Chapter 11

An Appraisal on in Vitro Conservation and Biotechnological Interventions in Spilanthes Acmella Murr.

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ABSTRACT

Spilanthes acmella Murr., commonly known as "Toothache plant" and "Akarkara" of the family Asteraceae, holds an important place in Indian and global scenario owing to its medicinal properties. Different pharmacological experiments in a number of in vitro and in vivo models have convincingly demonstrated the ability of Spilanthes to exhibit anti-inflammatory, antimalarial, antioxidant, antimicrobial and diuretic activities, lending support to the rationale behind several of its traditional uses. Spilanthes virtues are, to a large extent, attributable to its chemical constituents viz. alkylamides, phenolics, caumarin and triterpenoids. Of these, the most studied group has been the alkylamides, which are abundant in this plant and one which remains the most sought after by scientists is an antiseptic alkylamide, (2E, 6Z, 8E)-deca-2,6,8-trienoic acid N-isobutyl amide, commonly known as spilanthol. Spilanthol has immense application in pharmaceuticals, food, health and body care products. The present review is an up-to-date and comprehensive analysis on in vitro conservation and biotechnological studies of Spilanthes. With this review authors aim to offer an experience background to researchers who intend to study on Spilanthes.

Keywords: Akarkara, In Vitro, Micropropagation, Spilanthes, Secondary metabolites

Abbreviation: BAP - 6-benzylaminopurine; 2, 4-D - 2,4-dichlorophenoxyacetic acid; IAA - Indole-3-acetic acid; Kinetin - N^6-furfuryladenine; 2-iP - 2-isopentenyl adenine; NAA - a-naphthalene acetic acid; GA_3 - Gibberellic acid, pCPA - para-Chlorophenoxyacetic acid; TDZ - Thidiazuron.

INTRODUCTION

Spilanthes acmella Murr**.** (Common names: Akarkara and Toothache plant) is an indigenous species belongs to the family, Asteraceae. This plant is

widely distributed in the tropical and subtropical regions and can be found in damp pastures, at swamp margins, on rocks near the sea and as a weed of roadsides. The genus *Spilanthes* contains 35 tropical species, of which around five species known so far (Anonymous 1989), growing in India: *S. acmella* Murr., *S. acmella* L. var oleraceae clarke, *S. calva* L., *S. paniculata* L. and *S. mauritiana* L. Among these, *S. acmella* Murr. and *S. acmella* L. var oleraceae Clarke are rare in occurrence. *Spilanthes* is a perennial herb upto 30-60 cm tall, with a prostrate or ascending branched cylindrical hairy stem and simple ovate opposite leaves without stipule. *Spilanthes* flowers are yellow, non-fragrant with five petals on long peduncles.

Flowers and leaves of the plant bear pungent taste and have been used as a spice for appetizers and as folk medicines for stammering, toothache, stomatitis and throat complains (Nakatani and Nagashima 1992; Ramsewak *et al*. 1999). This plant also possesses immune-modulatory, antioxidant and insecticidal biological properties (Ramsewak *et al*. 1999; Pandey and Agrawal 2009; Guiotto *et al*. 2008; Matthias *et al*. 2008; Prachayasittikul *et al*. 2009). Furthermore, *Spilanthes* is one of the active constituents in compositions for acute- or long-term cure of microbial infections, particularly, oral pathogenic microorganisms (Adler 2006). It is also effective against blood parasites at extremely low concentration and is poisonous to most invertebrates whereas harmless to the vertebrates (Watt and Brayer-Brandwijk 1962). In addition, its extract is an active constituent of beauty care cosmetics such as fast acting muscle relaxant to accelerate repair of functional wrinkles (Belfer 2007). The plant extract is also used as a nutritional supplement for taste improvement as a sweetener with high sweetness devoid of distasteful savour that does not affect the taste or odour of foods or drinks (Miyazawa *et al*. 2006). These properties can be attributed to several secondary metabolites present in the genus such as alkylamides, phenolics, coumarin and triterpenoids which are therapeutic and account for its use in traditional medicines all over the world, since long (Prachayasittikul *et al*. 2009).

With the worldwide increasing demand for *Spilanthes* derived products, there has been a concomitant increase in the demand for raw material. However, the increasing human and livestock populations have affected the status of wild plants. In fact, the pace of depletion has outpaced the pace of conservation. Although propagation by seed in *Spilanthes* is a predominant technique, it does not ensure pathogen-free plants. Dependence on season and slow germination rates are some of the other major limiting factors in conventional propagation (Pati *et al*. 2006; Dobránszki and da Silva 2010). Moreover, propagation by seeds is also limited because of the highly heterozygous nature of the plant due to protandry, which prevents self-pollination (Reddy *et al*. 2004). Many small, bright colour flowers are aggregated into capitulum (flower

head) which make them attractive to insects, thus, passing the way for entomophily. The genetic variation due to insect pollination may result into high heterogeneity in quality and quantity of chemical makeup of the plant.

To fulfill the increasing demand for this important medicinal plant, a constant source of planting material is required which could be utilized as a ready stock to meet the demand of the pharmaceutical industries for the production of bioactive compounds from them. In this context, plant cell cultures have been proved very valuable for the large scale propagation of plants and production of high value secondary metabolites owing to the consistency in quality and quantity of the desired product. The present Chapter reports the detailed in-depth information on pharmacological significance and appraisal on conservation *of Spilanthes* species through biotechnological interventions.

