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EFFECT OF SILVER NITRATE ON CALLUS CULTURES OF *SPILANTHES ACMELLA* MURR - AN ENDANGERED TOOTH ACHE MEDICINAL HERB

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ABSTRACT

Efficient protocols for callus production from *Spilanthes acmella* were evaluated in the presence of ethylene antagonist $AgNO_3$ in a callus inducing M S media supplemented with various concentrations of plant growth hormones (IAA, 2,4-D, NAA and BAP). Addition of 0.2, 0.4 & 0.6 mg/L $AgNO_3$ to the M S media significantly increased regeneration frequency and also produced more organogenic callus when compare to non-supplemented media. Among various concentrations administrated 0.2 and 0.4 mg/L $AgNO_3$ were found to be most promising in producing 100% callus. Callus inducing media supplemented with 1.0mg/L NAA, 0.1 mg/L BAP And 0.4 mg/L $AgNO_3$ found to be best suitable to produce green organogenic callus from hypocotyl explants of *Spilanthes acmella.* Administering M S media with high concentration of Auxins (2, 3 mg/L) promoted root formation rather than callus initiation.

KEYWORDS: Callus, Ethylene inhibitor, $AgNO_3$, Asteraceae, Spilanthol, Auxins.

INTRODUCTION

With the 'Herbal Medicine' conscious constraint, man has shifted his attention for plant derived medicines to address many life threatening diseases and disorders. Unfortunately, due to multi fold uses of plant drugs and their ever increasing demand, many medicinal herbs are becoming endangered and extinct. One such valuable ornamental cum threatened medicinal herb is *Spilanthes acmella,* (L.) Murr (Rao *et al,* 1983). This plant belongs to the family Asteraceae, well known as `"toothache plant", and also commonly used as a spice. It is native to the tropics of Brazil. *S. acmella* has been well documented for its uses as

antimalarial (Pandey and Agrawal *et al*, 2009), antibacterial (*Prasad and Seenaya*, 2000) antifungal (Sabitha and Murthy, 2006, Sharma *et al*, 2012), larvicidal (Amer and Malhothra, 2006), anti-inflammatory (Chakraborthy *et al*, 2004, Wu et *al*, 2008) and immunomodulating properties (Savadi *et al*, 2010). In addition, *in vitro* results on anti-obesity (Ekanem *et al*, 2010) and antioxidant (Tanwar *et al*, 2010) activities for *S. acmella* are encouraging. It has long history of use as a folklore remedy, e.g. for tooth ache, rheumatism and fever. Medicinal activities are mainly due to the presence of an alkaloid spilanthol (*N*-isobutyl-2, 6, 8-decatrienamide) (Sharma *et al*, 2010). Recently, scopoletin has also been detected in *S. acmella* flower buds (Prachayasit ikul *et al*, 2009 and *Singh et al*, 2010). Spilanthol also showed anti-ageing activity by inhibiting contractions in subcutaneous muscles, notably those of face and can be used as an anti-wrinkle product. Many anti-ageing products are available in commercial market as registered brands (Gatuline®, SYN®-COLL, ChroNOline™) containing spilanthol. Such wide spectrum uses of spilanthol makes it a wonder drug for pharmaceutical sector.

Despite able production of large amount of seeds, very poor germination percentage, low and short period viability of them often make the traditional conservation methods to fail to meet the world class pharmaceutical demand. So, it has become imperative to develop alternate methods for large scale propagation, conservation and optimized production of secondary metabolites. Many micropropagation protocols had been developed previously to propagate this plant in large scale from leaf and axillary bud explants (Pandey *et al*, 2009, Saritha *et al*, 2008). Suitable Spilanthol extraction techniques from micropropagated plants were also developed (Pandey *et al*, 2011). But, until now not much concentration was given to develop callus cultures which are direct source for easy extraction of secondary metabolites. In vitro raised callus, besides being a major source for drugs, also serves as raw material for Germplam preservation, long term sub cultures and to produce somatic embryos for artificial seeds synthesis. Callus is also a good source of genetic variability and adventitious shoot formation (Doods and Roberts, 1982).

