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DOES THE SILVER NITRATE INFLUENCE THE VARIOUS ENERGY SOURCES IN MICROPROPAGATION OF *SOLANUM VIARUM* DUNAL – AN IMPORTANT ANTI CANCER MEDICINAL PLANT?

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ABSTRACT

Solanum viarum, a native species of Argentina, commonly known as tropical soda apple, due to unrestricted exploitation for its bio active medicinal compounds is becoming as one among the threatened and endangered species in India. Though the *in vitro* micropropagation is the best promising alternative for conservation, energy sources used and excess production of ethylene in the media are the major constrains. To overcome the above said situation for the first time we had made an attempt study the effect of $AgNO_3$, a natural inhibitor of Ethylene on various energy sources like Glucose, Sucrose, Fructose and Maltose (1-6%). Incorporation of $AgNO_3$ (0.4mg/L) in to the culture medium of various levels of different energy sources of *Solanum viarum* has significantly influenced regeneration frequency, shoot proliferation and also rooting at different Auxin concentrations. There is an astonishing noticeable three folds increase caused by presence of silver nitrate in proliferation of shoots and roots observed after careful study of statically analysed data. Though the explants responded appreciably maximum at 4% Glucose with maximum mean number of shoots (42.8 ± 0.21) and 85% of regeneration frequency, their longevity over sub culturing was observed to be not promising when compare to 3% Sucrose (36 ± 0.37 Mmns and 100% Rf) which maintained the vigor over sub culturing on the same media in the presence of silver nitrate and also the maximum mean shoot length 10.9 ± 0.11cm was observed at these same concentration. 1 mg/L and 2mg/L IAA was found to be best concentrations for rooting With maximum mean number of roots (40.2 ± 0.13) and maximum mean root length (11.28 ± 0.14) respectively.

KEYWORDS: Carbohydrates**,** Silver nitrate, Shoot proliferation Ethylene inhibitor, regeneration frequency.

INTRODUCTION

Solanum viarum is one of the most important multipurpose medicinal plant belongs to the family Solanaceae and serves as a major source for Solasodine, a Glycoalkaloid constituent of solanum sps. Solasodine has been recognized as the potential alternative to an anti cancer precursor diosgenin which share a characteristic conversion to 16-dehydro pregnenolone acetate, the first step in steroid synthesis. Solasodine has been extensively exploited pharmaceutically in large scale to synthesis steroid hormone to treat cancer (Trouillas et al, 2005), Addison's disease, rheumatic arthritis, chronic asthma, leukemia, obesity, palsy and in skin diseases (Pingle & Dhyan sagar,1980) also used in preparing anti – fertility (Everist, 1981) and anti- inflammatory steroidal drugs (Pandurangan et al, 2011). Despite of economic importance, this plant becoming endangered and threatened by over exploitation by pharmaceutical industries, urbanization, desertification, industrial development and attack by numerous parasites. Though each plant produces 40 to 50 thousands seeds per annum, due to prolonged seed dormancy many times they fail to germinate. So to overcome this problem and also in order to meet the world's pharmaceutical industries demand, there is a great need to establish an *in vitro* plant regeneration system to enhance their productivity and sustainability. Some plant regeneration procedures through organogenesis, protoplast culture, Ploidy breeding (Thejovathi et al, 1996) were established by previous researchers. Despite many efforts, the underlying major problem in these procedures is a low frequency of regeneration, less number of multiple shoots and longevity in cultures.

The expression of cellular totipotency relies mainly on the phytohormone Ethylene which influences regeneration frequency, embryogenesis, shoot regeneration and rooting on which the entire concept of *in vitro* tissue culture depends. Ethylene is a gaseous hormone produced in closed vessels of *in vitro* tissue cultured tubes, where there will be no or limited gaseous exchange. It's a simple hydrocarbon (C_2H_4), but has maximum impact on growth, cellular differentiation, fruit ripening and senescence in plants when it's concentration as low as 0.01µL L^{-1} (Reid, 1995). So excess accumulation of gases directly influences the successes of *in vitro* regeneration. Ethylene suppresses the growth and morphogenesis of explants depending on the species and stage of the culture (Kumar et al). To subside these lacunae various Ethylene inhibitors like $AgNO_3$ have been used in media by many previous peer

scientists. According to literature silver masks the activity of ethylene and acts as a strong inhibitor. In some species silver nitrate improved callus proliferation (Fei et al, 2000), shoot organogenesis(Emi et al,2012), microspore embryogenesis (Khandakar et al 2013), *in vitro* flowering (Pratheesh et al, 2011). Hence the effect of silver nitrate on systematic propagation of this medicinally important endangered *Solanum viarum* was under taken for the first time Micropropagation of medicinally important plants is mainly influenced by several medium growth supplements, among those carbohydrates play an important role. In *in vitro* cultures carbohydrates serves as not only energy sources but also as osmotic agent (Lipovska and Konranodona, 2004). The type and levels of exogenous carbohydrate supplements play a dynamic role in plant growth and multiplication (Hossain et al., 2005). In addition, sugar sensitive plant gene which plays an important role in cellular adjustment to critical nutrient availability, might show carbohydrate modulated gene expression at various levels (Koch, 1996).