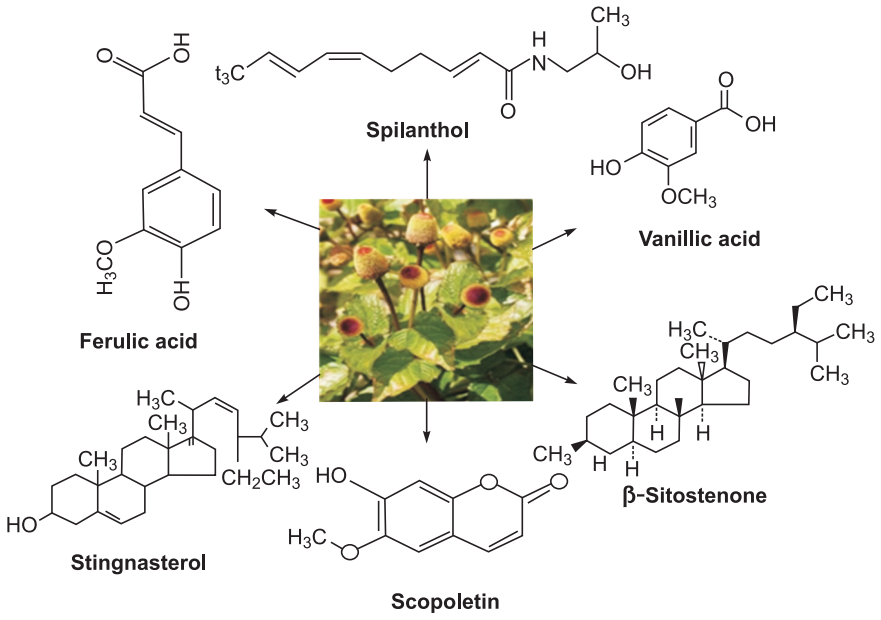
PHARMACOLOGICAL APPLICATIONS

Spilanthes has been used as folk medicine since ancient times to cure severe toothache, infections of throat and gums, stomatitis, paralysis of tongue, and psoriasis (Anonymous 1989). *Spilanthes* extract is also effective against malarial parasites, specifically malarial spirochetes, either as a prophylactic or as a treatment for malarial paroxysms (Richard 1996). In Amazon, it has been used for the treatment of tuberculosis by laymen (Storey and Salem 1997). This plant is well known for antiseptic, analgesic, and diuretic properties (Jondiko 1986; Ramsewak *et al*. 1999). Ratnasooriya *et al*. (2004) investigated the diuretic potential of *Spilanthes* using rats. Chakraborty *et al*. (2004) evaluated the anti-inflammatory and analgesic activity of the aqueous extract of *S. amcella*. The extract showed dose dependent significant inhibition of prostaglandins which are involved in the late phase of acute inflammation and pain perception.

Recently, Prachayasittikul *et al*. (2009) evaluated antimicrobial, antioxidant and cytotoxic activities of *Spilanthes* extracts. They showed that fractions from the chloroform and methanol extracts can inhibit the growth of many microorganisms e.g. *Corynebacterium diphtheriae* (NCTC 10356) and *Bacillus subtilis* (ATCC 6633). In addition, studies on *Spilanthes* immune stimulating properties are available, which find applications in local buccal mucosa preparations indicated for painful mouth tissues and minor mouth ulcers. Besides, *Spilanthes* is well known for larvicidal, and insecticidal properties (Ramsewak *et al*. 1999; Saraf and Dixit 2002; Pandey *et al*. 2007).

The above properties are mainly due to the presence of a wide array of compounds with varying structural patterns, such as alkylamides (spilanthol), phenolics (ferulic acid and vanillic acid), coumarin

(scopoletin) and triterpenoids, like β-sitostenone and stigmasterol (**Scheme 1**) (Prachayasittikul *et al*. 2009). Of these, the most abundant principle is antiseptic alkylamide, (2E, 6Z, 8E)-deca-2,6,8-trienoic acid N-isobutyl amide, commonly known as spilanthol (Khadir *et al.* 1989). The analgesic activity of spilanthol has been attributed to an increased gamma-aminobutyric acid (GABA) release in the temporal cerebral cortex (Rios *et al*. 2007).



Scheme 1 Chemical structures of medicinal compounds present in *Spilanthes acmella.*

Interestingly, spilanthol has been demonstrated to inhibit nitric oxide (NO) production in a murine macrophage cell line, to efficiently down-regulate the production of inflammatory mediators interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF-α), and to attenuate the expression of cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS) (Wu *et al*. 2008). Other investigations have also confirmed the down regulation of some pro-inflammatory cytokines by bioactive alkylamides under various experimental conditions (Cech *et al*. 2006; Guiotto *et al*. 2008; Wang *et al*. 2008; Woelkart and Bauer 2007). These findings suggest that spilanthol can be a useful inhibitor of inflammatory mediators and is a potential new lead compound for COX-2 selective non-steroidal anti-inflammatory drugs (NSAIDs). Besides, spilanthol is very effective even at low doses against eggs and pupae. In pupae, it seems to work on nervous system as evident by abnormal movement like jerks, spinning and uncoordinated muscular activity. This suggests that the drug disturbs nerve conduction

somewhere. The mortality of pupae in short span of time upon exposure to the drug also indicates that spilanthol greatly disturbs the ongoing processes of histolysis and histogenesis. Spilanthol has been found harmless to majority of vertebrates and lethal to invertebrates (Watt and Brayer- Brandwijk 1962). In addition to this, spilanthol clinical trial has also been done for anti-aging property. It stimulates, reorganize and strengthen the collagen network, notably those of the face, and can be used as an anti-wrinkle product.

Among others, the scopoletin (coumarin) and ferulic acid (phenolics) found in this plant are reported to be of immense pharmacological interests (Prachayasittikul *et al*. 2009). Scopoletin is a phytoalexin, its production is mainly seen upon pathogenic infection and is considered as an important defense mechanism against bacteria and fungi (Smith 1996). It has attracted the most attention because of its use in cardiovascular disease, and antitumor and antithyroid treatment. In addition to this, scopoletin also possesses antioxidant, antimicrobial, anti-inflammatory, antipyretic and hepatoprotective properties. Ferulic acid is most highly regarded for its antioxidant property. Additionally, it exhibited a wide range of therapeutic effects against cancer, diabetes, cardiovascular and neurodegenerative diseases. Because of the immense medicinal value of *Spilanthes*, the plant is being overexploited by the local population as well as pharmaceutical companies. It is, therefore, imperative to develop more efficient methods for its conservation and large scale propagation.