Under *in* vitro conditions, optimum expression of Totipotency of any plant cell is clearly manifested by supplying exogenous plant growth hormones in the media. Along with external supplemented hormones certain endogenously produced and accumulated hormones such as ethylene in the culture vessels engender certain detrimental effects on cellular expressions. Ethylene is basically a fruit ripening hormone produced in gaseous state and accumulated in

closed culture vessels. Effect of ethylene in inducing friable embryonic callus had been studied in many plant species. These past reported studies clearly depicts the negative impact of ethylene on callus formation (Chalutz and Devay, 1969, Zobel and Roberts, 1978), high rates of callus necrosis (Adkins *et al.,* 1990). However, Ethylene action under in vitro conditions can be suppressed by adding of inhibitors like $AgNO_3$, $CoCl_2$. Addition of $AgNO_3$, in the culture media had shown better impact on callus cultures when compare to $CoCl_2$ in *Solanum Viarum* (Sujana and Naidu, 2015). Supplementation of $AgNO_3$ increases type II callus production from immature embryos in maize (Songstad *et al.,* 1991). Incorporation of silver nitrate in the medium of *Brassica oleracea* callus significantly improved growth and allowed long term callus cultures (Williams *et al.,* 1990).

In the present investigation we have made an attempt to develop most reliable protocol for callus culture to overcome certain recurrent constraints in the large scale production of raw material for secondary metabolites, thus helping in the conservation and sustainable utilization of this endangered herb for medicinal purposes.

MATERIAL AND METHODS

Plant material

Spilanthes acmella plants were collected from G.K.V.K. Agriculture University, Bengaluru and grown in the botanical garden of P.V.K.N. Govt. College, Chittoor. Healthy seeds were collected from dried flowers and used for initiating callus cultures.

Aseptic inoculation of seeds

Callus cultures were initiated from hypocotyl explants of *Spilanthes acmella*. Seeds were surface sterilized with 0.4% Bavistin for 1 minute to avoid the fungal contamination and thoroughly wash it with sterilized distilled water. Then, seeds were soaked in 0.1% $HgCl_2$ for 2 minutes followed by thorough wash with sterilized double distilled water to remove traces of all sterillants. Sterilized seeds were transferred to autoclaved M S media (Murashigae and Skoog, 1962) supplemented with 2mg/L BAP. The pH of the media was adjusted to 5.7 before autoclaving at 121^0C for 15 minutes. These cultures were maintained at 25 ± 2^0C under 16 hrs light and 8 hrs darkness for 3 weeks under aseptic culture conditions.

Callus induction

Callus cultures were initiated from hypocotyl explants which were raised from aseptic seeds after 3 weeks of inoculation. Hypocotyl segments (3mm in length) were placed on the callus

inducing media containing 0.1mg/L BAP and Different concentrations of IAA (1,2,3mg/L), 2,4-D (1,2,3 mg/L), NAA (1,2,3), $AgNO_3$ (0.2, 0.4 & 0.6 mg/L). All the hormonal concentrations were made with aqueous solution and added to the media before autoclaving. Five replicates were maintained for all the experiments and each experiment was carried out twice at 25 ± 2^0C under 16 hrs light and 8 hrs darkness. Growth proliferation and nature of callus growth were recorded after 4 weeks of observations. Intensity of callus was represented as + Very low, ++ Low, +++ High, ++++ Very High.

RESULTS AND DISCUSSION

Effect of various concentrations of $AgNO_3$ and growth hormones on callus initiation from hypocotyl explants

Experimental observations of hypocotyl explants response for callus induction at various concentrations of Auxins (IAA, 2,4-D, NAA) and $AgNO_3$ along with 0.1mg/L BAP were recorded in Table 1.

Table: 1 Effect of different concentrations of AgNO3 and growth hormones on callus initiation from leaf explants of *in vitro* grown *Solanum viarum*. Data represent mean ± SE of 5 replications.

Plant growth regulator (mg/L)				$AgNO_3$ Con (mg/L)	Regeneration frequency%	Nature of callus formation	Intensity formation
NAA	2,4-D	IAA	BAP				
1	-	-	0.1	-	65	Pale, Fragile	+
1	-	-	0.1	0.2	100	Pale Yellow, Fragile	++
1	-	-	0.1	0.4	100	Pale Green, nodular	++++
1	-	-	0.1	0.6	90	Pale yellow, profused	++++
2	-	-	0.1	-	75	Pale. fragile	+
2	-	-	0.1	0.2	95	Pale yellow,hairy	+
2	-	-	0.1	0.4	100	White, Hairy	++
2	-	-	0.1	0.6	80	White robust hair	+
3	-	-	0.1	-	65	Pale, fragile	+
3	-	-	0.1	0.2	90	Pale yellow, hairy	+
3	-	-	0.1	0.4	100	White nodular, hairy roots	++
3	-	-	0.1	0.6	65	Dense robust roots	+
-	1	-	0.1	-	80	Pale, Fragile	+
-	1	-	0.1	0.2	100	Light green, fragile	+++
-	1	-	0.1	0.4	100	Dark brown, shoot tips	++++
-	1	-	0.1	0.6	85	Dark Brown, Compact	++++

