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Effect of temperature and media supplements on slow growth conservation of medicinal plant *Spilanthes acmella*

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ABSTRACT: *Spilanthes acmella* (L.) Murray is a major source of spilanthol, an alkamide exhibiting various biological and pharmacological activities. *In vitro* slow growth was attempted by culturing *in vitro* nodal explants on MS medium supplemented with different concentrations of osmotic agents mannitol and sorbitol and ABA (abscisic acid) as well as on 1/2MS followed by storage at 15±2 and 26±2°C for 2, 4, 6 and 8 months without any subculture. Storage conditions were evaluated by percent survival, number and length of shoots and number of leaves. Post storage revival of cultures after every conservation period was evaluated by re-growth on MS media supplemented with 0.5mg/l BAP at 26±2°C.

Slow growth treatments significantly improved survival with maximum percent survival on MS+2% mannitol at 15±2°C at the end of 8 months. Of the two temperatures, 15±2°C gave a significant reduction in growth. Cultures stored at 26±2°C did not survive more than 5 months. Among different treatments, 2% mannitol followed by 2% sorbitol proved effective in slowing growth of the cultures in terms of shoot number and length. Half MS salts and addition of ABA to the media did not result in slowing down growth. Moreover, shoot number, length and leaf number decreased with increase in the storage duration. Re-growth with maximum percent survival was observed in plants stored on MS+2% mannitol at 15±2°C. However, higher concentrations of osmotic agents proved deleterious for survival as well as re-growth of the plants.

Key words: *Spilanthes*, slow growth, conservation, osmotic agents, temperature, storage

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INTRODUCTION

Spilanthes acmella (L.) Murray (Asteraceae) is a rich source of the alkamide spilanthol which is known to be an effective insecticidal and larvicidal compound (Saraf & Dixit 2002, Mbeunkui *et al.* 2011). It can, therefore, be developed into a potent antimalarial agent. *S. acmella* is also well-reported to possess diuretic, vasorelaxant, anti-inflammatory, antimicrobial, immunomodulatory, analgesic and aphrodisiac activities (Tiwari *et al.* 2011). Several preparations of *S. acmella* like Declatone neck antiwrinkle cream, Sinus support formula "intensify" and "Spilanthes supreme" – an antiviral formula are commercially available. Moreover, *S. acmella* has been reported as an acutely endangered plant species (Sharma & Shahzad 2013) and conventional propagation of *Spilanthes* is not sufficient due to poor vegetative propagation and low rate of germination (Rios-Chavez *et al.* 2003) which is posing a hurdle in coping with the ever-increasing industrial demands. Furthermore, the biosynthetic pathway of spilanthol is still unknown. Therefore, *in vitro* conservation can be a mode of germplasm storage to ensure maximum vigour and a disease-free state.

In vitro conservation can be performed by normal *in vitro* culture preservation, cryopreservation and slow growth conservation. Normal preservation and cryo-

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Fig. 1: Slow growth conservation of *S. acmella*. **A-G**: Storage at 15±2^{0}C on control (6Mo), 2% mannitol (8Mo), 2% sorbitol (8Mo), 4% mannitol (6Mo), 4% sorbitol (8Mo), ½ MS (6Mo) and ABA (4Mo), respectively. **H-N**: Storage at 26±2^{0}C on control (2Mo), 2% mannitol (4Mo), 2% sorbitol (4Mo), 4% mannitol(4Mo), 4% sorbitol(4Mo), ½ MS (4Mo) and ABA (4Mo) respectively.

Table 1. Percent survival during slow growth conservation

Conservation Temperature	Treatments	Percent Survival			
		2 Mo	4 Mo	6 Mo	8 Mo
15±2^{0}C	**MS**	100	87.5	12.5	0.0
	2% mannitol	100	97.5	70	**52.6**
	4% mannitol	100	78.6	43	0.0
	2% sorbitol	100	97.5	75	48
	4% sorbitol	100	89.4	38	10
	½ MS	100	81	36	0.0
	0.1 mg/l ABA	100	56	0.0	0.0
26±2^{0}C	**MS**	100	0.0	0.0	0.0
	2% mannitol	100	64	0.0	0.0
	4% mannitol	95	15	0.0	0.0
	2% sorbitol	95	18	0.0	0.0
	4% sorbitol	91.6	29	0.0	0.0
	½ MS	90	33	0.0	0.0
	0.1 mg/l ABA	94	10	0.0	0.0

