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RESEARCH ARTICLE

Silver thiosulphate enhance *in vitro* regeneration of *Centella asiatica* (L.) -An important antijaundice medicinal plant

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Abstract: A study was conducted to investigate the effect of ethylene inhibitor silver thiosulphate on *in vitro* shoot regeneration by using nodal explants of *Centella asiatica* (L.). In this experiment, when STS was added into MS medium, it was found that the vitrified, stunted shoot formation was inhibited. In addition silver thiosulphate (STS) enhanced the shoot regeneration frequency and mean shoot number. MS medium fortified with lower concentration of STS (8.9 µM l^{-1}) was favoured the high frequency of regeneration (80%) and induce maximum number of shoots (6.8 ± 0.42) with maximum shoot length (5.14). STS range in between (4.4-44.4 µM l^{-1}) was used throughout the study. It was also noticed that the further increase in the concentrations of STS which extremely inhibited shoot induction and with a diminution of regeneration response (50%) and even the explants were unsuccessful to survive on the media. Ethylene inhibits the shoot morphogenesis and also affects the root formation. Ag+ ions inhibit ethylene action on regeneration. The best rooting response was noticed when micro shoots were transferred to rooting medium supplemented with IBA (2.5 mg l^{-1}).

Keywords: *Centella asiatica* (L.); Silver thiosulphate; Ethylene inhibitor; Nodal explants; *In vitro* regeneration.

Abbreviations: STS: Silver thiosulphate; BAP: 6-Benzyl amino purine; IAA: Indole-3-acetic acid; IBA: Indole-3-butyric acid; NAA: Naphthalene acetic acid.

Introduction

Centella asiatica (L.) Urban is a stoloniferous perennial herb belonging to the family *Apiaceae* or *Umbelliferae*. It is a slender creeping plant rooting at the nodes. In India it is commonly known as 'Indian pennywort' 'Jal bramhi' 'Mandookaparni' and 'Gotukola'. It has been used in the treatment of jaundice, measles, hepatitis, syphilus, smallpox and rheumatism (Prajapati et al., 2006). Consumption of *C. asiatica* have been identified for many years in treating numerous kind of diseases such as gastrointestinal disease, gastric ulcer, asthma, wound healing and eczema (Brinkhaus et al., 2000). Also consists of glycosides as brahmoside, indocentelloside and leaves are rich in caro tenoids, higher levels of natural antioxidants compounds like vitamin E, vitamin C, total carotenes, total xanthophylls, tannins and total phenolics. The herb was reported as antidiabetic, antiviral, antiulcer, antibacterial, antitumour and high memory enhancing activity (Vasantharuba et al., 2012). This plant is conventionally propagated by cuttings and seeds. This type of propagation cannot meet the increasing demand. This plant used as the raw material for the preparation of pharmaceutical, dermaceutical and aroma therapeutical products. *In vitro* techniques have been found to be mounting use in the conservation of threatened plants in modern years and this development is likely to continue as more species face hazard of extinction and to be useful in the proliferation of large number of endangered plants and potential herbal plants of healthcare applications.

The broad effectiveness of ethylene inhibitors at promoting shoot regeneration in several plant species has been reviewed (Kumar et al., 1998) and silver nitrate is the most usually used source of silver ion which could impede with ethylene incorporation at its receptor sites (Beyer 1979). The silver ions of STS will inhibit the ethylene produced by explants. The beneficial effects of silver ions have been widely reported (Chi et al., 1990). Furthermore, the addition of silver ions to the medium was reported to obviously enhance regeneration frequency and the number of shoots per explants in *Stevia*

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rebaudiana (Bertoni) (Preethi et al., 2011) *Asclepias curassavica* (SH Reddy et al., 2012), *Mentha piperita* (Sujana and Naidu, 2011) and *Solanum nigrum* (Sridhar et al., 2011). According to previous data, vitrification of shoots is associated to an elevated ethylene production which was related to hypolignification. Toting up of silver ions acts as inhibitor for stunted (Ziv 1991). *In vitro* culture of plant cells, tissues or organs on a medium augmented with selective agents offers the opportunity to select and regenerate plants with desirable characteristics including tolerance to stress (Purohit et al., 1998).

