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Antagonistic Effect Of Silver Nitrate And Cobalt Chloride Against Ethylene Action To Enhance In Vitro Regeneration Potency Of Solanum Viarum Dunual

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Abstract: ***This investigation had been carried out to evaluate the influence of ethylene inhibitors such as silver nitrate ($AgNO_3$) and cobalt chloride ($COCl_2$) on in vitro propagation of Solanum viarum. Exogenous administration of ethylene inhibitors such as, silver nitrate ($AgNO_3$) and cobalt chloride ($COCl_2$) at the concentration of 1mg/L each, resulted in the maximum tissue response in terms of shoot length (7.26 ±0.09 cm) and number of shoots (17.3 ± 0.9) after 8 weeks of culturing the leaf explants in 2mg/L BAP, 0.1 mg/L NAA supplemented Murashige and Skoog medium. The results also demonstrated the significant improvement in root regeneration by developing maximum roots per explant (20.2 ± 0.28) of in vitro raised shoot lets, cultured in the MS media supplemented with $AgNO_3$ (1mg/L) and $COCl_2$ (1mg/L) along with 1mg/L IAA The maximum root lengths(7.04 ± 0.04) were recorded in the media supplemented with 1mg/L $AgNO_3$ and 1mg/L $COCl_2$ along with 1mg/L NAA. These in vitro regenerated protocols developed using ethylene inhibitors could be used for possible micropropagation and plant transformation in Solanum viarum.***

Keywords: Ethylene inhibitors, Direct shoot regeneration, Silver nitrate, Cobalt chloride, Rhizogenesis.

Abbreviations:
$AgNO_3$ Silver nitrate
$COCl_2$ Cobalt chloride
BAP Benzyl Amino Purine
IAA Indole Acetic Acid
NAA Napthalene Acetic Acid
Rf Regeneration frequency

I. INTRODUCTION

Solanaceae family significantly known for accommodating number of medicinal plants which are rich in Anti cancer & Anti diabetic steroids like lactones, glycosides, alkaloids and flavanoids. *Solanum* L. (1753), the largest genus in the family and one of the broadest genus of the angiosperms, with 1,328 species distributed across the whole world. Solasodine is one of the major Glyco alkaloid constituent of solanum sps, have been recognized as the potential alternative to diosgonin which share a characteristic conversion to 16-dehydro pregnenolone acetate, the first step in steroid synthesis. Maiti and Mukharjee have screened 43 species of Solanum from India for steroidal alkaloid especially Solasodine. One among them is *Solanum viarum*, native species of Argentina, commonly known as tropical soda apple. It is a profusely branched, prickly herbaceous perennial plant which serves as the major source of Solasodine. Lot of efforts

have been made to grow this plant *in vitro* to exploit commercially as the secondary metabolite glycoalkaloid solasodine produced in this plant is used 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). Due to unrestricted exploitation for its bio active medicinal compounds this plant becoming as one among the threatened and endangered species in India.

The *in vitro* micropropagation is the best promising alternative for conserving this plant. But invariably, certain hormones are getting accumulated rather than deliberate addition, due to unavoidable closed environment of tissue culture vessels. Excess accumulations sometimes show antagonistic effect on plants regeneration. Ethylene is one such gaseous hormone which is receiving a lot of attention recently in pioneered tissue culture research programmes. It is a simple hydrocarbon (C_2H_4), but has maximum impact on growth, cellular differentiation, fruit ripening and senescence in plants even at concentrations as low as 0.01µL L^{-1} or 10^{-6} % v/v (Reid, 1995). Influence of ethylene in tissue culture propagation had been well documented previously. Ethylene suppresses the growth and morphogenesis of explants depending on the species and stage of the culture (Kumar *et al.*, 1998a). It has been reported previously, that ethylene is known to suppress the plant regeneration (Robinson's & Adams, 1987), cell differentiation (Charaibi *et al.*, 1991) as well as general growth of the plant (Pua, 1993). To overcome the above said situation for the first time we had made an attempt to study the effect of $AgNO_3$ and $COCl_2$, ethylene inhibitors to enhance the shoot and root proliferation in *Solanum viarum.*