-	2	-	0.1	-	70	Pale, Fragile	+
-	2	-	0.1	0.2	95	Pale yellow, fragile	++++
-	2	-	0.1	0.4	100	light brown, hairy	++++
-	2	-	0.1	0.6	75	Dense, Hairy	+
-	3	-	0.1	-	65	Pale, Fragile	+
-	3	-	0.1	0.2	90	Pale brown, fragile	+
-	3	-	0.1	0.4	100	Light brown, robust roots	+
-	3	-	0.1	0.6	70	Robust hairy roots	+
-	-	1	0.1	-	70	Pale, Fragile	+
-	-	1	0.1	0.2	100	Pale green, fragile	++++
-	-	1	0.1	0.4	100	Dark green, nodular	++++
-	-	1	0.1	0.6	90	Pale Green, Fragile	+++
-	-	2	0.1	-	65	pale, fragile	+
-	-	2	0.1	0.2	95	Pale yellow, hairy	+
-	-	2	0.1	0.4	95	Dense hairy	++
-	-	2	0.1	0.6	80	Dense, robust hairy	+
-	-	3	0.1	-	65	Pale, fragile	+
-	-	3	0.1	0.2	90	Pale yellow hairy	+
-	-	3	0.1	0.4	90	Dense hairy	+++
-	-	3	0.1	0.6	70	More dense hairy	+

Intensity of the callus: + Very low, ++ Low, +++ High, ++++ Very High.

Callus initiation was started from the cut ends of the hypocotyl explants at all concentrations after one week of inoculation. Initially explants were enlarged in size and started to grow as irregular mass from the cut edges. But the growth rate was observed to be different at various concentrations of Auxins and $AgNO_3$. Callus growth was found to be rapid and vigorous in $AgNO_3$ supplemented media when compared to $AgNO_3$ lacking media. In the $AgNO_3$ absentia, callus cultures were observed to produce pale, fragile, stunted growth structures at all concentrations of Auxins. This stunted pale growth of callus might be due to partial or complete suppression of cellular Totipotency by accumulated ethylene hormone in the culture vessels. These results are corroborated with callus induction reports in *Solanum viarum* (Sujana *et al*, 2017), buffalo grass (Fei *et al*, 2000).