However, the use of silver thiosulphate has not yet been reported in *C. asiatica* Therefore in this context, the present communication was assessed to investigate the efficacy of silver ions on induction, regeneration to produce adventitious direct shoot organogenesis of *C. asiatica* from field grown nodal explants.

Materials and methods

Collection of plant material and surface sterilization

Young *C. asiatica* plants were collected from herbal garden, Department of Biotechnology, Dravidian University, Kuppam, Andhra Pradesh, India. Axillary buds were collected from the young sprouts of the stock plants were selected as explants. These explants were washed under running tap water for 10 mins, followed by immersing in liquid detergent solution 5 % (v/v) Tween 20, for 15 mins. and then washed under running tap water. The explants were surface sterilized with 0.4 % (w/v) bavistin, a systemic fungicide (BASF India Ltd.,) and then with 70% (v/v) ethanol for 90 sec. The explants were surface sterilized with 0.1 % (w/v) $HgCl_2$ (Merck India) for 1-3 minutes and thoroughly washed with sterile double distilled water for thrice to remove the traces of $HgCl_2$ before inoculation.

Culture medium and culture conditions

MS medium (Murashige and Skoog 1962) fortified with STS (4.4 - 44.4 µM l^{-1}) and sucrose 3.0 % (w/v) alone was used throughout the study. MS basal medium supplemented with various plant growth regulators at different concentrations either alone or in combination were used as control. The p^H of the medium was adjusted to 5.8 and gelled with addition of 0.8 % (w/v) agar. Molten medium was dispensed approximately 15 ml into culture tubes (25 × 150 mm) and closed with non-absorbent cotton plugs. The medium was autoclaved at 15 lbs / sq inch pressure and 121^0C for 20 mins.

All the cultures were incubated in an *in vitro* culture room maintained at 26 ± 2 ^{0}C temperature and 55 - 60 % relative humidity with a photo period of 16 hrs day light and 8 hrs dark with a light intensity of 3000 lux provided by cool white fluorescent tubes. All the cultures were transferred to fresh culture medium after 21 days of culture duration for the development of *in vitro* rooting.

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.

All the experiments were conducted with a minimum of 20 explants. All assays were repeated at least three times. The experimental data were statistically analyzed by one-way ANOVA using the DMRT (Duncan's Multiple Range Test) ($P < 0.05$) and were presented as the mean average ± standard error (SE).

Results and discussion

Impact of ethylene inhibitor STS on regeneration of C. asiatica from nodal explants

Nodal explants when cultured on MS basal media supplemented with STS, bud breaking and induction was observed. Surprisingly STS at low concentrations that were used had a positive effect on regeneration and induce shoot formation (Figure 1c). Maximum frequency of shoot regeneration (80 %), shoot number (6.8 ± 0.42) with mean shoot length (6.20 ± 0.43) were observed at 8.9 µM l^{-1} STS when used (Figure 1a). Lower concentrations of STS favored the

high frequency of regeneration and more number of shoots per explant. The optimum range of STS concentrations was recorded between 8.9 - 44.4 µM l^{-1} was used in the present study. The shoot development was increased when STS concentrations changed from 4.4-8.9 µM l^{-1}. But thereafter shoot number and regeneration was decreased with increase in the STS concentrations. MS basal medium supplemented with BAP (2.5 mg l^{-1}) was used as control (Figure 1b).