Silver and cobalt are heavy metals, not regarded as essential elements, but considerably effects the growth and development of plants in various degrees depending on the concentration of these transition metals in the surrounding media (Palit *et al*., 1994). Few experimental studies in the past shown that ethylene inhibitors such as $AgNO_3$ and $COCl_2$ had played significant role in callus proliferation (Fei *et al*,2000), shoot organogenesis(Park *et al*,2012), microspore cultures (Hays *et al.*,2000) microspore embryogenesis (Khandakar *et al*., 2013), *in vitro* flowering (Pratheesh *et al*, 2011) and also in plant regeneration (Ahmed., 2014). Hence, in the present investigation we aimed to evaluate the effect of various concentrations of $AgNO_3$ and $COCl_2$ supplemented in the MS media to develop possible improved protocol for micropropagation of *Solanum viarum.*

II. MATERIALS AND METHODS

PLANT MATERIAL

Mature and healthy seeds collected from ripen and dried fruits of *Solanum viarum* plant were washed under running tap water for 10 min to remove adherent fruit tissues, dried juice and also to avoid fungal contamination during *in vitro* culturing. Decontamination of the seeds was performed by soaking the seeds in 0.4 mg/L (w/v) bavistin for 1 min and in 0.1% mercuric chloride for 2 min followed by rinsing the seeds in the double distilled water to remove the traces of all sterilants.

Under aseptic conditions seeds were inoculated on autoclaved MS media (Murashige and Skoog, 1962) basal medium supplemented with 3% sucrose and gelled with 0.8% agar. Prior to autoclaving at 121° C for 20 min, the pH of the medium was adjusted to 5.8 and cultures were maintained at 25± 2°C under 16 hrs light and 8 hrs dark for 8 weeks. The primary shoots developed were further subcultured *in vitro* under aseptic culture conditions as mentioned above in 2mg/L BAP and 0.5 mg/L IAA supplemented MS medium. Healthy and uniform leaves were excised from in vitro sub cultured shoots and used as explants for further experiments.

MS MEDIA SUPPLEMENTED WITH DIFFERENT CONCENTRATIONS OF $AGNO_3$, $COCL_2$ AND VARIOUS PHYTOHORMONES TO INDUCE DIRECT ORGANOGENESIS

Uniform sized leaves were transferred to modified MS regeneration media supplemented with different gradient doses of $AgNO_3$ and $COCl_2$ (0.5 – 6.0 mg/L) at 2mg/L BAP, 0.1 mg/LNAA and 0.1 mg/L IAA. All these different concentrations were maintained separately by following the same culture conditions of mother culture. Number of shoots and shoot lengths were recorded after 8 weeks of inoculation and 5 replications were maintained for each concentration.

ROOTING AND ACCLIMATIZATION

After sufficient elongation shoots were excised and inoculated in the rooting media supplemented with different concentrations of NAA and IAA for root organogenesis in 1mg/L silver nitrate ($AgNO_3$) and 1mg/L cobalt chloride ($COCl_2$) supplemented M.S media. 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 and 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 5 replicates of each treatment after 8 weeks of observation.

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

III. RESULTS AND DISCUSSION

EFFECT OF DIFFERENT CONCENTRATIONS OF SILVER NITRATE AND COBALT CHLORIDE ON PLANT REGENERATION FREQUENCY

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Our experimental results represented in table1, evidently specify the variation in regeneration frequency according to the type and concentration of ethylene inhibitors, supplemented in the MS medium. The lowest regeneration frequency was observed from leaf explants cultured in the absence of ethylene inhibitors. Optimum regeneration (90%) was observed in the media either supplemented with 2mg/L $AgNO_3$ or $COCl_{2;}$ however the maximum regeneration frequency was observed when the media was supplemented with both the ethylene inhibitors at 1mg/L concentration. This might be due to cumulative effect of Ag^+, CO^+ ions present in the media to suppress the ethylene biosynthesis as well as its action.

INFLUENCE OF ETHYLENE INHIBITORS ON SHOOT PROLIFERATION

External administration of silver nitrate ($AgNO_{3)}$ and cobalt chloride ($COCl_2$) in the *in vitro* culture medium found to enhance the regeneration potency more effectively by developing longer shoot lengths and also higher number of shoots per explant of *Solanum viarum.* Though *in vitro* grown leaf explants at 2mg/L concentration $AgNO_3$ and $COCl_2$ improved shoot regeneration individually, the maximum number of shoots were (17.3 ± 0.9) were obtained when MS media was supplemented with both $AgNO_3$ and $COCl_2$ at 1mg/L concentration along with 0.1 mg/L NAA followed by 2 mg/L $AgNO_3$ at o.1 NAA mg/L (14.4 ± 0.71).