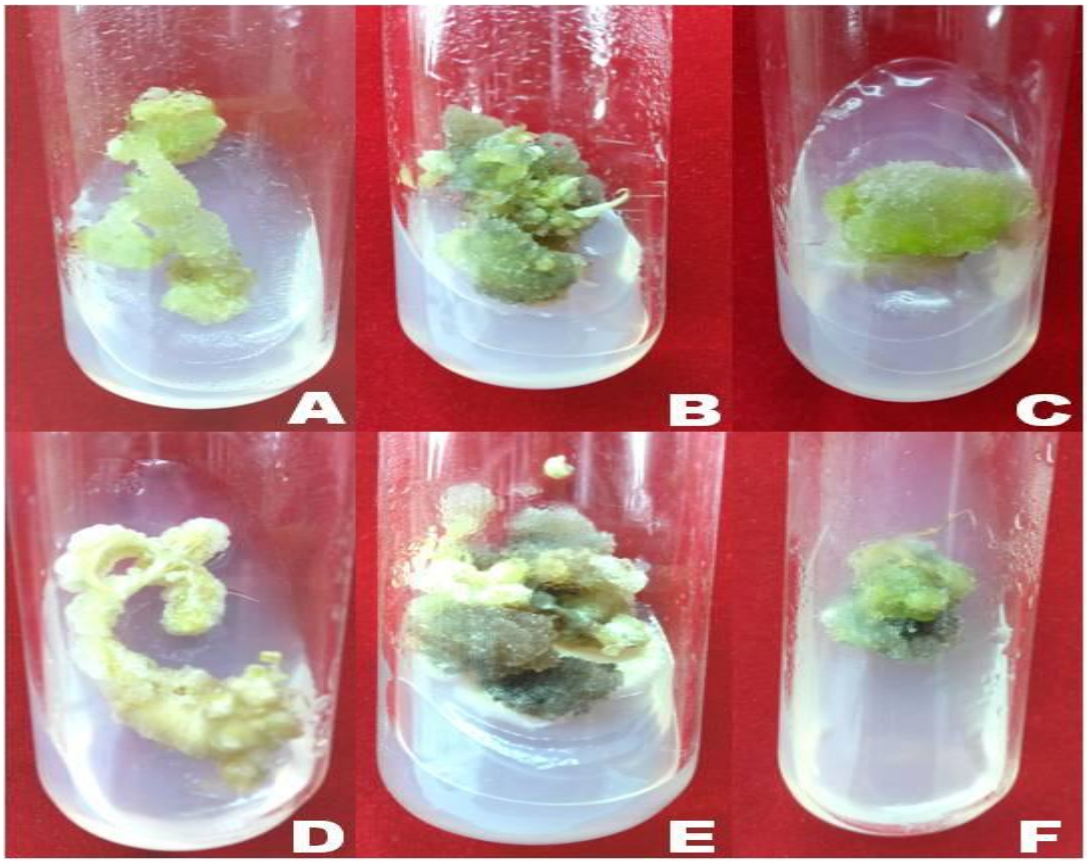

Fig – 1: Effect of $AgNO_3$ on Callus proliferation from hypocotyl explants of *Spilanthes acmella* cultured on MS media containing 0.1 mg/L BAP and (A) 1mg/L IAA + 0.4mg/L $AgNO_3$, (B) 1mg/L 2,4 - D + 0.4mg/L $AgNO_3$, (C) 1mg/L NAA + 0.4mg/L $AgNO_3$, (D) 1mg/L IAA + 0.6mg/L $AgNO_3$, (E) 1mg/L 2,4 - D + 0.6mg/L $AgNO_3$, (F) 1mg/L NAA + 0.6mg/L $AgNO_3$.

Callus formation at 0.2 and 0.4 mg/L $AgNO_3$ in all tested concentrations of Auxins (Fig:1) found to be effective in promoting in terms of increased regeneration frequency, callus size and nature (texture). Whereas at 0.6 mg/L $AgNO_3$ supplementation media callus growth was suppressed. This may be due to toxic effects of metal ions on cellular totipotency expression and some previous research reports support this observation (Takuma *et al*, 2000).

Variations in Auxin concentrations at 0.1 mg/L BAP also had shown implicit impacts on callus growth and texture. 1 mg/L Auxins found to be better in callus induction when compared to 2 & 3 mg/L concentrations. Higher concentrations of Auxins (2, 3 mg/L) had provoked rapid robust rhizogenesis (Fig:2) at all concentrations of $AgNO_3$. This may be due to very high Auxin and Cytokinin ratio (De, 2008) in the culture media.