IMPLEMENTATION OF CONSERVATION STRATEGIES IN *SPILANTHES*

There are mainly two strategies for medicinal plants conservation i.e. *in situ* conservation and *ex situ* conservation. *In situ* conservation focuses on maintenance of plant species in their natural environment where they have developed their unique characteristics through the protection of the environment itself. On the other hand *ex situ* approach involves plant conservation outside the native habitat and is generally used to protect populations in danger of extinction. *In situ* technique is idealistically the most appropriate method of plant conservation that allows plants to interact and co-evolve with other component of ecosystems. However, besides being time consuming due to slow growth and multiplication process (Vinoth and Ravindhran, 2013), the technique is highly vulnerable to natural calamities, like forest fires, extreme climate conditions and destruction by diseases and disasters.

Ex situ conservation techniques can be used to complement *in situ* methods and in a few cases is It is the only option. Approaches to *ex situ* conservation comprise methods, like seed storage, field genebanks

and botanical gardens. DNA and pollen storage also indirectly contribute to *ex situ* conservation of plant genetic resources. Among the different *ex situ* conservation techniques, seed storage is the most appropriate technique for long-term conservation of plant genetic resources. However, conventional seed storage strategies are not promising for a large number of important medicinal plants which produce recalcitrant seeds that do not survive dehydration and lose viability in a short period of time (Vinoth and Ravindhran, 2013). Moreover, propagation by seeds is also limited in *Spilanthes* because of the highly heterozygous nature of the plant due to protandry, which prevents self-pollination (Reddy *et al*. 2004). Field genebanks offer easy access to conserved material but they have risk of destruction by natural calamities, pests and diseases. Hence, *in vitro* conservation through biotechnological interventions is the safest and efficient alternative for medicinal plant conservation. Biotechnology offers an opportunity to utilize plant cell, tissue or organ by growing them *in vitro* to get large number of plants and desired medicinal metabolites.

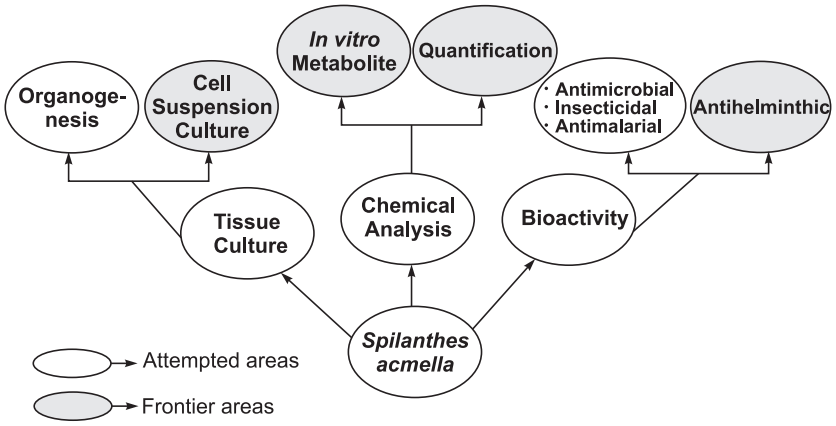
Conservation of *Spilanthes* Species by in Vitro Tissue Culture Methods

In vitro tissue culture is defined as the culture of different somatic cells, tissues or organs of plants under controlled *in vitro* conditions with the aim of producing a large number of identical progeny plants in a relatively short span of time compared to conventional propagation methods. Some of the distinct advantages are as follows: (1) plant material can be obtained at anytime independent of flowering period and seed is not always required for propagation (2) virus free plants can be obtained by in vitro meristem culture (3) true-to-type plants can be obtained where elite genotypes are needed (4) large scale propagation may be achieved within a short time frame (5) recalcitrant or immature seeds can also be utilized to produce large number of plants, and (6) the in vitro methods need small storage space compared to the conventional methods. Moreover, *in vitro* culture techniques are very useful for the species with reproductive abnormalities and/or have extremely reduced population.

In vitro conservation starategy involves the following steps- **Stage 0:** the preparative stage, involving germplasm collection, **Stage 1:** explant establishment, which involves the use of sterile, viable cultures, **Stage 2:** the multiplication stage, where large number of propagules are produced, **Stage 3:** the plantlet production where in vitro shoots or embryos are teated with appropriate growth regulators to produce entire plantlets, and **stage 4:** hardening of plantlets, which involves the acclimatization of plantlets to survive *in vivo* conditions.

Despite the profound economic value of *Spilanthes*, very little scientific work is done in this species. Tissue culture studies on *S. acmella* are in its infancy and so far only eight reports are available on micropropagation

of this species (Table 1). The already attempted and future possible biotechnological interventions in *Spilanthes* are presented in **Scheme 2.** Brief overviews of two tissue culture techniques *viz*. axillary and adventitious shoot proliferation, involved in rapid multiplication of *Spilanthes*, are presented below:



Scheme 2 Biotechnological interventions in *Spilanthes acmella*.

Axillary Shoot Proliferation

Axillary shoot proliferation is a most popular approach for true to type clonal propagation of plants because it favors more uniformly diploid plants where the bud present at priori on the initial explant grows into a shoot. Thus, it guarantees that the characteristics of the source plant are conserved (Rao and Venkateswara 1985). Moreover, considerable gains in metabolite production can be achieved by clonal propagation of plus trees. Till date, a few workers have reported clonal propagation of *Spilanthes* plant.