0.0=Plant did not survive.

preservation are not reasonable due to the requirement of frequent subculture (Peschke & Phillips 1992) and a high-specification experimental technique respectively which increase the labour and cost. However, slow growth can be achieved by reducing the culture temperature, modifying the culture media with supplements of osmotic agents, growth inhibitors, or by removing growth promoters (Dodds & Roberts 1995). Such *in vitro* slow growth conserved cultures makes germplasm available at any time for international distribution (Wilkins & Dodds 1983). Therefore, the present work for the first time evaluated the influence of temperature, osmotic agents, growth inhibitor (ABA) reduction of MS salts and storage duration on slowing the growth and post storage revival of the culture for developing a slow growth conservation system for the plant.

MATERIAL AND METHODS

Plant material. Nodal explants were taken from healthy mother plants growing in the Pt. Ravishankar University campus, Raipur, Chhattisgarh. Sterilised explants were inoculated on MS (Murashige & Skoog 1962) medium supplemented with 0.5 BA (benzylaminopurine) for initiation of culture followed by two subsequent subcultures on MS enriched with 1mg/l kinetin.

Slow growth treatment. Nodal explants dissected from aseptically raised cultures were then used for slow growth treatment by inoculating on sterilised twenty millilitres of slow growth media which comprised MS medium supplemented with mannitol and sorbitol at concentrations varying between 2% and 4% w/v. Also, MS media with half MS salts and MS supplemented with 0.1mg/l ABA was used. All media had 3% w/v sucrose and 0.8% w/v agar without any growth regulators. Inoculated cultures were tightly plugged and maintained for up to 2, 4, 6 and 8 months without transfer onto fresh medium in growth

Table 2. Overall effect of temperature on shooting of *S. acmella* under storage conditions

Temperature	Shoot No. (Mean±SE)	Shoot Length(cm) (Mean±SE)	Leaf No. (Mean±SE)
15±2^{0}C	2.19a±0.05	2.07a±0.08	4.94a±0.09
26±2^{0}C	2.20a±0.06	2.66b±0.12	5.38b±0.12

All values are mean±standard error; means followed by different letters differ significantly at 5% as analysed by Duncan Multiple Range Test using SPSS.

Table 3. Overall effect of different treatments on shooting of *S. acmella* under storage conditions.

Treatments	Shoot No. (Mean±SE)	Shoot Length (cm) (Mean±SE)	Leaf No. (Mean±SE)
Control	1.75b±0.07	4.14c±0.22	4.94bc±0.17
2% mannitol	3.32d±0.12	1.69b±0.07	5.72de±0.18
2% sorbitol	3.20d±0.12	1.76b±0.12	6.16e±0.21
4% mannitol	1.72b±0.06	0.67a±0.04	3.69a±0.17
4% sorbitol	2.02c±0.05	0.78a±0.04	4.60d±0.17
½ MS	1.32a±0.05	4.20c±0.12	5.29b±0.16
0.1 mg/l ABA	1.38a±0.06	3.99c±0.12	4.99c±0.15

All values are mean±standard error; means followed by different letters differ significantly at 5% as analysed by Duncan Multiple Range Test using SPSS.

Table 4. Overall effect of conservation periods on shooting of *S. acmella* under storage conditions.

Conservation Period	Shoot No. (Mean±SE)	Shoot Length(cm) (Mean±SE)	Leaf No. (Mean±SE)
2 months	2.25a±0.07	2.08b±0.12	5.37b±0.12
4 months	2.11a±0.06	2.37b±0.11	5.03b±0.12
6 months	2.04a±0.12	2.08ab±0.14	4.90ab±0.15
8 months	2.61b±0.13	0.81a±0.10	4.48a±0.20

All values are mean±standard error; means followed by different letters differ significantly at 5% as analysed by Duncan Multiple Range Test using SPSS.

chambers under a 16 hour photoperiod with fluorescent light and a photon flux of approximately 52 μmol $s^{-1}m^{-2}$ at 26±2 and 15±2°C.

Re-growth and establishment of plantlets. Re-growth of survived plantlets was performed on fresh MS medium with 3% w/v sucrose and 0.8% w/v agar supplemented with 0.5mg/l BA after every storage period, under standard culture room conditions at 26±2°C. Percent survival was recorded as a measure of plant recovery after a period of 28 days. Proliferated plants with well-developed roots were hardened and finally potted in soil.

