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Influence of Bavistin and Silver Thiosulphate on *in Vitro* Regeneration of *Asclepias curassavica* (L.) Using Nodal Explants

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ABSTRACT

The effect of bavistin and the ethylene inhibitor (silver thiosulphate) on shoot regeneration using nodal explants of *Asclepias curassavica* (L.) has been investigated. Among the different concentrations studied, highest number of shoots was obtained on MS media with 200 mg/L bavistin. Among the varying concentrations (10 - 100 µM/L) of silver thiosulphate tested, highest number of shoots was obtained on MS medium amended with 60 µM/L silver thiosulphate without growth regulators. This study also establishes the stronger cytokinin like activity of bavistin. Effect of different growth additives like coconut milk, ascorbic acid and casein hydrolysate were tested on direct shoot regeneration. Among the different growth additives tested casein hydrolysate showed better and reproducible result at 0.025% in combination with 3 mg/L KN + 0.5 mg/L NAA. Antioxidants, activated charcoals and polyvinyl pyrrolidone were used to remove phenolics. Activated charcoal removed the phenolic exudates completely at 0.025% and prevented the browning of media and thus enhanced the frequency of regeneration (85%). The microshoots developed through *in vitro* regeneration were transferred to rooting media containing IBA alone and in combination with KN and the highest number of roots was observed on MS medium with IBA 1 mg/L + 0.2 mg/L KN.

Keywords: Bavistin; Regeneration; Asclepiadoideae; Silver Thiosulphate

1. Introduction

Asclepias curassavica (L.) (Tropical milkweed) is an erect, evergreen sub shrub belonging to the sub family Asclepiadoideae, in Apocynaeceae family [1]. Its root extracts are widely used as an emetic and laxative. A decoction of the plant is used as an abortifacient. Roots are of medicinal importance (termed "Pleurisy root") and are used as an expectorant for pneumonia, lung problems, treat warts, fever, ringworm and bleeding. In general, Asclepiadoideae plants are source of cytotoxic and cardiac glycosides consisting of highly valuable products of medicinal importance. World wide shift towards herbal medicinal inclination over synthetic pharmaceuticals has resulted in overexploitation of number of plants with medicinal values. Several species, of known important drugs sources are being exploited a great deal as they are the only source of these drugs and in their effectiveness in production. Although India harbours rich plant diversity, with ever increasing population and deforestation have adversely affected their status; medicinal plants in particular [2].

Endemicity, restricted distribution, small population, inaccessible areas and anthropogenic pressure have caused decline in wild population of many species making their status as rare [3]. It is imperative that viable strategies ought to be implemented to conserve the surviving population at least, the critically important medicinal species from further loss. The conventional approaches to conservation and preservation include the *in situ* and *ex situ* conservation strategies. However, for many rare species, *in situ* preservation is not a feasible option owing to increasing human disturbance. Under such circumstances *in vitro* regeneration is undoubtedly an efficient means of *ex situ* conservation of plant diversity [4,5].

With the advent of biotechnological approaches, culturing plant cells and tissues have turned out to be easier and a boon for conserving and propagating valuable, rare

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and endangered medicinal plants. At present, tissue culture methods are used in almost 600 companies throughout the world to produce more than 500 million units annually from 50,000 varieties of plants [6]. The global biotechnology business is estimated to be round 150 billion U.S. dollars, of which 50% - 60% is in agribusiness and the annual demand of tissue culturally raised products constitutes about 15 billion US dollars with an annual growth rate of about 15 per cent [7].

Antimicrobial agents (antibiotics and fungicides) are generally used in plant tissue culture media to eliminate microorganisms that are present in explants or arise as laboratory contaminants. Several of such agents are reported to effect *in vitro* cell culture and plant regeneration. Certain agents like carbendazim, fenbendazole and imazalil were found to be least toxic to plant cells and had a broad spectrum fungicidal activity [8]. Ag^+ ions of the ethylene inhibitor silver thiosulphate inhibit activity of ethylene in a various plant species [9]. The ethylene inhibiting effect of Ag^+ is due to an interference believed with ethylene binding [10]. The positive effect of Ag^+ ions suggests that ethylene produced by inoculated explants inhibits shoot organogenesis [11]. The beneficial effects of ethylene inhibitors on organogenesis for plant regeneration have been widely reported [12-14]. The influence of Bavistin and silver thiosulphate has been well documented in many medicinal plants such as *Mentha piperita* [15], *Stevia rebaudiana* [16] and effect of Bavistin with adenine sulphate has been studied in *Picrorhiza scrophulariiflora* [17].

