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EFFECT OF SILVER NITRATE AND DIFFERENT CARBON SOURCES ON *IN VITRO* SHOOT MULTIPLICATION OF *SPHAERANTHUS INDICUS* (LINN.)-AN IMPORTANT ANTIJAUNDICE MEDICINAL PLANT

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ABSTRACT

An attempt has been made to study the influence of different carbon sources such as sucrose, fructose, glucose and maltose (1-6%) and ethylene inhibitor silver nitrate (0.4 mg/l) along with Kn (1.0 mg/l) and NAA (0.1 mg/l) was investigated for the growth of multiple shoots from axillary bud or nodal explants of S. indicus. The regeneration frequency, growth and multiplication rate were highly influenced by the type and concentration of carbohydrates and $AgNO_3$ used. The utmost number of shoots (29.1) was obtained on MS medium augmented with 3% fructose and 0.4mg/l $AgNO_3$ when compared to media devoid of silver nitrate. In the lack of carbon sources there is no regeneration was originate. Observations of the shoot cultures developed on media containing one of these carbon sources indicated that 3% fructose was the preferential carbohydrate for the production of multiple shoots followed by sucrose, maltose and glucose from nodal explants of S. indicus. In vitro shoots were then excised from the shoot clumps and transferred to the rooting medium containing NAA, IBA (1.0-2.0 mg/l) and $AgNO_3$ (0.1-0.6 mg/l). The well rooted plantlets were then separated from the culture tubes and transferred into sterile soil + vermiculate (1:1) in green house. Finally the hardened plants were transferred to the field environment for utmost survivability.

KEY WORDS

Axillary bud explants, Carbon sources, Micropropagation, Silver nitrate, Sphaeranthus indicus

INTRODUCTION

Sphaeranthus indicus (Linn.) is one of the important herbaceous medicinal plants belonging to the family Asteraceae. It is commonly known as 'Boddasoram' in Telugu and 'East Indian globe thistle' in English. The plant is much branched, aromatic and grows up to 30 – 60 cm height. It is mainly found in paddy fields and damp situations in the plains all over India, Ceylon, Malaysia, China, Africa and Australia ascending to an altitude of 1500 m in the hills, especially as a weed in the paddy fields [1]. *S. indicus* has long been used in the indigenous medicine. All parts of the plant find medicinal uses. The herb is bitter and hot with a sharp sweet taste and the juice of the plant is styptic and said to be useful in treating jaundice, diseases of the spleen, elephantiasis, anemia, pain in the uterus and vagina, epileptic convulsions, leukoderma, dysentery, hemicranias (Ayurveda). The powdered seeds and roots are given as an anthelmentic. The bark powder mixed with whey is a valuable remedy for piles. Flowers are credited with alternative, depurative and tonic properties. The oil obtained from the root is aphrodisiac, used in prolapsus ani (Unani) [2]. The whole herb is used in ayurvedic preparations to treat epilepsy and mental disorders [3] and hepatitis [4].

In micropropagation sugars serve as major carbon source to provide optimal environment for plant regeneration. Hence, it is essential to add the primary metabolite carbohydrate to tissue culture media for *in vitro* plant propagation. In general the regeneration frequency increases with increasing the concentration of carbon sources until an optimum is reached and decreases at higher concentration. Thus the *in* vitro plantlets require a carbohydrate supply to meet the energy requirements [5]. The involvement of ethylene in plant tissue growth and differentiation has been widely investigated. Application of ethylene

precursors and/or inhibitors has shown that ethylene may often have diverse effects in similar tissue culture systems. Although it has been reported that ethylene may promote callus growth [6], it generally appears to inhibit shoot regeneration [7]. Silver nitrate ($AgNO_3$), a potent inhibitor of ethylene action [8], was shown to promote regeneration in *Brassica campestris* [9] and *Helianthus annuus* [10]. Therefore, the aim of the present study was to determine the effect of silver nitrate and different carbon sources such as sucrose, glucose, fructose and maltose on *in vitro* shoot regeneration from nodal explants of *Sphaeranthus indicus.*

MATERIALS AND METHODS

Source of plant material

The plant material of *Sphaeranthus indicus* (Linn.) for the experiment was collected from the Herbal garden of Dravidian University, Kuppam, Andhra Pradesh, India, and also from the fields in the surroundings of the Dravidian University campus.