For the first time, Haw and Keng (2003) attempted *in vitro* clonal propagation of *Spilanthes* by axillary shoot proliferation. The aseptic axillary buds formed multiple shoots within five weeks when cultured on MS medium supplemented with BAP (8.8 µM) and NAA (0.54 µM). The addition of IBA had no significant effect on the multiple shoot formation. However, the study lack crucial information on percent culture response, the rate of proliferation in recurrent cycles of shoot multiplication, frequency of rooting, etc., and transplantation was not attempted. Recently, a report (Singh and Chatuvedi 2010) on systematic clonal propagation by nodal segment culture is published whereby, detailed description on *in vitro* shoot multiplication, rooting and hardening are described. In this study, nodal explants of *S. acmella* bearing two opposite axillary buds were cultured on MS basal medium or basal medium supplemented with BAP,

Table 1 In vitro conservation protocol standardized in Spilanthes acmella

S. No.	*Explant*	*Direct/Indirect Organogenesis*	*Culture Medium*		*References*
			Shoot Multiplication	*Rooting*	
1.	Hypocotyls	Not Mentioned	MS + BAP (2.2 µM) + NAA (0.54 µM)	½ MS + IBA (4.9 µM)	Saritha et al. 2002
2.	Axillary buds	Direct	MS + BAP (8.8 µM) + NAA (0.54 µM)	Not Mentioned	Haw and Keng 2003
3.	In vitro leaves	Direct	MS + BAP (13.2 µM) + IAA (5.7 µM)	½ MS + IBA (4.9 µM)	Saritha and Naidu 2008
4.	In vitro leaves	Indirect	MS + BAP (10.0 µM) + NAA (1.0 µM)	½ MS + IBA (0.1 µM)	Pandey and Agrawal 2009
5.	Shoot tips	Direct	Growth regulator-free full-strength MS liquid medium		Singh et al. 2009a
6.	Nodal segment transverse thin cell layer sections	Direct	MS + BAP (10.0 µM)	MS basal medium	Singh et al. 2009b
7.	Nodal segments	Direct	MS + BAP (5.0 µM)	½ MS + Sucrose (5%)	Singh and Chaturvedi 2010
8.	Leaf disc	Direct	MS + BAP (5.0 µM) + IAA (5.0 µM)	½ MS + Sucrose (5%)	Singh and Chaturvedi 2012

Kinetin or 2-iP at 3.0 µM concentration Although on basal medium, the frequency of bud-break was appreciable (64%), and incorporation of BAP to the basal medium had further improved the incidence of bud-break and promoted multiple shoot formation on the nodes (2 shoots/axillary buds). While BAP favoured 100% shoot-bud induction from axillary buds, only 20% response was observed on Kinetin supplemented medium, and 2-iP was noted as being inhibitory for axillary bud proliferation. The addition of a low concentration of GA_3 (1.0 µM) to the BAP supplemented medium promoted multiple shoot formation in 70% of the cultures but the shoots remained weak and stunted. On the other hand, a single shoot with long internodes was developed from axillary buds in 100% cultures when NAA (1.0 or 5.0 µM) was added to BAP containing medium.

Irrespective of the treatments, excessive adventitious root proliferation was observed either at the base of the explant or all over the surface of the *in vitro* developed shoot.

Since the shoot multiplication rate was higher with the addition of BAP, it was further evaluated at a concentration range of (1.0-15.0 µM) to promote shoot proliferation with no adventitious roots. The rate of shoot multiplication and length of shoots varied with the concentration of the BAP. At its optimum level (5.0 µM), 20.3-fold shoot multiplication occurred every 5 weeks with no adventitious root formation. On MS + BAP (5.0 µM), bud-break occurred within a week and an average of 2.9 shoots per axillary bud were formed within 4 weeks in 100% cultures. The shoots grew well and attained a length of 4.9 cm with 3.5 nodes (or 7 axillary buds) per shoot after 5 weeks (Fig. 1A). Interestingly, at all lower concentration of BAP (1.0-3.0 µM), the incidence of excessive adventitious root proliferation was observed from all over the surface of the *in vitro* developed shoots and the explants as well. Moreover, lower concentration of BAP supported multiple shoot formation with long internodes (Fig. 1B). There was a marked decline in the growth of shoots and the rate of shoot multiplication with the increasing concentration of BAP; the node was crowned with several newly formed small shoots difficult to count. BAP at 15.0 µM, resulted in stunted shoots with pale leaves (Fig. 1C).

The hand sections of the nodal region of the shoots in culture revealed the presence of a ring of lignified xylem cells which fluoresce bright yellow when stained with acridine orange. Multiple (2-3 numbers) adventitious roots (arrow marked) were originated from these lignified tissues (Fig. 1D, E); in Fig. 1D, an axillary bud can also be viewed at one side. Thus, MS + BAP (5.0 µM) proved optimum for shoot multiplication. At the end of the passage each shoot was cut into single node segments and planted on the fresh medium of the same composition. Each node again produced multinodal, multiple shoots after 5 weeks. Thus, signified the effectiveness of *in vitro* methodology for true-to-type plant regeneration of *Spilanthes*.

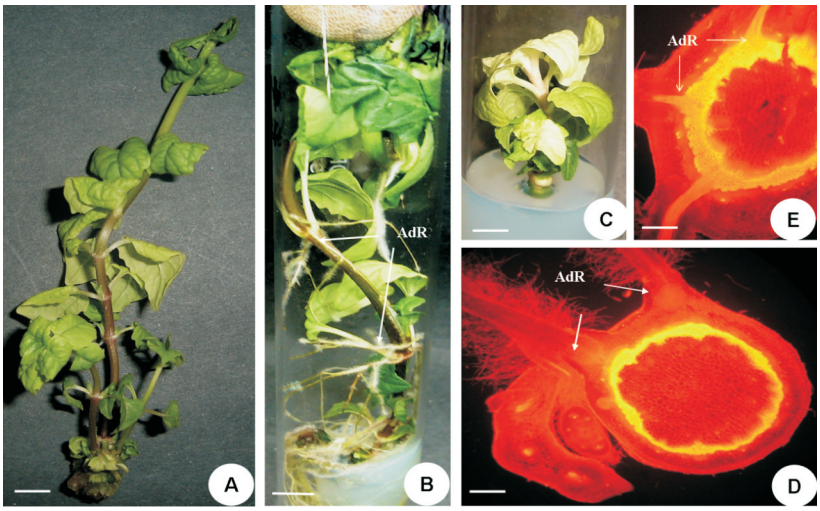


Fig. 1 Nodal Segment Culture (a) A nodal segment on MS + BAP (5.0 μM), showing long, multinodal shoots, after 5 weeks of culture initiation (bar = 0.5 cm); (b) A nodal segment on MS + BAP (3.0 μM), after 8 weeks of culture initiation, showing a shoot with long internodes and with adventitious roots all over its surface (bar = 1.1 cm); (c) A nodal segment on MS + BAP (15.0 μM), after 8 weeks of culture initiation, showing stunted shoots with pale colored leaves (bar = 0.33 cm); (d) An anatomical section of nodal segment from b, stained with acridine orange, showing differentiation of adventitious root (AdR) and position of axillary bud (AxB) (bar = 0.5 mm); (e) Same as d, showing origin of adventitious root differentiation (AdR) at different points (bar = 0.5 mm).