The positive role of different growth additives coconut milk, ascorbic acid and casein hydrolysate has been reported extensively in the *in vitro* propagation of *Gymnema sylvestre* [18]. Antioxidants like polyvinyl pyrrolidone and activated charcoal have been used to reduce the phenolic exudates in a variety of plant tissue culture protocols. Hence the present study was aimed in understanding the influence of these growth additives as well as antioxidants in the in vitro shoot multiplication of *A. curassavica*. However no such attempts have been made in *A. curassavica.* Hence, the present study was aimed to comprehend the influence of bavistin and silver thiosulphate on *in vitro* shoot regeneration from nodal explants of *A. curassavica*.

2. Materials and Methods

In the present study, *A. curassavica* seeds were collected from Tirumala hills, Tirupati, Andhra Pradesh (A.P.) during March, 2005. The seeds were germinated in the garden, Department of Biotechnology, S.V. University, Tirupati, A.P. Actively growing shoots with five to six nodes were used as explants. The explants were washed under running tap water for 5 - 10 minutes, presoaked in liquid detergent (1% tween 20) for 1 - 5 minutes and surface sterilized in 70% ethanol for 60 seconds and Mercuric chloride (0.1%) for 1 - 5 minutes. Then they were rinsed with sterile double distilled water for 4 - 5 times. After trimming the cut ends, the explants were blotted on sterile filter paper discs. Nodal explants of *A. curassavica* were cultured on MS media supplemented with Bavistin (fungicide) at different concentrations ranging from 10 - 400 mg/L containing 3% (w/v) sucrose. The media was solidified with 0.8% (w/v) agar. The pH of the medium was adjusted to 5.8. In addition, to trace the effect of silver thiosulphate on direct shoot regeneration, the nodal explants of *A. curassavica* were cultured on MS media supplemented with silver thiosulphate at different concentrations (10 - 100 μM) along with growth additives and antioxidants. The effect of different growth additives and antioxidants were tested on shoot regeneration from nodal explants of *A. curassavica*. Effect of different concentrations of growth additives such as coconut milk (CM) and casein hydrolysate (CH) and antioxidants like activated charcoal (AC), poly vinyl pyrrolidone (PVP) and Ascorbic acid (AA) were studied. Different adjuvants were tested in combination with various cytokinins at their optimum concentrations either individually or in combination with different auxins. Experiments were set up in a completely randomized design and each treatment had 4 replicates of 5 explants each. All data are statistically analyzed by Analyses of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT).

3. Results and Discussion

Bavistin in direct shoot regeneration appeared to have much stronger cytokinin-like activity. Bavistin is a systemic fungicide that belongs to benzimidazole family. Benzimidazoles are a group of organic fungicides with systemic action that are extensively used in agriculture [19]. It has been reported that the molecular structure of methyl benzimidazole carbamate (or) carbendiazim has some resemblance to kinetin, adenine and to many other cytokinins based on adenine [20]. In addition, it has also been demonstrated that these compounds can have beneficial effects on the physiology of the plant [21] for example, benzomyl (carbamate of methyl-N-butyl-carbamyl-benzimidazole), one of the most effective and extensively used benzimidazoles, has a cytokinin activity in soy and radish [22,23].