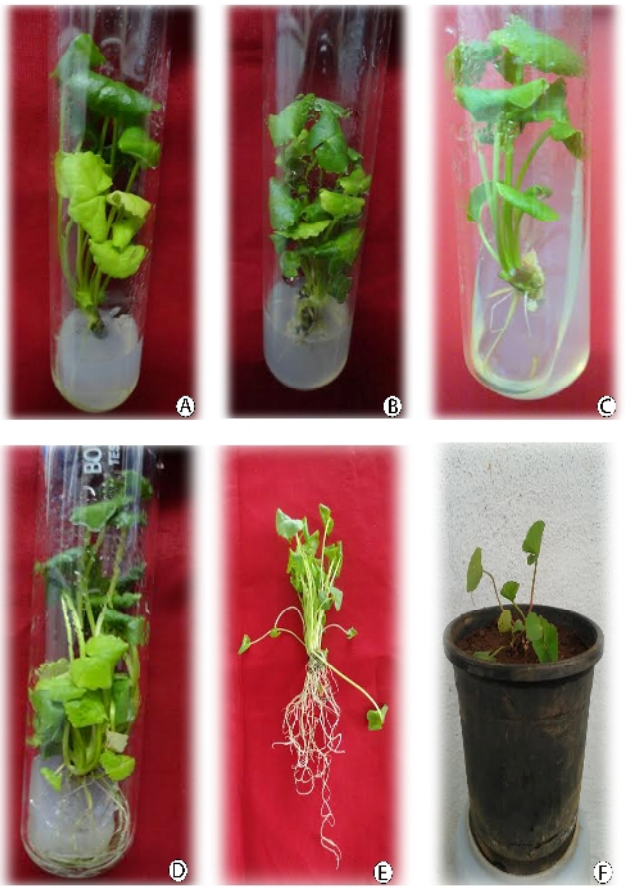

Figure 1: Effect of an ethylene antagonist STS on *in vitro* regenerationof *Centella asiatica* (L.) from nodal explants.

a. Shoot regeneration on MS basal medium augmented with STS (4.4 µM l^{-1}).
b. Shoot regeneration on MS basal medium with STS (8.9 µM l^{-1}).
c. Multiple shoot regeneration on MS basal medium with supplemented with BAP (2.5 mg l^{-1}) (Positive control).
d. *In vitro* rooting of regenerated micro shoots on half strength MS medium with IBA (2.5 mg l^{-1}).
e. Tissue cultured plantlet ready for hardening.
f. Plantlets transferred to earthen pots containing soil and vermiculite in 1:1 ratio.

One of the most imperative factors of physical atmosphere in plant tissue culture is ethylene (C_2H_4), a gaseous plant hormone that plays an important role in plant growth and development. Ethylene inhibits shoot morphogenesis and formation of shoots. Ethylene is produced during *in vitro* cell division and consequently its accumulation in tissues directs the depletion on oxygen. Ethylene reduces the frequency of adventitious shoot regeneration from explants and retards growth of the shoots. Silver ions acts as competitive inhibitors of ethylene action rather than inhibiting ethylene synthesis (Zhang et al., 2001). The silver ions hinder ethylene action in wide variety of ethylene induced responses in plants by reducing the receptor functional capacity to bind ethylene (Beyer 1979; Yang and Hoffman 1984). Addition of silver nitrate favoured shoot morphogenesis (Harsh Pal et al., 2000). Ethylene inhibitors such as STS and $AgNO_3$ inhibit the binding of ethylene during cell division. STS enhancing *in vitro* shoot regeneration of *Apricot* leaves are also reported (Burgos and Albuquerque 1984). The use of silver nitrate in plant regeneration had been reviewed (Kumar et al., 1998). Other species as *Sinningi speciosa* (Lodd.) Hiern have also been found to be affected by $AgNO_3$ (Eui-Ho Park et al., 2012).