Preparation and sterilization of plant material

Actively growing shoots with 5-6 nodes were excised from 2-3 months old field grown mature plants raised from the seeds. After leaf excision, the axillary buds were dissected from the shoots and washed in running tap water for 10-15 min. then in 5% (v/v) Tween-20, a liquid detergent for 5 min. followed by continuous washing in distilled water until all the traces of detergent was removed. Then the explants were soaked in 0.4% (w/v) bavistine for 10 min. and finally surface decontamination of the explant was performed by passing through a solution of 0.1% Mercuric Chloride (w/v) for 2 min. The optimal exposure time and optimal concentration of the surface sterilent was determined after several initial trials. Surface sterilization was followed by 4-5 rinses in sterile distilled water. The cut ends of the explants were further trimmed and axillary buds/nodes (1.0 - 1.5 cm) were prepared. Then the explants were blotted on sterile filter paper discs to absorb excess of water before planting them vertically on agar gelled MS media in culture vessels.

Culture medium and culture conditions

The shoots formed *in vitro* in different media were separated aseptically and axillary buds excised from the regenerated shoots were used as explants as a prerequisite for the present study. The excised axillary buds were inoculated on MS medium [11] containing various concentrations of different sugars, Kn, NAA and $AgNO_3$. 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 dispensing the media into the containers (15ml for 25 x 150 mm test tubes) 0.8% (w/v) agar was added to the media and melted. Culture vessels containing media were sterilized at 15 lbs. pressure in an autoclave at 121°C for 15-20 min. 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 *Sphaeranthus indicus* were maintained in a culture room at temperature of 24 ± 2° C and 55-65% RH with 16 h/8 h photoperiod at a photon flux density of 3000 lux or 50-70 Em^{-2} s^{-1} provided by cool white fluorescent tubes. Sub culturing was carried out at regular intervals of thirty days. Visual observations of the cultures were taken for every transfer and the effects of different treatments were quantified on the basis of percentage of cultures showing response.

Data collection and statistical analysis

Visual observations were recorded on the frequency in terms of number of cultures responding for axillary shoot proliferation; shoot development, number of shoots per explant, average length of the regenerated shoots, number of roots per shoot and average root length.

Despite scarcity and limitations encountered with the plant material, for most of the treatment a minimum of 10 replicates were used. All the experiments were repeated at least twice/thrice and the cultures were observed at regular intervals. The qualitative data were subjected to statistical analysis by using standard error (SE±) for shoot length, rate of shoot multiplication and then number of roots per shoot.

RESULTS AND DISCUSSION

Efffect of $AgNO_3$ and different carbon sources on *in vitro* plant regeneration from nodal explants

In this study different types and concentrations of carbon sources were tried along with $AgNO_3$ (0.4mg/l) to study their effect on shoot multiplication from

axillary bud explants of *Sphaeranthus indicus*. Among the different carbohydrates tested, fructose performed well followed by sucrose, maltose and glucose. The maximum shoot number (29.1±0.16) was recorded at MS medium supplemented with 3% of fructose along with $AgNO_3$ (0.4mg/l), Kn (1.0mg/l) and NAA (0.1mg/l). Highest frequency of shoot regeneration was observed both at 3% of sucrose (98%) and 3% of fructose (95%) (Table-1 and Fig-1). Least number of shoots (1.4±0.34) was observed in media augmented with 6% of glucose. In all the concentrations tested and observed the second highest mean number of shoots was noticed at 3% of sucrose (23.7±0.20). But, there is no regeneration was observed in absence of carbohydrates in the media. Similarly supplementation of $AgNO_3$ (0.4mg/l) along with carbon sources into the media had increased shoot proliferation over three folds when compared to control.

In plant tissue culture continuous supply of carbohydrates is essential, since the photosynthetic activity of *in vitro* plant tissue culture is reduced due to low light intensity, high humidity and limited gaseous exchange [12]. Although sucrose has been the carbohydrate of choice in the vast majority of work on *in vitro* shoot induction and shoots development, it is not always the most effective carbon source for regeneration [13]. The growth and multiplication of shoots *in vitro* are affected by many factors, one of which is the concentration and type of exogenous carbon source added to the medium [14].

The carbon sources serve as energy and osmotic agents to support the growth of plant tissues. Due to the presence of low levels of carbon dioxide concentration present in *in vitro* conditions, an appropriate type and concentration of sugar is needed to promote seed germination and regeneration of plant [15]. But, in the present study fructose along with $AgNO_3$, Kn and NAA was performed well to produce maximum number of shoots. Similar enhancement was observed in *Coffea canephora* [16] where a carbohydrate along with AgNO3 increases somatic embryo production. However, there are many reports that different carbon sources are proved to be better for *in vitro* propagation like in *Stevia rebaudiana* [17], in *Asparagus* [18], in cucumber [19], but still now there are no reports on the effect of different carbon sources along with along with $AgNO_3$ on multiple shoot formation in medicinal plants.