Experimental Design. For each treatment 40 replicates were used and after every storage period 10 replicates were taken out and percent survival, shoot number, length and leaf number calculated, and tested for regrowth. Data were then subjected to statistical analyses and means were compared for similarity using Duncan's multiple range test (DMRT) at 5% significance using SPSS 16.00.

RESULTS AND DISCUSSION

Percent survival. Slow-growth conservation (Fig. 1.) approaches were attempted using osmotic agents, ABA

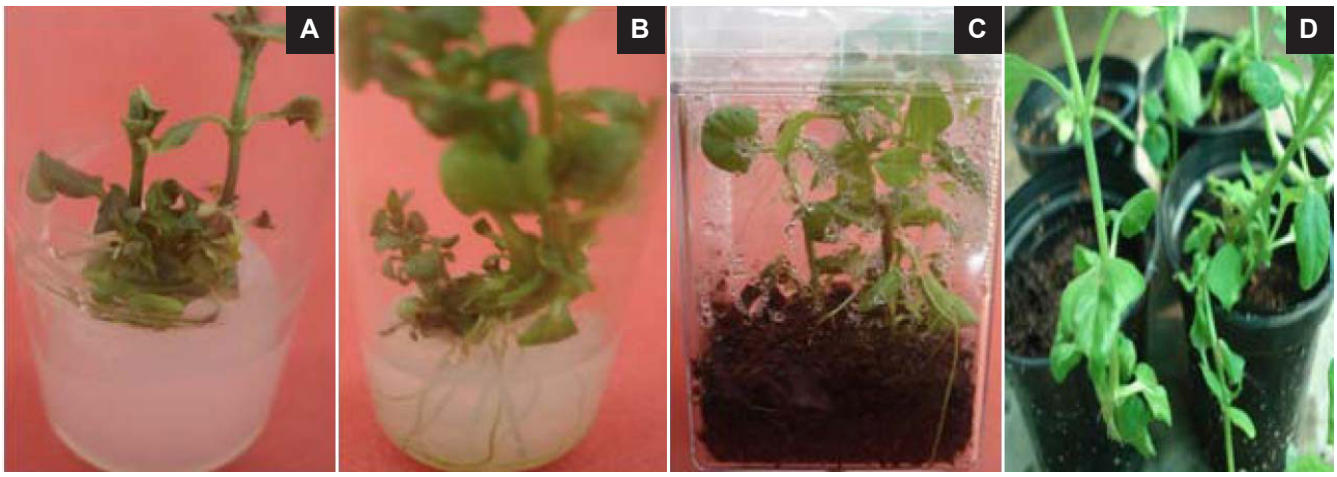

Fig. 2: Regrowth of conserved cultures after 8 Mo. **A**-2% mannitol, **B**-2% sorbitol **C**-Hardening **D**-planting in pots.

Table 5. Effect of temperature, treatments and period of conservation on percent survival of *in vitro* slow growth conserved S. *acmella* after regrowth.

Conservation Temperature	Treatments	Conservation period			
		2 Mo	4 Mo	6 Mo	8 Mo
15±2⁰C	**MS**	100	97	62	0.0
	2% mannitol	100	100	95	**68**
	4% mannitol	100	98	56	42
	2% sorbitol	100	100	89	**59**
	4% sorbitol	100	99	58	46
	½ MS	100	91	52	0.0
	0.1 mg/l ABA	100	90	0.0	0.0
26±2⁰C	**MS**	100	0.0	0.0	0.0
	2% mannitol	100	100	0.0	0.0
	4% mannitol	98	100	0.0	0.0
	2% sorbitol	100	100	0.0	0.0
	4% sorbitol	100	100	0.0	0.0
	½ MS	95	86	0.0	0.0
	0.1mg/l ABA	92	80	0.0	0.0

0.0=Plant did not survive.

growth retardant and half MS salts in MS media with storage at two temperatures 26±2 and 15±2°C. Maximum percent survival of 52.6% was found in MS+2% mannitol at 15±2°C followed by 48% in MS+2% sorbitol at 15±2°C at the end of 8 months (Table 1). However, MS+4% sorbitol showed only a 10% survival at the end of 8 months while MS+4% mannitol survived only up to 7 months, with 15% survival, which shows that a higher concentration of osmotic agents was deleterious for plant survival. Similar findings were also reported by Bekheet (2000) on *Asparagus officinalis* L. and Sarkar & Naik (1998) on potato micro plants. However, cultures stored on media with ABA and reduced MS salts did not survive beyond 4 and 6 months respectively. Moreover, cultures stored at 26±2°C did not survive after 5 months and the overall percent survival was also found to decrease rapidly after 5 months with increase in storage period, which may be due to desiccation and nutrient depletion of the media that proved detrimental during storage.