It was well thought-out that the increase in shoot regeneration frequency by silver ions is caused by the interruption of an ethylene signal transduction pathway (Zhang et al. 1998). Ethylene swelling in the culture causes troubles for shoot induction (e.g. stoloniferous shoots, decreasing leaf surface, dry weight and chlorophyll) and regeneration in tissue culture. Therefore, inhibition of ethylene action using ethylene antagonists such as STS can direct to normal growth of shoots which consequently increases regeneration efficiency. It was reported that ethylene inhibits cell division and cytodifferentiation in lettuce pith explants (Perl et al. 1988). As the same way, cell division frequency was reduced by 95% in ethylene treated *Pisum sativum* (Apelbaum and Burg 1972). But in contrast to these results the addition of STS did not prove any encouraging effect on the regeneration of *Prunus avium* (Matt and Jehle 2005).

Table 1: Effect of different concentrations of STS on regeneration of *C. asiatica* from nodal explants.

STS (µM l^{-1})	Shoot regeneration frequency (%)	Mean no. of shoots/explant	Mean no. of shoot length (cm)
-	-	-	-
4.4	64	3.2 ± 0.12^{c}	3.96 ± 0.42^{b}
8.9	80	6.8 ± 0.42^{e}	5.14 ± 0.81^{d}
13.3	75	4.1 ± 0.38^{d}	4.40 ± 0.92^{c}
17.8	60	2.9 ± 0.81^{c}	3.84 ± 0.87^{b}
22.2	80	2.6 ± 0.49^{b}	4.68 ± 0.43^{e}
44.4	70	2.0 ± 0.65^{a}	3.10 ± 0.48^{a}

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

Effect of different auxins on rooting of in vitro raised plantlets of C. asiatica on half strength MS medium

Data collection was performed after 4 weeks of inoculation. *In vitro* derived micro shoots (4.0 – 6.0 cm) were removed from culture tubes and separated from shoot clumps and sub cultured on the half strength MS medium augmented with different auxins such as IBA, NAA and IAA. Among all concentrations tested shoot proliferation with roots (80 %) was noticed on MS medium with IBA. The maximum number of roots (32.4 ± 0.48) with root length (5.2 ± 0.91cm) was recorded on IBA (2.5 mg l^{-1}). (Figure 1d) and (Table 2) respectively.

Among the auxins employed for rhizogenesis, growth hormone IBA facilitates maximum rooting efficiency. IBA was more effective than NAA and IAA in promoting rooting of a wide variety of plants, and it is used commercially for rooting of many plant species worldwide. The effectiveness of IBA on rooting has been reported in *Thapsia garganica* (Makunga et al., 2003). Half-strength MS with IBA showed good number of roots compare to full-strength MS medium. MS salt concentration has been carried out for *in vitro* rooting of *Hemidesmus indicus* (Misra et al., 2003).

Table 2: Effect of different concentrations of auxins on rooting of *in vitro* derived micro shoots of *C. asiatica* (L.) on half strength MS medium**.**

Plant growth regulators (mg l^{-1})			Frequency of root formation (%)	Mean no. of roots/shoot	Mean length of root (cm)	Callus
IBA	IAA	NAA				
0.5	-	-	65	7.2 ± 1.20^{b}	3.0 ± 0.24^{bc}	-
1.0	-	-	70	9.6 ± 1.46^{d}	4.2 ± 0.15^{de}	-
1.5	-	-	75	14.8 ± 1.52^{f}	$4.9 \pm 0.16^{g\,hi}$	-
2.0	-	-	85	20.2 ± 0.60^{h}	6.4 ± 0.32^{j}	-
2.5	-	-	100	22.5 ± 0.21^{i}	7.2 ± 0.25^{k}	-
3.0	-	-	80	16.4 ± 0.23^{g}	5.0 ± 0.35^{c}	-
-	0.5	-	70	5.3 ± 1.30^{a}	3.12 ± 0.51^{ab}	-
-	1.0	-	75	8.6 ± 1.63^{c}	3.8 ± 0.16^{d}	-
-	1.5	-	85	12.1 ± 1.59^{e}	4.0 ± 0.67^{ef}	-
-	2.0	-	90	16.2 ± 1.67^{g}	4.86 ± 0.21^{ghi}	C^{+}
-	2.5	-	80	19.8 ± 1.89^{h}	5.2 ± 0.16^{hi}	-
-	3.0	-	70	14.6 ± 0.80^{f}	$4.78 \pm 0.32^{f\,g}$	-
-	-	0.5	65	7.2 ± 2.32^{b}	2.25 ± 0.31^{a}	-
-	-	1.0	60	$9.5 \pm 2.83^{c\,d}$	3.21 ± 0.16^{c}	-
-	-	1.5	85	12.6 ± 2.26^{e}	$4.75 \pm 0.18^{g\,h}$	C^{+}
-	-	2.0	70	9.8 ± 2.09^{d}	2.42 ± 0.26^{ac}	C^{+}