Only a limited number of plant cell lines have been isolated which are autotrophic when cultured in vitro. Autotrophic cell lines are those which are capable of synthesizing their own carbohydrates as energy source directly from carbon dioxide assimilation (Bergman 1967, Larosa et al, 1981). But these lines show very slow growth (Fukuma & Hilderbrandt, 1967), especially in the successesful ambient atmosphere where in the CO_2 is low. Growth of these autotrophic cell lines always depend on the enriching CO_2 concentrations in the culture tubes during the photoperiod and also reducing or eliminating sugar from the medium, to optimize in vitro environment. Neither of the conditions can be controlled in *in vitro* propagations. The presence of excess of carbohydrates in tissue culture media specially inhibits chlorophyll formation and photosynthesis making autotrophic growth less feasible.

In plant tissue culture continuous supply of carbohydrate is essential, since the photosynthetic activity of in vitro plant tissue is reduced due to low light intensity, high humidity and limited gaseous exchange (Kozai, 1991). However it was suggested by many earlier workers that successes of *in vitro* propagation not only depends on the choice of media but also on the type of the carbon sources (saccharides) and different additives which enhances the positive response of the explants in the culture media.

Among all carbohydrates sources sucrose found to be more advantageous due to its efficient uptake across plasma membrane (Fuents). Sucrose 2-5% is the most popular carbohydrate used with Tissue cultures (Bridgen, 1994). Sucrose also found to show better regeneration

than other carbon sources in cork oak (Ramano et al, 1995), in Linum (Cunha, 1999). However it's also reported by many other researchers that the different carbon sources like Glucose, Fructose and Maltose are also proved to be better carbohydrate sources for in vitro propagation. Fructose have been reported to be effective in preventing hyperhydricity and helps in production of adventitious shoots in Almonds (Rugin et al),also achieved maximum number of shoots in mulberry (Vijay chitra), in *Solanum nigram* (Sredhar et al, 2011), in *Menta piperata* (Sujana et al). Another major carbon source in culture media proved to be Glucose by R. K Jain in case of *Rosa indica*, and by Hisashi & Yasuhiro in *prunus mume*.

The present study was taken up to study the affect of different carbon source in the presence of 0.4 mg/L $AgNO_3$ which enhanced the plant growth on in vitro proliferation, with particular reference to culture longevity and affect of different Auxins on enhanced rooting of *Solanum viarum.*

MATERIALS AND METHODS

This investigation has been conducted in Plant Biotechnology laboratory at Department of Biotechnology, Dravidian University, kuppam. A.P. India, between February, 2014 to December, 2014.

Plant material

Healthy seeds were pooled from ripen and dried fruits of Solanum viarum plant grown in Mulika vanam of Dravidian University, Kuppam. Matured seeds were washed under running tap water for 10 minutes to remove adherent fruit tissues and dried juice, to avoid fungal contamination while culturing. Then the seeds were soaked in 0.4% bavistin (W/V) for 1 minute and 0.1% $HgCl_2$ for 2 minutes. Finally seeds were sterilized with sterile distilled water to remove the traces of all sterilants.

Under aseptic conditions seeds were inoculated on autoclaved M.S media (Murashige & Skoog, 1962) basal medium. Prior to autoclaving at 121^{o}c for 20 minutes the P^{H} of the medium was adjusted to 5.7. Cultures were maintained at 25 ± 2^{o}c under 16hrs and 8hrs dark for 8 weeks. The primary shoots developed further sub cultured in vitro under aseptic conditions as mentioned above in 2mg/l BAP supplemented M.S media. Healthy and uniform leaves were excised from in vitro sub cultured shoots and used as explants for further experiments.

M.S media supplemented with various carbon sources

Uniform sized leaves were transferred to M.S media supplemented with 2mg/L BAP, 0.5mg/L IAA and 0.4mg/L $AgNO_3$ and also supplemented with various concentrations (1-6%) of different carbon sources viz., Glucose, Sucrose, fructose and Maltose maintained separately. All culture conditions were maintained as mentioned above.