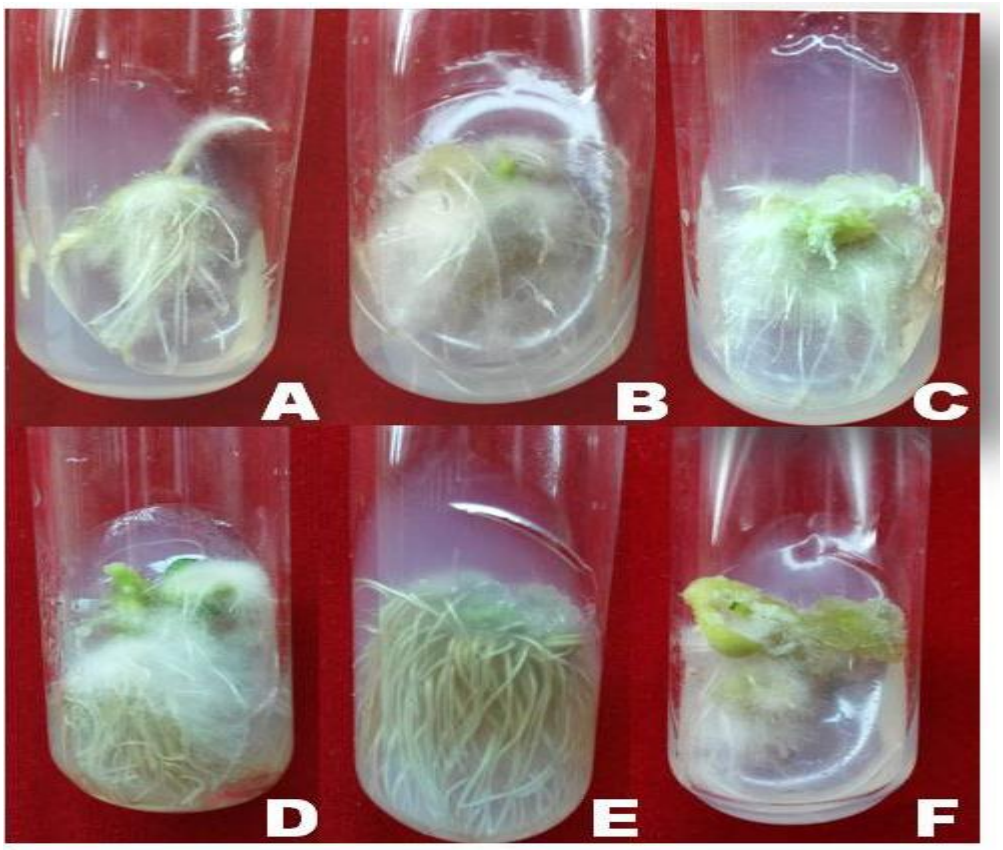

Fig – 2: Effect of $AgNO_3$ on Callus proliferation from hypocotyl explants of *Spilanthes acmella* cultured on MS media containing 0.1 mg/L BAP and (A) 2mg/L IAA + 0.4mg/L $AgNO_3$, (B) 2mg/L 2,4 - D + 0.4mg/L $AgNO_3$, (C) 2mg/L NAA + 0.4mg/L $AgNO_3$, (D) 3mg/L IAA + 0.4mg/L $AgNO_3$, (E) 3mg/L 2,4 - D + 0.4mg/L $AgNO_3$, (F) 3mg/L NAA + 0.4mg/L $AgNO_3$.

Best stimulation of callus growth in terms of 100% regeneration frequency and green nodular texture was observed to be at 1 mg/L NAA, 0.4 mg/L $AgNO_3$ and 0.1 mg/L BAP. Various concentrations of IAA were found to be less proliferative when compare to NAA & 2,4-D. Supplementation of 1mg/L 2,4- D at 0.2 mg/L $AgNO_3$ concentration found to be productive, whereas at 0.4 & 0.6 mg/L $AgNO_3$ concentration formed dark brown compact callus . This might be due to active involvement of 2,4-D in polymerization of phenols, alkaloids & other chemicals to produce brown colour products (De,2008). This brown callus on further sub culturing did not show any growth. 0.4 mg/L $AgNO_3$, 0.1 mg/L BAP at all 1mg/L Auxin concentrations observed to produce green nodular callus with shoot tips. This green colour may be attributed to development of chloroplastids, which on further sub cultures proliferated to produce multiple shoots.

The exact mechanism of $AgNO_3$ ameliorating action on callus initiation and proliferation is still unclear. However, from the previous studies on antagonistic action of $AgNO_3$ by peer scientists it is thought that $AgNO_3$ blocks ethylene receptors (Bayor, 1976) or silver ions thought to disturb the ethylene ion binding site (Rodriguez *et al*, 1999). Sometimes over accumulation of ethylene in culture tubes is auto inhibitory for further production of ethylene itself (Yang *et al.*, 1984). We can assume that $AgNO_3$ might be supplying threshold level of hormones to cells for unorganized cellular growth upon continuous interaction with oozed endogenous hormones from cut ends of explants, accumulated and exogenously supplied hormones. We can also presume that silver ions (Ag^+) might have non-competitively blocked the ethylene perception by altering conformation of ETR1 (Ethylene receptor in cell walls) upon replacing Cu^+ in the cofactor (Zhao *et al*, 1999). Further molecular level studies have to be taken up for better understanding of $AgNO_3$ impact on callus proliferation.

CONCLUSION

Spilanthes acmella, an important medicinal herb, becoming extinct and endangered due to its commercial exploitation and low natural propagation responses. In our present investigation, a suitable protocol had been developed for callus production which can serve as direct source of secondary metabolite spilanthol. Nonetheless, threshold level supply of exogenous hormones and endogenously produced hormones besides incorporation of $AgNO_3$ into the callus inducing media of *Spilanthes acmella,* significantly improved the callus initiation and proliferation. Further molecular level investigations are required to examine the factors influencing the quantitative and qualitative production of spilanthol under in vitro conditions.

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