Shooting response during the storage period. There were considerable effects of slow-growth conditions on mean shoot number, length and leaf number.

Effect of temperature - Of the two storage temperatures, 15±2°C was significantly effective in slowing the growth of cultures in terms of shoot length and leaf number with mean shoot length 2.07±0.08 (Table 2) which was in accordance with previous reports that the reduction of incubation temperature is very effective in prolonging the sub-culturing cycle by reducing the growth rate (Westcott 1981).

Effect of treatments - Mean shoot number and length were higher in MS+2% mannitol and sorbitol than 4% concentrations which were significantly lower than the control (Table 3). Similar results were found in *Plumbago indica* where mannitol supplement to the culture medium resulted in reduced growth in terms of shoot length (Charoensub & Phansiri 2004). Conversely, Lata *et al.* (2010) found 2 to 4% mannitol supplements inadequate for *in vitro* conservation of *Podophyllum peltatum* L. Decreasing MS salts to half and addition of ABA did not result in any significant reduction in growth.

Effect of storage duration - Increase in the storage period increased mean shoot and leaf number up to 4 months, after which they decreased rapidly at the end of 8 months due to depletion and desiccation of the media (Table 4).

Regrowth after storage. Re-growth of the conserved plantlets (Fig. 2) showed maximum percent survival when stored with MS+2% mannitol followed by 2% sorbitol at 15±2°C at the end of 8 months and minimum with MS+4% mannitol at 15±2°C (Table 5). This shows that a higher concentration of osmotic agents is lethal for survival and re-growth of the plantlets. Therefore, a low concentration of osmotic agents together with low temperature resulted in growth suppression which helped the good recovery of plants.

CONCLUSION

It is concluded that a concentration of 2% of osmotic agents together with a low temperature of 15±2°C is suitable for growth suppression which helped the survival of plants in culture up to 8 months without any subculture, followed by good recovery on fresh medium. Therefore, a slow-growth protocol is an effective method with reduction of labour and cost for *in vitro* conservation of *S. acmella*.

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Botanica SERBICA

REZIME

Efekat temperature i sastava medija na spori rast i konzervaciju lekovite biljke *Spilanthes acmella*

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Spilanthes acmella (L.) Murray je spilantola, alkamida koji ima značajne biološke i farmaceutske aktivnosti. Čuvanje ove biljke u uslovima *in vitro,* odnosno nodalnih eksplantata na MS medijumu u koji su dodavane osmotski agenti manitol, sorbitol, apscisinska kiselina, koje su čuvane 15±2 i 26±2°C tokom 2, 4, 6 i 8 meseci bez subkulturisanja.

Način čuvanja procenjivan je na osnovu procenta preživljavanja, broja i dužine izdanaka, i broja listova. Oživljavanje nakon perioda konzervacije procenjivan je na osnovu oporavka na MS medijumu obogaćenog sa 0.5mg/l BAP at 26±2°C.

Tretman sporog rasta značajno povećava preživljavanje sa najvaćim procentom preživljavanja na MS+2% manitola na 15±2°C na kraju osmog meseca. Niža temperatura od 15±2°C značajno smanjuje prirast. Kulture čuvane na 26±2°C nisu preživljavale duže od 5 meseci. Manitol a potom i sorbitol u 2% koncentraciji su efektivno usporavali rast kultura meren brojem i dužinom izdanaka. Duplo smanjena jačina MS medijuma ili dodavanje ABA nije uticalo na usporavanje rasta. Ponovno prorastanje sa maksimalnim procentom oporavka konzerviranih biljaka uočeno je na MS+2% manitol na 15±2°C. Viša koncentracija osmotskih agenasa uticala je negativno na preživljavanje i oporavak tretiranih biljaka.

Ključne reči: *Spilanthes*, spori rast, konzervacija, osmotski agenti, temperatura, čuvanje