Similar to the multiple shoot regeneration, mean shoot length also was influenced by various concentrations of silver nitrate and cobalt chloride. The maximum mean shoot length was observed in the media supplemented 1mg/L $AgNO_3$ and $COCl_2$ along with 0.1 NAA mg/L(7.68 ± 0.13cm) followed by 2mg/L $AgNO_3$ along with 0.1 NAA mg/L (7.26 ± 0.09 cm). Surprisingly minimum mean shoot length was observed in media supplemented with 6mg/L $AgNO_3$ (1.3 ± 0.12 cm) where in the un required callus formation was also observed to be very high.

In the study, the gradual increase in the concentration of both silver nitrate and cobalt chloride in the medium initially provoked good morphogenic response but at higher concentrations significantly depressed the shoot proliferation. Nevertheless, plantlets developed in the presence of 0.5 and 1 mg/L $AgNO_3$ and $COCl_2$, they were small, weak, slightly yellowish and wrinkled. When the concentrations of these ethylene inhibitors raised (2mg/L) in the medium, an improvement in the morphogenesis recorded wherein developed plantlets grew strong, with large healthy leaves with maximum shoot lengths. At higher concentrations of $AgNO_3$ and $COCl_2$ (4 and 6 mg/L) supplementation, we can see callus formation along with fewer number of shoots, leaves having larger internodes from leaf explants, but the explant response was found to be higher than the control group where no ethylene inhibitors are added to the media. Poor morphogenic response in $AgNO_3$ and $COCl_2$ absentia in the MS media may be related to ethylene biosynthesis or metabolism. Lesser response at low concentrations of ethylene inhibitors possibly due to lower concentration of Ag^+, CO^+ ions present in the media which might not be sufficient to supress either ethylene biosynthesis or it's action. Perhaps at higher concentration, heavy dose of metal ions may be toxic to promote shoot or root proliferation.

From the previously reports regarding different aspects of plant micropropagation of various plant species, $AgNO_3$ treatment had shown very high positive response when compare to $COCl_2$ supplementation in *in vitro* cultures (Kabir *et al*., 2013; Sharma *et al.*,2008; Buzzy *et al.*, 2006).. For the first time our results clearly depicts the synergistic significant effect of both the ethylene inhibitors on shoot proliferation.

INFLUENCE ON RHIZOGENESIS OF *IN VITRO* GROWN SHOOTLETS

Response of *in vitro* raised shoots upon culturing in $AgNO_3$ and $COCl_2$ supplemented MS media along with different concentrations of NAA and IAA was represented in table 2. The results indicate the marked increase in the production of larger number of roots and their length over 2 folds in control in the absence of ethylene inhibitors. The maximum number of roots (20.2 ± 0.28) were obtained in the media supplemented with 1mg/L $AgNO_3$ and 1mg/L $COCl_2$ at 1mg/L IAA with 100% regeneration frequency followed by 1mg/L $AgNO_3$ and 1mg/L $COCl_2$ at 1mg/L NAA (15.2 ± 0.28) with 95% regeneration frequency. Whereas the maximum root length was observed at 1mg/L NAA along with 1mg/L $AgNO_3$ and 1mg/L $COCl_2$ (7.04 ± 0.04 cm).

Most probably the accumulated ethylene in the closed vessels may be one of the important factors to cause recalcitrant morphogenesis in *Solanum viarum.* Silver ions are reported to inhibit ethylene action (Bayer, 1976).Whereas cobalt inhibit ethylene biosynthesis (Roustant *et al.* , 1986). In plants (Yang and Halfman, 1984) ethylene is a simple olefin exists in gaseous state, produced from S- Adenosyl methionine (SAM) through the intermediate 1- aminoacyle propane-1-corboxylie acid (ACC). The ACC will be converted into ethylene in the presence of the enzyme ACC oxidase which can be blocked by cobalt ions there by arresting the ethylene production. Various views and experimental evidences have been put forth to explain the silver ion's capability in blocking ethylene receptors (Bayer, 1976) to make plants insensitivity to ethylene. Silver ions are thought to perturb the ethylene ion binding site (Rodriguez *et al.*, 1999). Binding process of ethylene to its binding site ETR 1 is mediated by Cu^+ cofactors. The gradual replacement of copper cofactors with Ag^+ brings about conformational changes in such way which continuously represses ethylene responses (Zhao *et al.*, 2002).