Rooting and Acclimatization

After sufficient elongation shoots were excised and inoculated in the rooting media supplemented with various concentrations of Auxins like IAA, NAA and IBA separately. The rooted plants were then transferred to poly cups containing vermiculite and sterile soil at 1:1 ratio, subsequently plantlets were transferred to the green house after proper acclimatization planted in the soil.

Data collection and Statistical analysis

Data of different variables like regeneration percentage, total number of shoots per plant, shoot lengths, total number of roots per plant and root lengths were collected from 20 replicates of each treatment after 8 weeks of inoculation.

The experimental data was statically analysed by one way ANOVA using DMRT (Duncan's multiple range test) ($P<0.05$) and were represented as the average±standard error.

RESULTS AND DISCUSSION

Preliminary experiments were conducted for selection of explants and best suitable (most significant) silver nitrate concentration to produce more number of shoots. The leaf explants and 0.4 mg/L was found to be more effective for *in vitro* propagation of *Solanum viarum.* After pursuing the observations depicted in the table 1 & 2 (Fig 1), we can clearly say that the type and amount of carbohydrates on multiple shoot proliferation and also different concentrations of Auxins on root regeneration are significantly influenced by silver nitrate in comparison with normal control.

Effect of silver nitrate on *In vitro* Regeneration frequency

The regeneration frequency which indicates the survival rate of explants has varied depending on the presence of silver nitrate and energy sources supplemented to the media. Silver nitrate supplied media showed maximum survival rate compared to control. The media free from energy source showed minimum (0%) regeneration where as media supplemented

with 0.4 mg/L $AgNO_3$ and 3% sucrose and 4% fructose showed maximum regeneration frequency as high as 100% and 98% respectively. The media without silver nitrate which is kept as control showed maximum of 90% only at 4% of sucrose and fructose. Among all carbohydrates, sucrose supplemented media grown plantlets showed better regeneration frequency ranging from 65 to 100%.

Effect of silver nitrate on multiple shoot regeneration

The different carbohydrate sources supplied to the media in the presence of 0.4 mg/L AgNO3 were significantly different at $P< 0.01$ with respect to mean number of shoots and mean shoot lengths. The reducing glucose in the presence of silver nitrate at 4% concentration gave significantly higher mean number of shoots(42.8 ± 0.21) followed by 3% sucrose (36 ± 0.37). The same concentrations of glucose (4%) and sucrose (3%) also showed maximum mean number of shoots (15 ± 0.41 and 13.2 ± 0.23) in the absence of silver nitrate in the culture medium. These results obviously indicate the significance of silver nitrate on various concentrations of carbohydrates. The presence of silver nitrate in the media had made the explants to respond at the maximum to achieve increased shoots proliferations over three folds.

Similar to the multiple shoot regeneration mean shoot length also was influenced by silver nitrate on various types and concentrations of carbohydrates. The maximum mean shoot length was given by $AgNO_3$ supplemented 3% sucrose (10.9±0.11cm) followed by 3% glucose (8.37±004cm). Surprisingly minimum mean shoot length was observed in $AgNO_3$ media supplemented 6% sucrose (1.26 ± 0.71cm) where in the un required callus formation was also observed to be very high.

Table 1: Effect of $AgNO_3$ on different carbohydrates sources on multiple shoot regeneration from leaf explants of *in vitro* grown *Solanum viarum* supplemented with 2.0 mg/L BAP and 0.5 mg/L IAA. Data represent mean ± SE of 20 replications. Callus formation: +: very low, ++: Low, +++: High