Terminal 3-4 cm long portions of shoots from 5-week-old cultures on MS + 5 μM BA were used for rooting. The rooting was positively correlated with the sucrose concentration in the medium. There were significant increase ($P<0.05$) in the percent rooting and the number of roots per shoot with increase in sucrose concentration. On ½ MS + 50 g l^{-1} sucrose, which proved to be the best rooting medium, 100% shoots formed more than 35 roots directly from the basal cut end of the shoots (Fig. 2A). On this medium roots appeared after 2 weeks and maximum response was observed after 4 weeks. Some of the roots had developed laterals. Rooted shoots from ½ MS + 50 g l^{-1} sucrose were transferred out of culture. The plantlets were acclimatized successfully with 88.9% survival rate (Fig. 2B). During *in vitro* hardening, shoots elongated, leaves turned greener, and their lamina expanded. Consequently, the plants seemed much healthier and grew more vigorously after *in vitro* hardening.

Adventitious Shoot Proliferation

Apart from the axillary shoot proliferation, a majority of reports (Saritha *et al*. 2002; Saritha and Naidu 2008; Pandey and Agrawal 2009; Singh *et al*. 2009b, Singh and Chaturvedi 2012) on *Spilanthes* deal with *in vitro* plant

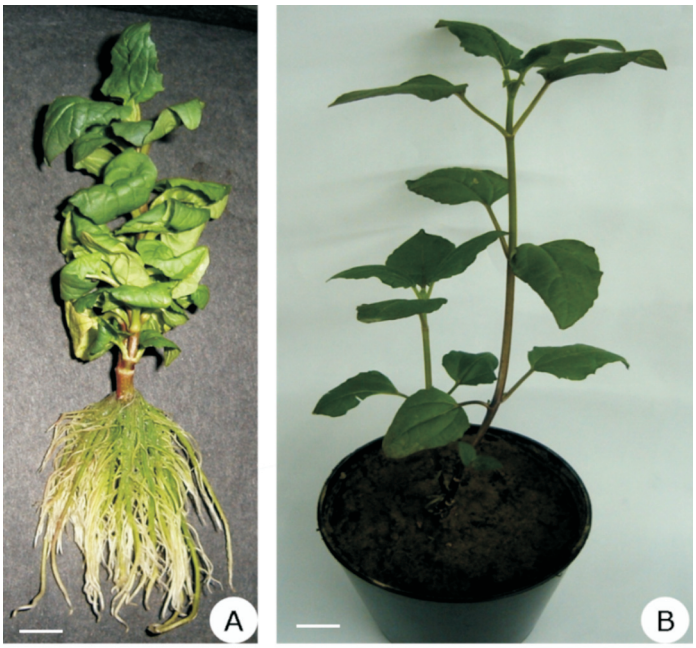


Fig. 2 Nodal Segment Culture Rooting and Hardening (a) A micropropagated shoot from MS + BAP (5.0 µM), rooted on ½ MS + Sucrose (50 g/l). Roots have developed directly from the basal cut end of the shoot (bar = 1.0 cm); (b) A hardened micropropagated plant, 1 month after transfer to soil (bar = 2.0 cm).

regeneration by adventitious shoot proliferation from seedling explants, leaf or hypocotyls or thin cell layer sections of nodal segment.

Saritha *et al*. (2002) were first to report the successful tissue culture of *Spilanthes*. They reported multiple shoot proliferation (10 ± 0.6) from hypocotyl explants of 1-week-old seedlings on MS medium supplemented with BAP (2.2 µM) and NAA (0.54 µM). About 95% of the *in vitro* developed shoots rooted on half strength (½) MS medium containing IBA (4.9 µM). After 6 years, Saritha and Naidu (2008) reported shoot regeneration from leaf explants obtained from the above mentioned multiple shoots. Maximum number of shoots per explants (20 ± 0.47) was recorded on MS medium containing BAP (13.2 µM) and IAA (5.7 µM). An anatomical study confirmed that shoot regeneration was via direct organogenesis. Micropropagation of *Spilanthes* by leaf-disc culture was also reported by Pandey and Agrawal (2009). They obtained green and compact callus on MS medium supplemented with BAP (10.0 µM) and NAA (1.0 µM), in 15 days. The fresh subculture of callus on the same medium resulted into the differentiation of an average of 12.9 shoot-buds, in 50% cultures after every 30 days. Elongation of shoot-buds occurred only if they were transferred to MS + BAP medium devoid of NAA. On MS + BAP (10.0 µM),

an average of 4.22 shoots and 15 shoot-buds per explants were obtained in 70.3% culture in 30 days. Shoots were rooted on ½ MS + IBA (0.1 µM) within 2 weeks. The plantlets were successfully hardened and established in soil where they flowered and set viable seeds.

Singh *et al.* (2009b) established *in vitro* propagation system of *Spilanthes* using nodal segment transverse thin cell layer (tTCL) culture system. MS medium fortified with BAP (5.0 µM) was optimal for shoot regeneration from tTCL. On this medium, the explant inoculated in the upright orientation exhibited a high frequency (97%) of shoot regeneration from the edge of the explants, and the highest number of shoots (an average of 31.5) per explant. In contrast to this, intact node (1.0-1.5 cm) cultured on MS + BAP (5.0 µM) had significantly lower shoot multiplication ability with only 4.5 shoots per responsive explants. Incorporation of Kinetin or NAA in BAP containing medium did not increase shoot multiplication from tTCL nodal segments. Rooting of shoots was achieved on growth regulator free full-strength MS medium. Singh *et al.* (2009a) also carried out plant regeneration from alginate encapsulated shoot-tips of *Spilanthes*. They accomplished the best gel complexation for encapsulation of shoot tips using sodium alginate and calcium chloride. From encapsulated shoot tips both shoots and roots emerged simultaneously on growth regulator-free full-strength MS liquid medium.