Presence of $AgNO_3$ in the media showed distinct significance in *in vitro* propagation. Among all the concentrations tested media devoid of $AgNO_3$ showed lesser response and media without energy sources showed no regeneration. From the results it is clearly emphasized that the presence of optimal concentration of $AgNO_3$ and carbon sources had increased shoot proliferation in *S. indicus*.

Effect of silver nitrate on *in vitro* rooting

Induction of rooting is an important step in the propagation of plant species. Healthy shoots developed *in vitro* (3.0 – 5.0 cm in length) were excised from shoot clumps and transferred for rooting to MS media containing different concentrations of auxins such as NAA and IBA (1.0 – 2.0 mg/l) along with silver nitrate (0.1 – 0.6 mg/l). The comparison of three different auxins in the aspect of rooting response by using $AgNO_3$ was represented (Table – 2). From the data obtained, it was observed that *in vitro* shoots grown on MS medium supplemented with 2.0 mg/l NAA and 0.4 mg/l $AgNO_3$ produced highest rooting percentage (95%), average roots per shoot (34.6±0.26) and average root length (13.5±0.36 cm) followed by MS medium supplemented with 2.0 mg/l IBA and 0.4 mg/l $AgNO_3$, which gave 85% rooting percentage, average root number (20.8±0.56) and average root length (8.56±0.32 cm). These results are in agreement with those obtained in *Rotula aquatica* [20]. Ma et al (1998) [21] demonstrated that the use of ethylene inhibitors such as $AgNO_3$ and $CoCl_2$ may promote root formation in apple.

There was no rooting in case of shoots inoculated on auxin free basal media at any strength. At all the concentrations tried, exogenous supply of $AgNO_3$ with auxins favoured root formation. In all the concentrations tested, root primordial appeared between 10 – 15 days of inoculation. It was observed that, as the concentration of auxins and silver nitrate increased after certain levels the rooting response and rooting number was decreased. Lower concentrations of auxins either NAA or IBA along with

$AgNO_3$ (0.4 mg/l) favoured the mean number of root formation and root length in *S. indicus*.

Acclimatization and hardening

The well rooted shoots were removed from the culture tubes and washed thoroughly to remove the traces of agar. The plantlets of *in vitro* grown *Sphaeranthus indicus* with well developed roots and shoots were transplanted to plastic bags containing autoclaved vermiculite and soil (1:1). About 90% of the transplanted plantlets survived after acclimatization and showed healthy growth without any morphological variations. Finally after one month the hardened plants were transferred to pots containing garden soil and sand (2:1) and were allowed to grow under nursery shade conditions. These plants were watered at 3 days intervals and were finally planted in field condition. All the plantlets were phenotypically indistinguishable from the parent plants.

CONCLUSION

It can be concluded that among the different carbon sources tested, fructose along with silver nitrate (0.4mg/l) showed better response followed by sucrose, maltose and glucose in terms of multiple shoot induction. Since fructose and sucrose are the better carbohydrate sources for *in vitro* shoot multiplication of *Sphaeranthus indicus*. However, further research is required to explore the effect of different carbon sources on *in vitro* plant regeneration.

ABBREVIATIONS

Kn	Kinetin
$AgNO_3$	Silver nitrate
NAA	α – naphthalene acetic acid
IBA	Indole – 3 – butyric acid
MS	Murashige and Skoog

Table-1: Effect of different carbon sources and $AgNO_3$ on multiple shoot induction from axillary bud explants of *in vitro* grown *Sphaeranthus indicus* supplemented with 1.0 mg/l Kn and 0.1 mg/l NAA. Observation: after 8 weeks, values are mean ± S.E of 10 replicates.