IV. CONCLUSION

Only sporadic reports on regeneration are available for perennial, profusely branched solasodine rich plant *Solanum viarum*. In view of economic importance and to avoid plant material depletion, in the present investigation an attempt had been made to develop a protocol for highly reproducible regeneration system with great frequency of multiple shoots to improve this medicinally important and endangered plant *Solanum viarum*. With the obtained results we can easily infer that *Solanum viarum* is probably one of the species sensitive to ethylene produced and accumulated during *in vitro*

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culturing. The findings from the study are undoubtedly of considerable relevance and are helpful to extend the research further more to molecular level in order to understand the exact mechanism involved in enhancement of plant regeneration by suppressing ethylene accumulated in closed culture vessels.

Sl no	Concentration of plant growth regulators (mg / L)		Conc of $AgNO_3$ (mg / L)	Conc of $COCl_2$ (mg / L)	Regener-ation frequency %	Mean number of shoots ± S.E	Mean value of shoot length ± S.E.(Cm)	Callus for ma-tion
1.	0.1	-	-	-	70	2.2 ± 0.47^{a}	2.1 ± 0.06^{a}	-
2.	0.1	-	0.5	-	82	4.2± 0.24^{a}	2.6 ± 0.26^{a}	-
3.	0.1	-	1	-	85	7.1 ± 0.88^{b}	4.8 ± 0.1^{c}	-
4.	0.1	-	2	-	90	14.4 ± 0.71^{de}	7.26 ± 0.09^{d}	-
5.	0.1	-	4	-	75	6.4 ± 0.62^{b}	2.7 ± 0.06^{ab}	+
6.	0.1	-	6	-	70	3.5 ± 0.71^{a}	1.4 ± 0.16^{a}	++
7.	-	0.1	-	-	65	2.6 ± 0.29^{a}	1.91 ± 0.47^{a}	-
8.	-	0.1	0.5	-	76	3.6 ± 0.42^{a}	2.99 ± 0.15^{b}	-
9.	-	0.1	1	-	80	6.3 ± 0.31^{b}	4.6 ± 0.18^{bc}	-
10.	-	0.1	2	-	90	13.6 ± 0.29^{d}	5.84 ± 0.11^{c}	-
11.	-	0.1	4	-	80	5.8 ± 0.64^{ab}	3.61 ± 0.17^{b}	+
12.	-	0.1	6	-	70	2.8 ± 0.73^{a}	2.6 ± 0.10^{ab}	++
13.	0.1	-	-	0.5	78	3.1 ± 0.54^{a}	2.98 ± 0.21^{ab}	-
14.	0.1	-	-	1	80	5.3± 0.57^{ab}	4.19 ±0.07^{bc}	-
15.	0.1	-	-	2	90	12.3 ± 0.24^{d}	5.45 ± 0.85^{c}	-
16.	0.1	-	-	4	70	5.1 ± 0.47^{ab}	2.58 ± 0.21^{ab}	+
17.	0.1	-	-	6	65	1.8 ± 0.13^{a}	1.3 ± 0.12^{a}	+
18.	-	0.1	-	0.5	80	3.8 ± 0.45^{a}	3.6 ± 0.10^{b}	-
19.	-	0.1	-	1	75	5.6 ± 0.15^{ab}	5.17 ± 0.23^{c}	-
20.	-	0.1	-	2	82	11.5 ± 0.28^{cd}	4.45 ±0.31^{bc}	-
21.	-	0.1	-	4	78	4.3 ± 0.71^{a}	3.06 ±0.11^{b}	-+
22.	-	0.1	-	6	75	2.7 ± 0.52^{a}	2.13± 0.9^{a}	+
23.	0.1	-	1	1	95	17.3 ± 0.9^{e}	7.68 ± 0.13^{d}	-
24.	-	0.1	1	1	90	13.7 ± 0.12^{d}	5.12 ± 0.15^{c}	-
25.	0.1	-	2	2	65	6.2 ± 0.65^{b}	3.29 ± 0.19^{b}	++
26.	-	0.1	2	2	60	3.6 ± 0.62^{a}	3.9 ± 0.23^{b}	++

Table 1: Effect of different concentrations of $AgNO_3$ and $COCl_2$ at 2mg/L BAP, 0.1 mg/LNAA and 0.1 mg/L IAA on direct multiple shoot regeneration from leaf explants of Solanum viarum. Data represent treatment means ±S followed by different letter(S) within column indicate significant differences according to ANOVA and DMRT test (P < 0.05). Intensity of the callus: +: Very low, ++: low