Recently, Singh and Chaturvedi (2012) reported morphogenesis from leaf disc cultures. They cultured leaf-disc explants of 5 mm size on a range of media. The treatments involved MS basal medium and varying concentrations and combinations of cytokinins and auxins, like BAP, TDZ, Kinetin, NAA, IAA, 2,4-D and pCPA. Leaf-disc cultures showed no response in the absence of growth regulators. The morphogenic response of leaf-discs was greatly influenced by the type of growth regulator used in the medium. On most of the media combinations, leaf-discs either callused or differentiated into shoots.

Among individual cytokinin/auxin treatments, incorporation of BAP at 5.0 µM to MS basal medium had induced direct adventitious shoot organogenesis. In contrast to this, no shoot proliferation observed from leaf-disc explants cultured on MS medium containing either other cytokinins, TDZ, Kinetin or any of the auxins, 2,4-D, NAA or pCPA, alone at tested concentration of 5.0 µM.

Since the shoot proliferation was obtained with the addition of BAP, it was further evaluated at a concentration range of (1.0-7.0 µM). The frequency of shoot proliferation declined at lower concentrations of BAP; BAP at 3.0 µM concentration induced shoot regeneration in 80.3% cultures with only one shoot per explant while no response was seen at BAP 1.0 µM. The higher concentration of BAP (7.0 µM) induced callusing in 100% explants and the callus remained non-regenerative. At its optimal concentration of 5.0 µM, BAP showed highest percentage (100%) of shoot

organogenesis with an average of 3.5 adventitious shoots, directly from the explants, without an intervening callus phase.

Further, a combined effect of cytokinin and auxin was evaluated on multiple shoot induction. Incorporation of NAA (1.0 and 5.0 µM) in the BAP containing medium did not show significant effect ($p<0.05$) on differentiation of number of shoot-buds per explant. Although 100% cultures showed regeneration in NAA containing medium, only one shoot-bud proliferated per explant. In comparison to BAP alone and BAP + NAA, addition of IAA to MS + BAP medium enhanced the number of shoot-buds per explant significantly ($p<0.05$). The best response was obtained on MS + BAP (5.0 µM) + IAA (5.0 µM) medium, which promoted differentiation of loads of shoot-buds per explants, directly, in 82.9% cultures. On this medium, the first visible change on the cultured leaf-discs was observed within the first week of culture. The leaf-discs explants enlarged, and adventitious shoot-buds appeared to arise directly from the cut ends (Fig. 3A-E). The developmental ontogeny of shoot organogenesis from leaf-disc explants were recorded by scanning electron microscopy which confirmed the direct differentiation of shoot-buds from the explants (Fig. 4 A-D).

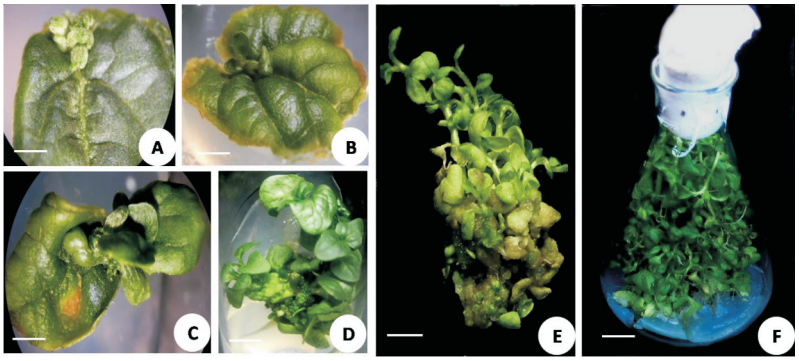


Fig. 3 Leaf Disc Culture Shoot Proliferation (a-b) Leaf-disc cultures on MS + BAP (5.0 µM), showing shoot bud initiation; (a) After one week (bar = 0.12 cm); (b) After two week (bar = 0.18 cm); (c) Same, after 3 weeks, showing development of foliar structures (bar = 0.23 cm); (d) Four-week-old leaf-disc culture showing well developed shoots (bar = 0.9 cm); (e) A five-week-old leaf-disc culture on MS + BAP (5.0 µM) + IAA (5.0 µM) showing differentiation of many shoot-buds and shoots (bar = 0.7 cm); (f) Same as e, 5 weeks after transfer to the same medium in 250 ml conical flask, showing proliferation of many well developed shoots (bar = 1.49 cm).

Though, numerous shoot-buds were differentiated from leaf-disc explants on MS + BAP (5.0 µM) + IAA (5.0 µM), however, only a few shoot-buds (5–6) developed into distinct shoots of 1.0–3.5 cm length over a period of 5 weeks and growth of rest of the adventitious