Energy source	Conc-entra-tion %	Regeneration Frequency (%)		Mean number of shoots		Mean shoot length (cm)		Callus formation	
		Without $AgNO_3$	0.4 mg/l $AgNO_3$	Without $AgNO_3$	0.4 mg/l $AgNO_3$	Without $AgNO_3$	0.4 mg/l $AgNO_3$	Without $AgNO_3$	0.4 mg/l $AgNO_3$
Control	**No ener-gy source**	-	-	-	-	-	-	-	-
Glucose	1	70	75	3.4±0.31	6.5±0.13	2.15±0.15	4.7±0.05	-	-
	2	74	80	4.8±0.46	7.5±0.22	3.6±0.32	5.8±0.44	-	-
	3	77	85	6.4±0.28	10.8±0.14	4.2±0.23	4.7±0.13	-	-
	4	85	90	8.0±0.37	13.3±0.31	3.1±0.14	3.9±0.04	-	-
	5	70	90	3.4±0.21	6.6±0.23	5.2±0.26	6.2±0.16	-	-
	6	65	86	1.4±0.34	2.3±0.87	6.7±0.19	7.5±0.52	-	-
Sucrose	1	70	83	4.4±0.19	9.2±0.14	3.0±0.25	4.0±0.31	-	-
	2	75	87	7.2±0.41	18.8±0.62	6.7±0.52	7.9±0.12	-	-
	3	85	98	14.7±0.56	23.7±0.20	5.3±0.41	5.1±0.11	-	-
	4	88	90	10.2±0.29	13.2±0.56	4.2±0.28	6.4±0.03	-	-
	5	79	85	6.4±0.57	7.3±0.28	4.6±0.35	8.6±0.15	-	-
	6	68	83	2.5±0.33	3.2±0.14	4.9±0.15	11.8±0.17	-	-
Fructose	1	55	75	9.4±0.31	10.6±0.28	3.2±0.13	6.2±0.21	-	-
	2	64	78	14.4±0.11	21.3±0.43	6.0±0.12	7.0±0.19	+	+
	3	80	95	28.4±0.38	29.1±0.16	5.4±0.22	4.4±0.34	+	+
	4	85	84	7.2±0.43	17.3±0.41	3.8±0.25	5.8±0.08	-	+
	5	73	80	5.4±0.31	9.1±0.51	4.7±0.52	3.7±0.16	-	-
	6	60	72	3.9±0.27	5.5±0.17	5.2±0.14	6.2±0.10	-	-
Maltose	1	63	70	4.6±0.53	7.4±0.61	4.3±0.31	6.3±0.53	-	-
	2	70	85	8.4±0.18	9.3±0.28	8.2±0.26	10.7±0.17	-	-
	3	75	83	7.0±0.47	13.0±0.18	4.3±0.24	8.3±0.46	-	+
	4	80	77	5.7±0.10	22.6±0.21	3.8±0.42	5.8±0.02	+	+
	5	76	70	3.4±0.62	8.5±0.53	4.5±0.28	6.5±0.18	-	-
	6	68	67	2.9±0.16	4.9±0.36	6.3±0.51	9.3±0.47	-	-

Intensity of callus: - = No callus, + = Very low

Table-2: Effect of $AgNO_3$ and different concentrations of NAA and IBA on *in vitro* rooting of *Sphaeranthus indicus* using MS medium. Observation: after 8 weeks, values are mean ± S.E. of 10 independent determinants.

Plant growth regulators (mg/l)		Concentration of $AgNO_3$ (mg/l)	Regeneration frequency (%)	Mean no. of roots/shoot	Mean root length (cm)
NAA	IBA				
1.0	-	-	65	7.6 ± 0.20	2.76 ± 0.31
1.0	-	0.1	75	11.9 ± 0.32	3.5 ± 0.19
1.0	-	0.2	80	15.5 ± 0.35	4.3 ± 0.20
1.0	-	0.4	85	19.5 ± 0.23	9.9 ± 0.73
1.0	-	0.6	75	8.5 ± 0.18	3.0 ± 0.34
2.0	-	-	70	9.6 ± 0.32	3.54 ± 0.28
2.0	-	0.1	80	13.9 ± 0.14	4.3 ± 0.37
2.0	-	0.2	85	17.4 ± 0.40	4.6 ± 0.29
2.0	-	0.4	95	34.6 ± 0.26	13.5 ± 0.36
2.0	-	0.6	80	20.3 ± 0.21	3.2 ± 0.35
-	1.0	-	60	6.7 ± 0.19	2.2 ± 0.14
-	1.0	0.1	70	11.5 ± 0.36	2.7 ± 0.15
-	1.0	0.2	75	14.5 ± 0.14	3.3 ± 0.32
-	1.0	0.4	80	17.9 ± 0.36	7.0 ± 0.41
-	1.0	0.6	72	9.0± 1.30	5.8 ± 0.1
-	2.0	-	65	9.5 ± 0.32	2.4 ± 0.28
-	2.0	0.1	70	12.4 ± 0.15	3.6 ± 0.30
-	2.0	0.2	78	15.4 ± 0.29	5.3 ± 0.61
-	2.0	0.4	85	20.8 ± 0.56	8.56 ±0.32
-	2.0	0.6	70	10.6 ± 0.30	3.1 ± 0.43

Fig-1: Multiple shoot initiation from axillary bud explants supplemented with A) MS+3% fructose, B) MS+3% sucrose, C) MS+4% maltose, D) MS+4% glucose, **E)** Initiation of roots from the regenerated shoots *in vitro* on MS medium + NAA (2.0mg/l) + AgNO3 (0.4mg/l), F**)** Plantlet showing elongated root system, G**)** Hardened plantlet in polybags containing soil and vermiculite in 1:1 ratio, H**)** Plantlet in field condition

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