In the present investigation Bavistin (fungicide) was used to study the effect on axillary bud proliferation in addition to its antifungal activity. Among the varying concentrations used for direct shoot regeneration, highest frequency of shoot regeneration of 70% was obtained at

250 mg/L Bavistin containing MS media. The results of the study indicated the differences in frequency of shoot regeneration, number of shoots/explants and length of shoot, the results are tabulated in **Table 1** with respect to Bavistin concentrations. Our study showed that at 250 mg/L concentration, Bavistin showed maximum shoot regeneration frequency and it was significant at $p < 0.01$. Similarly number of shoots/explants and length of shoots showed statistically signification results at concentrations 150, 200 and 250 mg/L compared to other concentrations of Bavistin (**Figure 1**). Earlier studies conducted in other plant systems have also shown that bavistin/fungicides [20] promote shoot regeneration. In present study, the influence of Bavistin in shoot regeneration of *A. curassavica* cultures is possibly due to its "cytokinin-like" activity, results are tabulated in **Table 1**. The breakdown products of Bavistin trigger shoot regeneration resulting in enhanced biosynthesis of endogenous cytokinins within the cultures.

The beneficial effects of the ethylene inhibitor silver thiosulphate (STS) on organogenesis have been widely

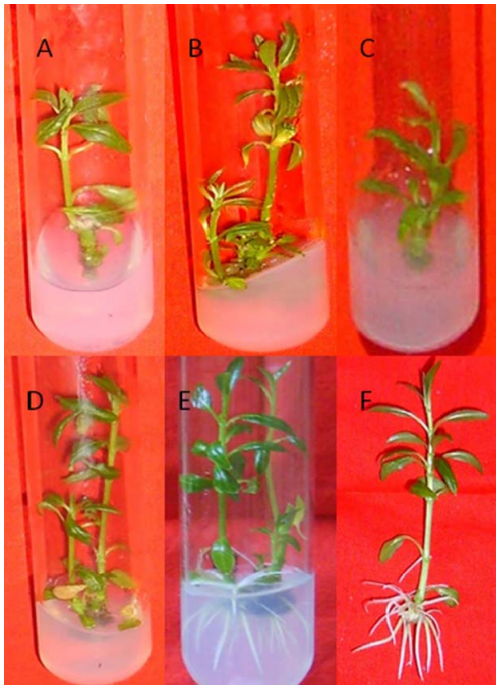

Figure 1. Axillary bud explants of *A. curassavica* cultured on MS medium supplemented with various concentration of Bavistin and Silver thiosulphate individually. (A) Shoot bud initiation from nodal explants on MS medium + Bavistin (100 mg/L); (B) Multiple shoot formation from nodal explants on MS medium + Bavistin (200 mg/L); (C) Shoot bud initiation from nodal explants on MS medium + STS (30 µM/L); (D) Multiple shoot formation from nodal explants on MS medium + STS (50 µM/L); (E) Rhizogenesis from *in vitro* cultured plants on MS media; (F) Well rooted plants ready for hardening.

Table 1. Effect of different concentrations of Bavistin on direct shoot regeneration from seedling nodal explants of *A. curassavica* in MS media. Observations: After 4 weeks.

Bavistin mg/L	Frequency of shoot regeneration (%)	No. of shoots/explant*	Length of shoots (cm)**
10	30.00^{a}	1.00^{a} (2.00)	3.20^{abc} (3.70)
25	35.00^{ab}	1.40^{abc} (2.40)	3.20^{abc} (3.70)
50	40.00^{bc}	1.20^{ab} (2.20)	3.60^{abc} (4.10)
100	45.00^{cd}	1.80^{bcd} (2.80)	3.80^{abc} (4.30)
150	50.00^{de}	2.20^{de} (3.20)	3.90^{bc} (4.40)
200	60.00^{fg}	2.60^{e} (3.60)	4.20^{c} (4.70)
250	70.00^{h}	2.00^{cde} (3.00)	4.00^{bc} (4.50)
300	65.00^{gh}	1.60^{abcd} (2.60)	3.80^{abc} (4.30)
350	60.00^{fg}	1.40^{abc} (2.40)	3.00^{ab} (3.50)
400	55.00^{ef}	1.00^{a} (2.00)	2.70^{a} (3.20)
CV%	12.84	34.59	34.97
CD@1%	5.33	0.74	1.15

*Values in the parenthesis are X + 1 transformed values; **Values in the parenthesis are X + 0.5 transformed values; The same letters indicate no significant difference at $p < 0.01$ (ANOVA and DMRT).

reported [11-14]. The Ag^{+} ions inhibit ethylene action in a wide variety of ethylene induced responses in plants. The ethylene inhibiting effect of Ag^{+} is believed to be due to an interference with ethylene binding [10]. The positive effect of Ag^{+} ions in shoot organogenesis suggests that ethylene produced by cultured explants inhibits shoot organogenesis of those explants [11].