Energy source	Conc %	Regene-ration %		Means Number of Shoots		Mean shoot length(cm)		Callus formation	
		Without $AgNO_3$	0.4mg/L $AgNO_3$	Without $AgNO_3$	0.4mg/L $AgNO_3$	Without $AgNO_3$	0.4mg/L $AgNO_3$	Without $AgNO_3$	0.4mg/L $AgNO_3$
Control	No carbohydrate	-	-	-	-	-	-	-	-
Glucose	1	50	65	3.8 ± 0.41	14.4 ± 0.3	2.64 ± 0.18	5.87 ± 0.06	-	-
	2	65	70	5.4 ± 0.49	22.4 ± 0.24	5.61 ± 0.04	6.8 ± 0.01	-	-
	3	73	76	7.8 ± 0.47	34.4 ± 0.19	7.71 ± 00.9	10.56 ± 0.10	-	-
	4	82	85	15 ± 0.41	42.8 ± 0.21	5.95 ± 0.15	7.23 ± 0.12	-	-
	5	70	85	4.8 ± 0.38	21.8 ± 0.41	3.61 ± 0.35	4.59 ± 0.11	-	-
	6	65	70	3.4 ± 0.62	17.6 ± 0.27	2.50 ± 0.32	3.63 ± 0.17	-	-
Sucrose	1	55	82	3.2 ± 0.25	16.8 ± 0.41	2.5 ± 0.13	4.8 ± 0.06	-	-
	2	66	90	6.2 ± 3.07	23.4 ± 0.48	3.07 ± 0.2	5.6 ± 0.05	-	-
	3	65	100	13.2 ± 0.23	36 ± 0.37	8.09 ± 0.16	10.9 ± 0.11	-	-
	4	90	96	10.8 ± 0.25	18 ± 0.37	5.4 ± 0.97	6.7 ± 0.04	-	-
	5	80	88	6.6 ± 0.44	4.4 ± 0.75	3.3 ± 0.29	3.3 ± 0.23	+	++
	6	60	65	3.2 ± 0.47	1.8 ± 0.22	3.17 ± 0.3	1.26 ± 0.71	+	+++
Fructose	1	50	65	5.4 ± 0.23	12.8 ± 0.61	4.43 ± 0.07	6.9 ± 0.18	-	-
	2	70	80	7.4 ± 0.42	14.8 ± 0.62	5.68 ± 0.12	7.6 ± 0.06	-	-
	3	85	95	9.8 ± 0.41	23.4 ± 0.43	6.55 ± 0.04	8.37 ± 0.04	-	-
	4	90	98	11.4 ± 0.34	17.2 ± 0.46	3.35 ± 0.12	5.23 ± 0.27	-	-
	5	80	92	6.4 ± 0.45	13.2 ± 0.53	2.88 ± 0.06	3.9 ± 0.24	-	-
	6	65	70	3.2 ± 0.47	12.8 ± 0.33	2.15 ± 0.05	4.7 ± 0.06	+	-
Maltose	1	45	68	3.2 ± 0.47	15 ± 0.41	3.23 ± 0.11	4.8 ± 0.12	-	-
	2	68	72	7.8 ± 0.30	33.8 ± 0.37	5.43 ± 0.05	6.7 ± 0.06	-	-
	3	70	85	7 ± 0.35	23.2 ± 0.34	4.26 ± 0.15	5.4 ± 0.05	-	-
	4	75	78	5.8 ± 0.35	15 ± 0.46	5.4 ± 0.16	7.6 ± 0.09	-	-
	5	78	85	3.8 ± 0.43	3.6 ± 1.09	2.62 ± 0.15	3.5 ± 1.06	+	++
	6	62	65	1.2 ±0.41	1.4 ±0.96	1.21 ± 0.27	1.54 ± 0.81	+	++

Effect of silver nitrate on *in vitro* Rooting

A marked difference with decline rate of rooting was observed when culture was supplemented with either lower or higher concentrations of Auxins. Similar to the other parameters under study, rooting of *in vitro* grown shoots also responded well in the presence of 0.4 mg/L $AgNO_3$ which was almost enhanced by 2 to 2.5 folds when compare to control media free from silver nitrate. Among various treatments proliferic rooting was observed in the presence of 0.4 mg/L $AgNO_3$ at 1mg/L IAA (40.2 ± 0.13) and 1mg/L IBA (35 ± 0.38) and the maximum mean root length was observed at 2mg/L (11.28 ± 0.14) and 1mg/L (9.45 ± 0.07) of IAA (Table2) respectively. But plenty of hairy roots emergence was observed when auxin concentration in the media increases.

Presence of silver nitrate in *in vitro* propagation showed marked significance in almost all parameters under present investigation. When media was devoid of energy source there was no growth at all and media without silver nitrate showed lesser response. This clearly explains that the presence of optimal $AgNO_3$ concentration at particular carbohydrate concentration has increased almost 3 to 4 folds in shoot proliferation and 2 to 2.5 folds in root proliferation at various concentrations of Auxins. This may be due to presence of silver nitrate, which might have suppressed the activity of excess of Ethylene present in the *in vitro* culture tubes and further promoted for easy translocation and assimilation of these energy sources available in the media by the explants resulting in cell division and leading to vigorous growth.