Sl no	Conc of plant growth regulators (mg / L)		R F (%) in 1mg/L $AgNO_3$ and 1mg/L $COCl_2$	R F (%) in Control	Mean number of roots± S.E in 1mg/L $AgNO_3$ and 1mg/L $COCl_2$	Mean number of roots± S.E in Control	Mean value of root length ± S.E.(Cm) in 1mg/L $AgNO_3$ and 1mg/L $COCl_2$	Mean value of root length ± S.E.(Cm) in Control
	NAA	IAA						
1.	0.5	-	80	60	12.4 ± 0.22^{cd}	5.6 ± 0.46bc	3.35 ± 0.04^{a}	2.28 ± 0.13^{a}
2.	1	-	95	80	15.2 ± 0.28^{de}	9.3 ± 0.85^{de}	7.04 ± 0.04^{d}	4.34 ± 0.11^{c}
3.	2	-	80	74	9.8 ± 0.14^{c}	4.5 ± 0.34^{b}	4.5 ± 0.11^{b}	3.8 ± 0.13^{b}
4.	3	-	75	65	3.4 ± 0.65^{a}	1.2 ± 0.25^{a}	3.5 ± 0.29^{ab}	3.1 ± 0.55^{b}
5.	-	0.5	85	70	11.6 ± 0.21^{c}	5.21 ± 0.63^{bc}	4.3 ± 0.11^{b}	3.89 ± 0.17^{b}
6.	-	1	100	86	20.2 ± 0.28^{f}	11.4 ± 0.54^{e}	6.45 ± 0.03^{c}	5.45 ± 0.12^{d}
7.	-	2	80	75	8 ± 0.41^{b}	4.7 ± 0.32^{b}	3.28 ± 0.14^{a}	3.21 ± 0.21^{b}
8.	-	3	75	70	5.6 ± 0.27^{a}	3.5 ± 0.71^{b}	2.16 ± 0.08^{a}	2.45 ± 0.06^{a}

Table 2: Effect of different concentrations of NAA & IAA on root organogenesis in M.S media supplemented with 1mg/L $AgNO_3$ and 1mg/L $COCl_2$ from in vitro grown shoots of Solanum viarum. Observation after 8 weeks: Values are mean ± S.E. of 5 replications. Data represent treatment means ±S followed by different letter(S) within column indicate significant differences according to ANOVA and DMRT test (P < 0.05).

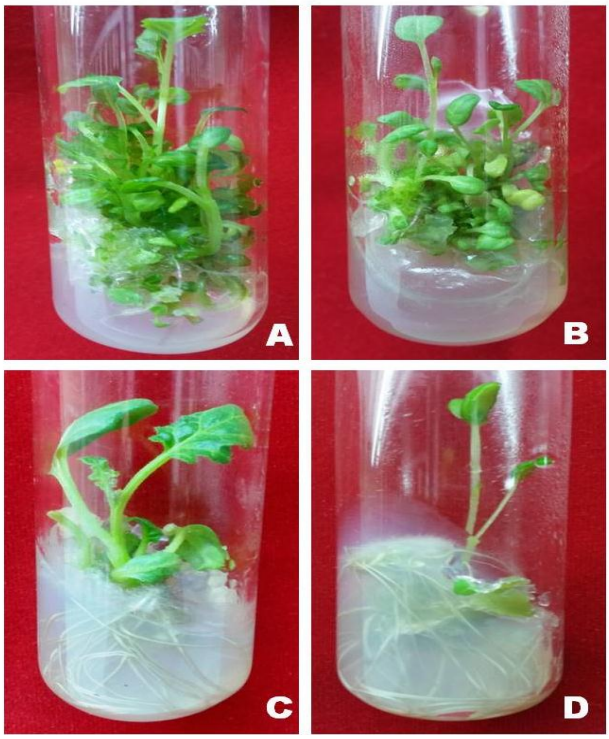

Figure 1: Effect of different concentrations of Silver nitrate, cobalt chloride and growth hormones on in vitro shoot and root organogenesis of Solanum viarum.

a. Multiple shoot formation from leaf explants on MS media supplemented with 1 mg/L $AgNO_3$, 1mg/L $COCl_2$ along with 2mg/L BAP and 0.1 mg/LNAA.

b. Multiple shoot formation from leaf explants on MS media supplemented with 2 mg/L $AgNO_3$ along with 2mg/L BAP and 0.1 mg/LNAA.

c. Rhizogenesis of in vitro raised shootlets on MS media supplemented with 1 mg/L $AgNO_3$ and 1mg/L $COCl_2$ along with 1 mg/L IAA.

d. *Rhizogenesis of in vitro raised shootlets on MS media supplemented with 1 mg/L $AgNO_3$ and 1mg/L $COCl_2$ along with 1 mg/L NAA.*

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