Experiments were conducted to scrutinize the effect of varying concentrations of ethylene inhibitor STS on shoot regeneration of *A. curassavica*. STS tested on direct shoot regeneration from nodal explants of *A. curassavica* was 10 - 100 µM/L in MS medium. Increasing the concentration of STS enhanced regeneration capacity of MS medium. The results of the study indicated the differences in frequency of shoot regeneration, number of shoots/explants and length of shoot, the results are tabulated in **Table 2** with respect to varying concentration of STS. Our study showed significant higher percentage of at 40, 70 - 100 µM/L concentrations of STS, maximum shoot regeneration frequency was obtained at 80 µM and it was significant at $p < 0.01$. Number of shoots/explants from 30 - 80 µM/L concentrations of STS produced significant results and length of shoots showed statistically signification results at concentrations of 30 - 80 µM/L compared to other concentrations of STS. Maximum shoot length 4.70 (5.20) at 50 µM/L concentrations was observed, results are tabulated in **Table 2**. Hence optimal

Table 2. Effect of silver thiosulphate on direct shoot regeneration from nodal explants of *A. curassavica* in MS media. Observations: After 4 weeks.

Concentration of silver thiosulphate in µM	Frequency of shoot regeneration (%)	No. of shoots/explant*	Length of shoots (cm)**
10	30.00[a]	1.00[a] (2.00)	2.70[a] (3.20)
20	50.00[b]	2.00[abc] (3.00)	3.80[b] (4.30)
30	60.00[c]	2.20[bcd] (3.20)	3.80[b] (4.30)
40	65.00[cd]	2.00[abc] (3.00)	4.50[bc] (5.00)
50	50.00[b]	3.20[d] (4.20)	4.70[c] (5.20)
60	60.00[c]	3.00[cd] (4.00)	4.50[bc] (5.00)
70	65.00[cd]	2.80[bcd] (3.80)	4.20[bc] (4.70)
80	70.00[d]	2.20[bcd] (3.20)	4.00[bc] (4.50)
90	65.00[cd]	2.00[abc] (3.00)	4.20[bc] (4.70)
100	70.00[d]	1.80[ab] (2.80)	4.00[bc] (4.50)
CV%	11.41	38.29	21.73
CD@1%	5.44	1.00	0.80

*Values in the parenthesis are X + 1 transformed values; **Values in the parenthesis are X + 0.5 transformed values; The same letters indicate no significant difference at $p < 0.01$ (ANOVA and DMRT).

concentrations of STS may prove to be a useful media supplement in plant tissue culture.

The presence of casein hydrolysate (0.025%) in the growth medium produces appreciable amount of callus from the basal cut end of the explant. A mean shoot number of 3.2 was observed at 3 mg/L KN + 0.5 mg/L NAA medium when 0.025% of casein hydrolysate was added. Casein hydrolysate did not showed any effect regarding shoot length in both KN and BAP containing media. Addition of ascorbic acid enhanced shoot number. The highest shoot number of 3.0 was achieved with 3 mg/L KN + 0.5 mg/L NAA when 0.01% of ascorbic acid was added to the medium. But a significant decrease in shoot length was observed on this medium compared to the medium without ascorbic acid. Addition of coconut milk had no significant effect on shoot morphogenesis. There was no enhancement of shoot number with addition of Coconut Milk to the media containing 2 mg/L BAP + 0.5 mg/L NAA and 3.0 mg/L KN + 0.5 mg/L NAA. A slight decrease in shoot number (2.5) was observed in 2 mg/L BAP + 0.5 mg/L NAA containing medium with addition of coconut water and slight increase in shoot number (3.2) was noted in 3 mg/L KN + 0.5 mg/L NAA medium and the results are tabulated in **Table 3**.

