's Multiple Range Test.

Table 2. Effect of basal media on adventitious root induction in *A. vulgaris*

Basal Media	Frequency of root induction (%)		Number of roots	
	Leaf	Root	Leaf	Root
B_5	82.7 b	82.1 b	6.8 b	7.4 bc
MS	98.2 a	96.9 a	8.9 a	10.8 a
SH	71.3 c	69.7 c	5.9 bc	8.9 b

* Data recorded after 30 days of culture, *Treatment means followed by different letters are significantly different from each other ($p<0.05$) according to Duncan's Multiple Range Test

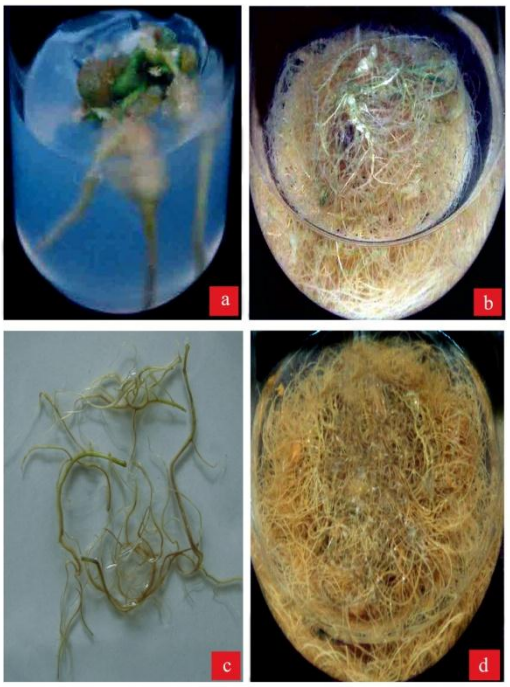

Fig. 1. Adventitious root induction and establishment of root cultures from leaf and root explants of *A. vulgaris*
a – Root initiation from leaf explant (2 weeks old), b – Biomass accumulation of leaf explant derived roots in suspension culture (4 weeks old),c – Root initiation from root explant (2 weeks old), d – Biomass accumulation of root explant derived roots in suspension culture (4 weeks old)

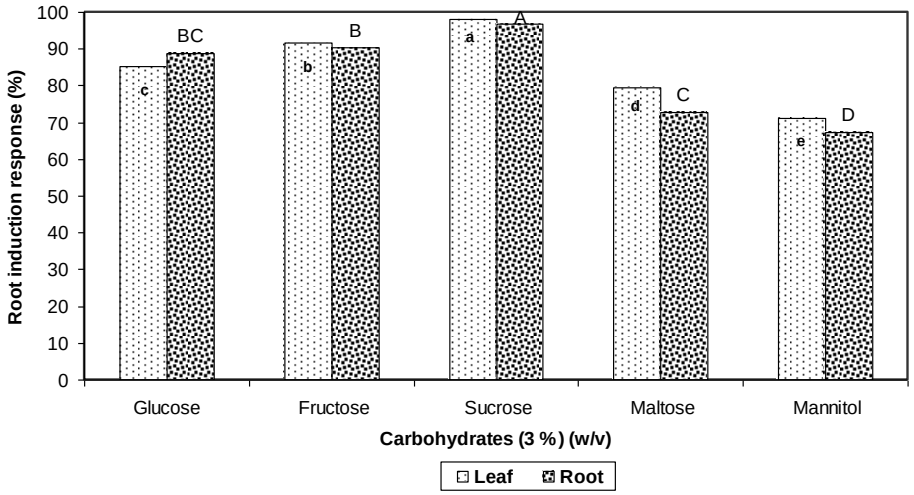

Fig. 2. Influence of different carbohydrate sources on adventitious root induction in *A. vulgaris*.
* Data recorded after 30 days of culture
*Treatment means followed by different letters are significantly different from each other ($p<0.05$) according to Duncan's Multiple Range Test

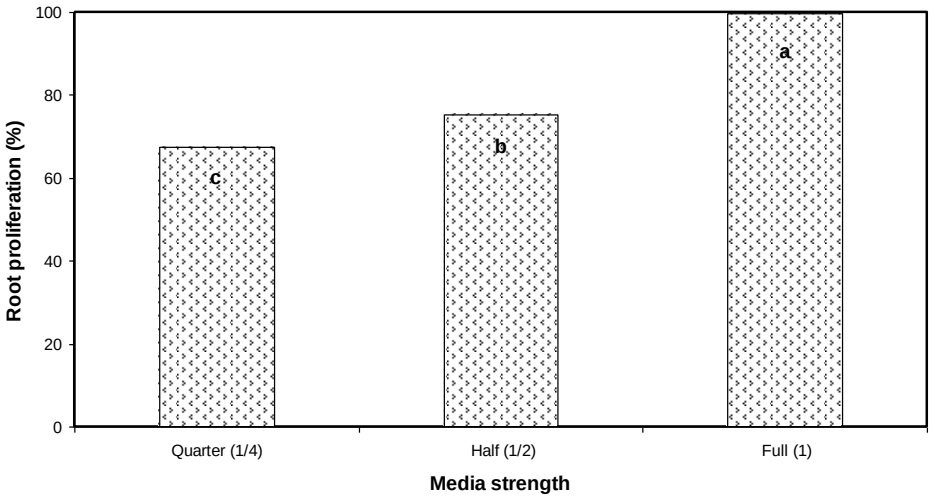

Fig. 3. Effect of MS media strength on root proliferation of *A. vulgaris* in liquid media.
* Data recorded after 4 weeks of culture
*Treatment means followed by different letters are significantly different from each other ($p<0.05$) according to Duncan's Multiple Range Test

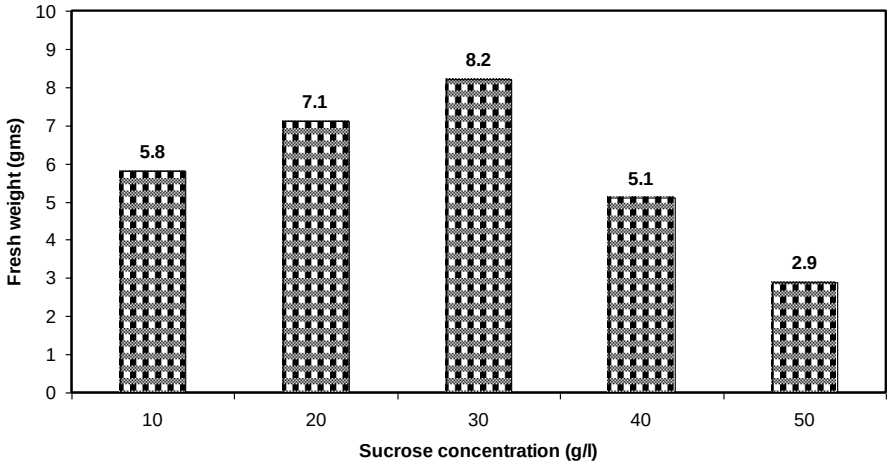

Fig. 4. Effect of sucrose concentration on biomass accumulation in root culture of *A. vulgaris*.
* Data recorded after 4 weeks of culture, *Treatment means followed by different numbers are significantly different from each other ($p<0.05$) according to Duncan's Multiple Range Test

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