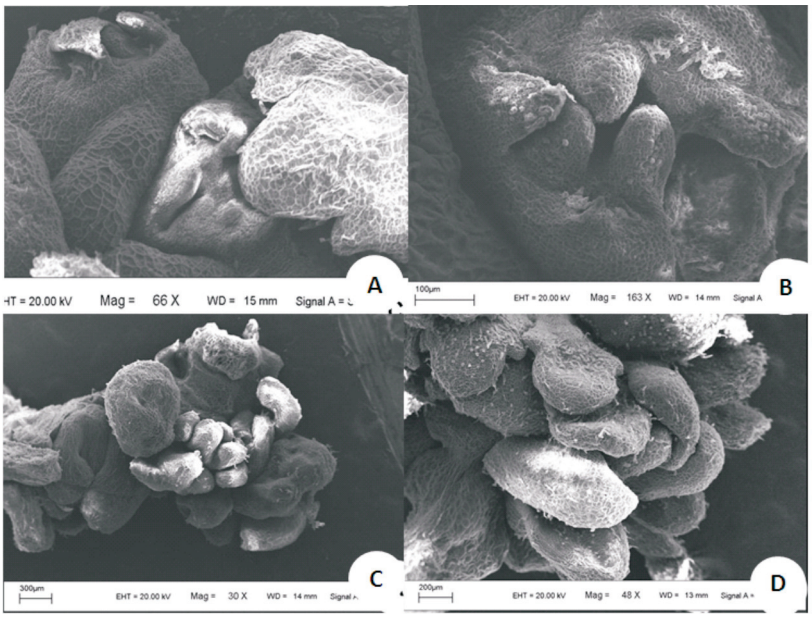


Fig. 4 Leaf Disc Culture Scanning Electron Microscopy (a-b) Scanning electron micrographs of one-week-old leaf-disc cultures showing initiation of shoot bud (A, bar = 246 µm; B, bar = 100 µm); (c) Scanning electron micrograph of 2-week-old leaf-disc cultures showing cluster of leaf primordial (bar = 300 µm); (d) Enlarged view of same (c) with distinct leaf primordial (bar = 200 µm).

shoot-buds remained arrested. To overcome this problem, after 5 weeks, distinct shoots were separated from leaf explants and utilized for rooting and remaining part, which was crowded with stunted shoots, was cut into 3–4 small segments, each with a bunch of 4–5 shoots. Thereafter, these segments were subcultured to 250 ml capacity flasks containing the fresh medium of MS + BAP (5.0 µM) + IAA (5.0 µM). With this arrangement, an average of 51 shoots was differentiated from each segment, in 5 weeks (Fig. 3F). In flask, shoots grew well and attained height of 4.5 cm with each shoot having an average of 4–5 nodes after 5 weeks.

Apart from the shoot proliferation, on few combinations, like MS + NAA (5.0 mM), MS + pCPA (5.0 mM), MS + BAP (7.0 µM) and MS + 2,4-D (5.0 mM), cultures responded initially for callusing but the calli did not survive after the first subculture. The best treatment in terms of sustained growth of calli, percent explants callused and the degree of callusing, was the combination of MS + BAP (5.0 mM) + NAA (1.0 mM) + 2,4-D (1.0 mM) (Fig. 5A-D).

The long shoots, from 5-week-old cultures on MS + BAP (5.0 µM) and MS + BAP (5.0 µM) + IAA (5.0 µM) were used for rooting. On ½ MS + sucrose (50 g l^{-1}), 100% shoots formed numerous roots directly from the

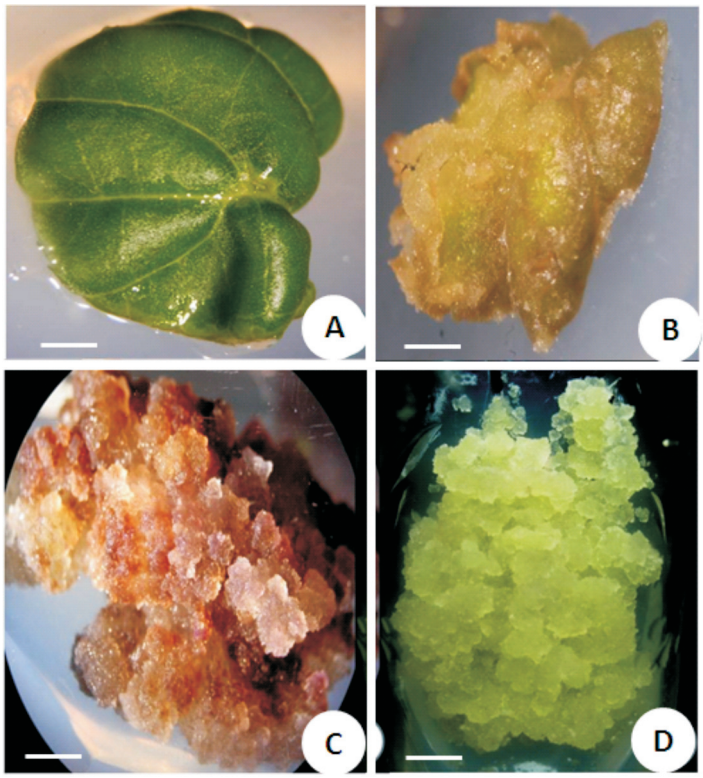


Fig. 5 Leaf Disc Culture Establishment of Callus Cultures (a) A leaf-disc at culture on MS + BAP (5.0 μM) + NAA (1.0 μM) + 2,4-D (1.0 μM) (bar = 0.1 cm); (b) A dedifferentiating leaf-disc explant of *Spilanthes* after 2 weeks of culture (bar = 0.16 cm); (c) A 5-week-old culture, showing proliferation of soft, friable and brownish callus from leaf-disc explants (bar = 0.29 cm); (d) A 5-week-old second subculture of a, showing healthy, fresh, friable and light green, massive callus ready for chemical analysis after three passages (bar = 0.33 cm).

basal cut end of the shoots**.** The plantlets were acclimatized successfully with 90% survival rate.

The ploidy stability of *in vitro* regenerants was determined by flow cytometry. Figure 6A,B shows representative histograms of field grown parent plant (control) and micropropagated plant derived from leaf-disc explants. Presence of single peak at around 200 channel position in histograms of micropropagated plants corresponded to those of control plant suggested that *in vitro* regenerants maintained ploidy stability and no changes in ploidy levels occurred during regeneration process.