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

Acclimatization and hardening

Well developed rooted plants were carefully removed from culture tubes and washed to remove the remnants of agar (Figure 1e). These healthy and cleaned rooted shoots were transferred to tray containing soil and vermiculate in 1:1 ratio for acclimatization (Chandra Sekhar Panathula et al., 2014). For a period of week the plants were kept in polythene membrane. After that the surviving plants were transferred to pots and maintained under greenhouse for hardening (Figure 1f).

Conclusion

From above study it was conclude that ethylene inhibitor STS does not show any negative effect on shoot regeneration. Thus this ethylene antagonist may be useful as media supplement to develop efficient protocols for *in vitro* regeneration as it favours the shoot formation. The protocol reported here offers an efficient method of conservation and multiplication of potent medicinal plant *C. asiatica*.

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References

Apelbaum, A., Burg, S.P. 1972. Effect of ethylene on cell division and deoxyribonucleic acid synthesis in *Pisum sativum*. *Plant Physiology*, 50: 117-24.

Beyer, E.M. 1979. Effect of silver ion, carbon dioxide and oxygen on ethylene action and metabolism. *Plant Physiology*, 63: 169-173.

Brinkhaus, B., Lindner, M., Schuppan, D., Hahn, E.G. 2000. Chemical, pharmacological and clinical profile of the East Asian medicinal plant *Centella asiatica*. *Phytomedicine*, 7: 427-428.

Burgos, L., Albuquerque, N. 1984. Ethylene inhibitors and low kanamycin concentrations improve adventitious regeneration from *Apricot* leaves. *Plant Cell Reports*, 3: 33-36.

Chandra Sekhar Panathula, Mahadev, M.D., Naidu, C.V. 2014. The stimulatory effects of the antimicrobial agents bavistin, cefotaxime and kanamycin on *in vitro* plant regeneration of *Centella asiatica* (L.)-an important antijaundice medicinal plant. *American Journal of Plant Sciences*, 5: 279-285.

Chi, G.L., Barfield, D.G., Sim, G.E., Pua, E.C. 1990. Effect of $AgNO_3$ and aminoethoxyivinylglycine on *in vitro* shoot and root organogenesis from seedling explants of recalcitrant *Brassica* genotypes. *Plant Cell Reports*, 9(4): 195-198.

Eui Ho Park, Hanhong Bae, Woo Tae Park, Yeon Bok Kim, Soo Cheon, Sang Un Park. 2012. Improved shoot organogenesis of *Sinningi speciosa* using silver nitrate and putrescence treatment. *Journal of Plant Omics*, 5: 6-9.

Harsh Pal Bias, Sudha, G., Suresh, B., Ravishankar, G.A. 2000. Silver nitrate influences *in vitro* root formation in *Decalepis hamiltonii*. *Current Science*, 79: 894-898.

Kumar, P.P., Lakshmanan, P., Thorpe, T.A. 1998. Regulation of morphogenesis in plant tissue culture by ethylene. *In Vitro Cellular & Developmental Biology-Plant*, 34: 94-103.