Surpassing of Glucose over Sucrose in the investigation could be explained, as the presence of sucrose might cause hypoxia and ethanol accumulation in cells due to quick metabolisation. But when it comes to longevity parameter under investigation, 3% Sucrose besides showing 100% regeneration percentage proved to be promising in repeated sub culturing. Whereas 4% Glucose, though appreciably extended with 85% survival rate and very high multiple shoots regeneration there was a problem of withering, yellowish lean shoots development in next sub culturing on the same media. Hence there is a prospect of using 3% Sucrose which had promoted more healthy shoots and high survival frequency (100%), instead of 4% of glucose for further investigations.

Table 2: Effect of $AgNO_3$ on different concentrations of NAA, IAA and IBA on root organogenesis from *in vitro* grown shoots of *Solanum viarum*. Observation after 8 weeks: Values are mean ± S.E of 5 replications. Intensity of the callus: +: Very low, ++: low, +++: medium.

Sl no	Concentration of plant growth regulators (mg / L)			$AgNO_3$ mg/L	Regeneration frequency (%)	Mean number of shoots ± S.E	Mean value of shoot length ± S.E.(Cm)	Callus formation
	BAP	NAA	IAA					
1	1	0.1	-	-	62	3.3 ± 0.34	1.4 ± 0.04	
2	1	0.1	-	0.4	75	5.8 ± 0.54	2.11 ± 0.09	+
3	1	0.5	-	-	70	4.5 ± 0.22	2.8 ± 0.45	
4	1	0.5	-	0.4	80	10.6 ± 0.56	3.26 ± 0.10	+
5	1	-	0.1	-	60	2.5 ± 0.67	2.26 ± 0.27	
6	1	-	0.1	0.4	70	7.4 ± 0.42	3.46 ± 0.06	+
7	1	-	0.5	-	75	5.2 ± 0.91	3.2 ± 0.80	
8	1	-	0.5	0.4	85	13.4 ± 0.41	4.02 ± 0.01	+
9	2	0.1	-	-	70	2.1 ± 0.21	3.6 ± 0.31	
10	2	0.1	-	0.4	80	10.4 ± 0.64	5.06 ± 0.09	+
11	2	0.5	-	-	80	6.1 ± 0.72	4.8 ± 0.78	
12	2	0.5	-	0.4	95	17 ± 0.38	7.57 ± 0.05	-
13	2	-	0.1	-	65	2.1 ± 0.21	3.6 ± 0.31	
14	2	-	0.1	0.4	85	9.8 ± 0.27	5.12 ± 0.11	+
15	2	-	0.5	-	80	6.8 ± 0.59	4.8 ± 0.72	
16	2	-	0.5	0.4	90	19 ± 0.36	6.01 ± 0.06	-
17	3	0.1	-	-	50	3.4 ± 0.84	2.1 ± o.32	
18	3	0.1	-	0.4	65	8.8 ± 0.28	3.22 ± 0.13	-
19	3	0.5	-	-	60	4.7 ±0.55	2.8 ±0.37	
20	3	0.5	-	0.4	75	11.6 ± 0.33	4.67 ± 0.15	-
21	3	-	0.1	-	50	1.8 ± 0.49	2.23 ± 0.02	
22	3	-	0.1	0.4	70	6.6 ± 0.44	3.34 ± 0.15	-
23	3	-	0.5	-	65	3.7 ± 0.73	3.1 ± 0.05	+
24	3		0.5	0.4	80	10.8 ± 0.62	4.8 ± 0.16	-

Fig: 1: Effect of various concentrations of carbohydrates on direct organogenesis of leaf explants of *Solanum viarum* cultured on M.S. medium supplemented with 2.0 mg/L BAP and 0.5 mg/L IAA.

(A) Initiation of shoots from leaf explants at 4% of glucose without $AgNO_3$.

(B) Initiation of shoots from leaf explants at 3% of sucrose without $AgNO_3$.

(C) Initiation of roots from *In vitro* grown shoots supplemented with 1mg/L IAA along with 0.4 mg/L $AgNO_3$.

(D) Initiation of roots from *In vitro* grown shoots supplemented with 1mg/L IBA along with 0.4 mg/L $AgNO_3$

CONCLUSIONS

This study points to the fact that presence of silver nitrate and selection of energy source does play an important role in the regulation of growth. It is also realized that greater mean number of shoots and their lengths may not be the true indicators. The culture's state of vigor, performance over time and survival also need due consideration. The presence of silver nitrate not only masks the ethylene activity but also at particular carbohydrate concentration might stimulate sugar sensitive gene to respond the maximum. This is a novel work taken up for the first time to study the cumulative effect of silver nitrate at various concentrations of energy sources to strengthen micropropagation and also unique of its kind. Further molecular level studies yet to be conducted to support this.

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