In Vitro Secondary Metabolite Production

Currently many secondary metabolites of medicinal values are solely obtained from the naturally grown whole plants which causes continued

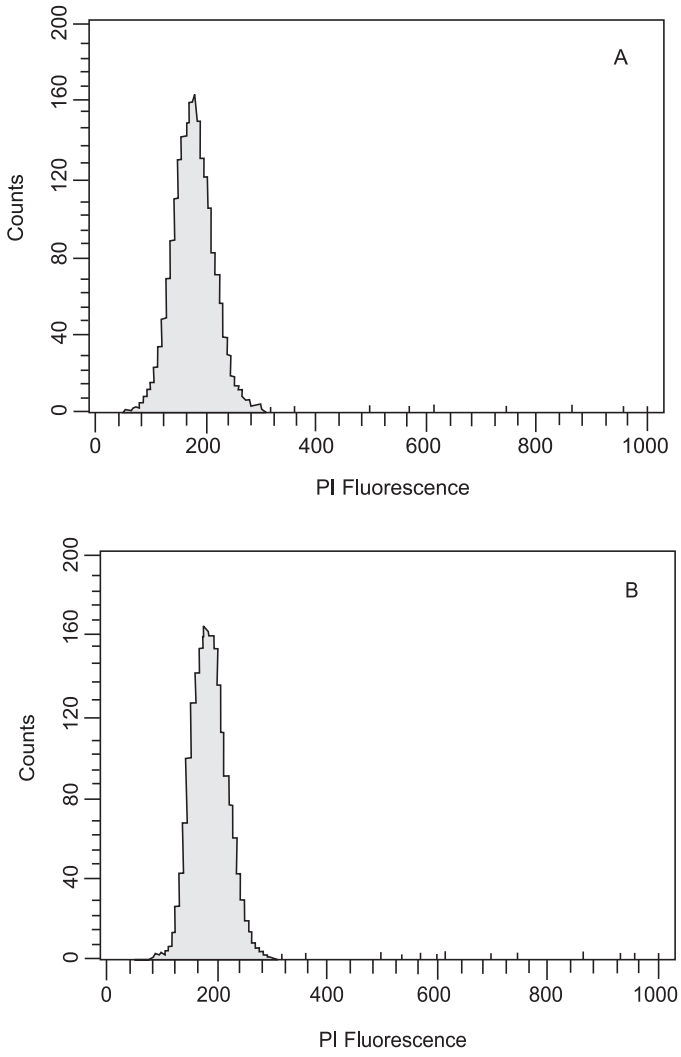


Fig. 6 Flow cytometric analysis (a) Representative flow-cytometric histogram of nuclei stained with propidium iodide and isolated from leaf tissues of field grown parent plant of *Spilanthes* (control); (b) Same, from micropropagated shoots derived from leaf-disc cultures of *Spilanthes*.

overexploitation or threatened or extinct of large number of plant species. Certainly, the development of alternative and complimentary methods over whole plant extraction would help in the conservation of large number of medicinally useful plant. In this perspective, plant cell culture technology shows promise for the large-scale production of high-value secondary metabolites of industrial importance in a short duration with reduced growth cycle. The production of pharmaceutically important metabolites by plant tissue culture methods offer a number of advantages such as:

(i) As we know that environmental fluxes cause alterations in type and quantity of metabolites, *in vitro* cultures will help to utilize the biomass and nullify the effect of seasonal variation on secondary metabolite content.

(ii) Also, studies on secondary metabolites require an in-depth understanding of biosynthetic pathways which is often difficult to conduct in whole plants but the biosynthetic activities can easily be monitored in particular cell types within a specific plant organ or at a certain time of season.

(iii) Cell cultures have a higher rate of metabolism than intact differentiated plants because the initiation of cell growth in culture leads to fast proliferation of cell mass and to a condensed biosynthetic cycle. As a result, secondary metabolite production can take place within a short cultivation time (about 2-4 weeks) with an added advantage of tunability. These cell cultures may further aid in scale up operations, for isolation of desirable compounds in bulk.

(iv) The plant cell cultures can serve as an attractive alternative option for producing important and novel metabolites all the year round without destroying the source plants and, thus, may help to improve the conservation status of *Spilanthes*.

In *Spilanthes*, so far, only two reports are available on *in vitro* metabolite production. First of all, Singh and Chaturvedi (2010) reported scopoletin accumulation in *in vitro* nodal segment derived plant. They have developed a novel HPLC method with fluorescence detector for the quantitative estimation of scopoletin in *S. acmella*, which can detect even a low concentration of scopoletin and could be easily implemented in routine practice. The results of this study showed that the scopoletin content of the nodal segment derived plants was 0.104 ± 0.03 mg/g DW of leaves which was comparable to that of the mother plant (0.101± 0.04 mg/g DW of leaves). Scopoletin biosynthesis was induced in several plant species upon infection by different pathogens (Matros and Mock 2004; Kai *et al*. 2006; Lerat *et al*. 2009) and is considered as an important compound that play an important role in defense mechanism against bacteria and fungi (Smith 1996). However, no quantification studies were performed in either of these reports. This is the first report on detection and quantification of scopoletin in *S. acmella*. The study revealed that even the uninfected leaves of *Spilanthes* could accumulate the scopoletin.

The same authors, after two year, observed spilanthol production from leaf of leaf disc derived plants. Interestingly, they noticed significantly ($p<0.05$) higher spilanthol production (3294.36 ± 12.4 mg/g DW) from leaf disc derived plants than from field grown plants. In the same study, callus cultures established from leaf disc accumulated low amount of spilanthol

(998.03 ± 15.6 mg/g DW) (Singh and Chaturvedi 2012). The study confirms the earlier reports which suggested that differentiated (organized and redifferentiated) cells and specialized organs generally produce most secondary products compared to dedifferentiated (unorganized) cells in cultures. (Rao and Ravishankar 2002; Tang *et al.* 2010).

CONCLUSION AND FUTURE THRUSTS

India though the hot-spot of medicinal plants is losing its plant diversity at a very high rate due to indiscriminate use of plant materials, population growth and habitat destruction. If the trend continues, the important medicinal plants will be lost or may reach an extinction. To avoid this situation, sincere conservation efforts are needed at mass scale to overcome the existing challenges. Large scale production of medicinal plants through tissue culture methods can reduce the existing ever-growing burden on wild population of medicinal plants and, thereby, may improve the conservation status of important medicinal plants like *Spilanthes*. The scientific research on *Spilanthes* suggests a huge biological potential of this plant. It is strongly believed that detailed information as presented in this chapter would be useful for the conservation and maintenance of large number plantations not only in Spilanthes but also in other equally important medicinal plants. This is probably one of the most promising lines of research for the coming year.

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