Makunga, N.P., Jager, A.K., Van Staden, J. 2003. Micropropagation of *Thapsia garganica*, a medicinal plant. *Plant Cell Reports*, 21: 967-973.

Matt, A., Jehle, J.A. 2005. *In vitro* plant regeneration from leaves and internodes sections of sweet cherry cultivars *Prunus avium* (L). *Plant Cell Reports*, 24: 468–476.

Misra, N., Misra, P., Datta, S.K., Mehrotra, S. 2003. Improvement in clonal propagation of *Hemidesmus indicus* R.Br. through adenine sulphate. *Journal of Plant Biotechnology*, 5(4): 239-244.

Murashige, T., Skoog, F. 1962. A revised medium for rapid growth and bioassays with to-

bacco tissue cultures. *Plant Physiology*, 15: 473-479.

Perl, A., Aviv, D., Galun, E. 1988. Ethylene and *in vitro* culture of potato: suppression of ethylene generation vastly improves protoplast yield, plating efficiency and transient expression of an alien gene. *Plant Cell Reports*, 7: 403-406.

Prajapati, N.D., Purohit, S.S., Sharma, A.K., Kumar, T. 2006. A Hand book of Medicinal Plants, Hindustan press, Agro bios, Jodhpur.

Preethi, D., Sridhar, T.M., Naidu, C.V. 2011. Effect of bavistin and silver thiosulphate on *in vitro* plant regeneration of *Stevia rebaudiana*. *Journal of Phytology*, 3(5): 74-77.

Purohit, M., Srivastava, S., Srivastava, P.S. 1998. Stress tolerant plants through tissue culture, In: Srivastava, P.S., (Eds.), Plant tissue culture and molecular biology: application and prospects. Narosa publishing house., New Delhi, pp. 554-578.

Salla Hemadri Reddy, Chakravarthi, M., Chandrasekara, K.N., Naidu, C.V. 2012. Influence of bavistin and silver thiosulphate on *in vitro* regeneration of *Asclepias curassavica* (L.) using nodal explants. *American Journal of Plant Science*, 3: 941-946.

Sridhar, T.M., Preethi, D., Naidu, C.V. 2011. Effect of silver thiosulphate on *in vitro* plant regeneration of *Solanum nigrum* (L.) - An important antiulcer medicinal plant. *Current Botany*, 2(7): 14-16.

Sujana, P., Naidu, C.V. 2011. Influence of bavistin, cefotaxime, kanamycin and silver thiosulphate on plant regeneration of *Mentha piperita* (L.) - An important multipurpose medicinal plant. *Journal of Phytology*, 3(5): 36-40.

Vasantharuba, S., Banumathi, P., Premalatha, M.R., Sundaram, S.P., Arumugam, T. 2012. Functional properties of *Centella asiatica* (L.): A review. *International Journal of Pharmacy and Pharmaceutical Sciences*, 4: 8-14.

Yang, S.F., Hoffman, N.E. 1984. Ethylene biosynthesis and its regulation in higher plants. *Annual Review of Plant Physiology*, 35: 155-189.

Zhang, F.L., Takahata, Y., Xu, J.B. 1998. Medium and genotype factors influencing shoot regeneration from cotyledonary explants of Chinese cabbage (*Brassica campestris* L. sp. *pekinensis*). *Plant Cell Reports*, 17: 780-786.

Zhang, P., Phansiri, S., Puonti, K.J. 2001. Improvements of *Cassava* shoot organogenesis by the use of silver nitrate *in vitro*. *Plant Cell, Tissue and Organ Culture*, 67: 45-54.

Ziv, M. 1991. Vitrification: Morphological and physiological disorders of *in vitro* plants, In: Debergh, P.C., Zimmerman, R.H. (Eds.), Micropropagation technology and applications. Kluwer academic publishers. Dordrecht, pp. 45-69.