Similar result was reported on other Asclepiadacean, *Hemidesmus indicus* by [24] where addition of 10% coconut water has resulted in highest number of shoots. It has been stated that ascorbic acid will increase the metabolic activity and accelerate the release of sugars [25], thereby enhancing the organogenic ability. Similar response with ascorbic acid was observed by [3,26-28]. More over ascorbic acid is a strong reducing agent that scavenges the free oxygen radicals present if any in the medium. Comparatively, the other growth adjuvants coconut water (10%) and casein hydrolysate (0.025%) were found ineffective in improving either shoot number (or) quality in the present investigation. This may support the view that for most tissue culture purposes, addition of undefined supplements containing amino acids may be unnecessary when correct balance of inorganic salts were present in the medium [29]. Callus formed at the cut end of the nodal explants readily turned brown and retarded the vigorous growth of shoot when they were cultured on medium with casein hydrolysate, possibly due to maladjustment of cells to the excessive organic nitrogen [18].

The antioxidants activated charcoal (0.025%) and polyvinyl pyrrolidone (0.025%) were used to remove phenolics. Activated charcoal subdued the phenolic exudates completely at 0.025% and prevented the browning of media and thus enhanced the frequency of regeneration (85%) data not shown. In absence of activated charcoal it was observed that media turned to brown in colour, prolonged cultures get contaminated. Comparatively other antioxidant (PVP) checked did not show much effect and frequency of regeneration was also less *i.e.*, 75%. The addition of activated charcoal having beneficial effects were reported in *Gymnema sylvestrae* [30], *Wattakata volubilis* [31].

In vitro derived shoots with a length of (3 - 5 cm) were excised and transferred to MS medium supplemented with different concentrations of auxins such as IBA, IAA and NAA (0.1 - 2.0 mg/l) alone and in combination with KIN. In all the concentrations tried, highest number of roots was observed on MS medium with IBA 1 mg/L + 0.2 mg/L KN. Highest rooting frequency (85%), with a maximum number of roots (12.2 ± 0.53) was observed in this combination, results are tabulated in **Table 3**.

4. Conclusion

From the results it is noticeable that fungicide bavistin, ethylene inhibitor silver thiosulphate does not shows any harmful effect on shoot regeneration of *Asclepias curassavica*. Thus these promissory compounds may be useful as a media supplement to develop efficient protocols for *in vitro* propagation as it favors the shoot formation. Casein hydrolysate can be used as an important growth factor that promotes shoot regeneration frequency of *Asclepias curassavica* in the present study. Activated charcoal

Table 3. Effect of different growth adjuvants on multiple shoot regeneration from nodal explants of *A. curassavica* in MS media supplemented with different growth regulators. Observations: After 4 weeks (The results are mean (SE±) of 20 independent determinations).

	Concentration of growth additive (%)	(%) of response	PGRs Concentration in mg/L			Average no of shoots formed Mean ± SE	Average length of shoot Mean ± SE (cm)
			BAP	KN	NAA		
CM	10	40	-	-	-	2.0 ± 0.00	1.7 ± 0.00
	15	40	-	-	-	2.0 ± 0.00	2.7 ± 0.00
	20	50	-	-	-	2.0 ± 0.00	2.5 ± 0.00
	10	65	2	-	0.5	2.5 ± 0.25	3.0 ± 0.46
	10	65	-	3	0.5	2.8 ± 0.56	3.5 ± 0.00
	10	50	-	-	0.5	1.2 ± 0.00	3.3 ± 0.52
AA	0.01	80	2	-	-	2.0 ± 0.00	2.0 ± 0.00
	0.01	85	2	-	0.5	2.4 ± 0.25	3.2 ± 0.56
	0.01	80		3	0.5	3.0 ± 0.00	2.0 ± 0.43
	0.025	82	2	-	0.5	2.0 ± 0.00	4.0 ± 0.00
CH	0.025	80	-	3	0.5	3.2 ± 0.56	2.9 ± 0.64
	0.025	60	-	-	0.5	1.2 ± 0.00	2.7 ± 0.74

CM = Coconut milk; AA = Ascorbic acid; CH = Casein hydrolysate.

removed the phenolic exudates completely and prevented the browning of media and thus enhanced the frequency of regeneration.

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