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Impact of organic farming on *Spilanthes acmella* Linn. an endangered important medicinal plant raised through tissue culture technique

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Abstract
Spilanthes acmella Linn belongs to family Asteracea is a multipurpose medicinal herb of tropical and subtropical regions. Due to its versatile properties the plant is in tremendous demand but due to its overexploitation and poor seed germination ability it has been depleted in its natural environment. An alternative method for its conservation and efficient rapid mass propagation is thus need of the hour. Therefore, the present work was aimed to develop an efficient *in vitro* propagation protocol for its rapid and large scale production. Different combinations of growth regulators in MS media were used to promote its shoot, and root formation. Maximum shoot proliferation was found in 0.5 mg/l of BAP in combination with 0.5 mg/l IBA. Regenerated shoots were rooted on MS medium supplemented with different concentration of auxins. The maximum rooting 90.5 % with 6.4 cm of root length was observed in MS media fortified with 0.5 mg/l IBA after 2 weeks of inoculation. Present study will help to propagate the medicinal plant for industrial herbal extraction of valuable pharmaceutical substances to be used in different diseases. This study will also help to execute further research on regeneration, transformation and hairy root induction of this important medicinal plant. The present research also focused on the methods and benefits of organic farming of the medicinal plant prevalent nowadays, although more better and profitable techniques can be introduced by carrying out further research in this regard. Hence it concludes that application of organic and biofertilzers acts as substitute of inorganic fertilizers in order to grow the medicinal and aromatic plants, should not be considered as a simple objective and short term benefits, but as a mean to improve the environmental conditions and human health. Therefore the present investigation was undertaken with the aim to prescribe the panacea by developing an effective method for rapid and large scale multiplication with protocol for effective organic farming to boost the vigour and other quantitative traits of the plant.

Keywords: *spilanthes acmella, in vitro* propagation, endangered, conservation, growth regulators, organic farming

Introduction
Spilanthes acmella L*inn***.** also known as Akarkara or Toothache plant belongs to the family Asteraceae is an important endangered medicinal plant. It is comprised of 60 species that are widely distributed in tropical and subtropical regions of the world, such as Africa, America, Borneo, India, Sri Lanka and Asia (Sahu *et al* 2011; Tiwari *et al* 2011) [25, 34]. In India, the genus *Spilanthe*s is represented by 7 species viz. *Spilanthes calva DC, S. paniculata DC, S. radicans Jacq, S. ciliate kunth, S. Acmella Linn. S. uliginosa Sw*., and *S. Oleracea*. *Spilanthes acmella* is one of the species is an annual or short-lived herb of about 40-60 cms tall and is grown in damp area (Tiwari *et al* 2011; Wongsawatkul *et al* 2008) [34, 35]. It has immense application in pharmaceuticals, food, health and body care products. Flowers and leaves have been used as a spice for appetisers and as folk medicine for stomatitis, for toothache, rheumatic (Wongsawatkul *et al* 2008) [35], throat complaints (Nakatani and Nagashima 1992) [17] as fresh vegetable (Tiwari *et al* 2011) [34] as well as spice for Japanese appetizer (Leng *et al* 2011) [12]. It also possesses anti-inflammatory, antioxidant, antimalarial antibacterial and antifungal properties due to the presence of a highly valuable biologically active compound spilanthol (Khadir *et al* 1989; Pandey *et al* 2009) [10, 19]. Because of its multifold uses *Spilanthes acmella* is being overexploited by the local population as well as pharmaceutical companies. Further due to the lack of vegetative propagation and low seed rate germination (Tiwari *et al* 2011) [34] this valuable plant species has declined fast and is listed as endangered (Rao *et al* 1983) [23]. Therefore the development of an efficient *in vitro propagation* protocol for its conservation and for the production of improved plants is an obvious reason.

In vitro propagation is an alternative method for meeting out the demand within in responsible time and obtain large number of consistently uniform, true to type plant and elite germplasm with in short span of time. Micropropagation method is specifically applicable to species in which clonal propagation is needed (Gamborg and Phillips 1995) [6]. There are considerable efforts made for *in vitro* plant regeneration of this valuable plant species (Saritha and Naidu, 2008; Singh *et al*. 2009; Yadav and Singh 2010) [27, 11]. But the present investigation is efficient, rapid and improved micropropagation methodology. Further, some elite shoots were also found *via* somatic embryogenesis with respect to the

height and other growth parameters.

Organic farming is a technique, which involves cultivation of plants in natural ways. It is a production system which avoids or excludes the use of synthetic preparations, artificial fertilizers, pesticides, growth accelerators and fodder additives. This process involves the use of biological materials, avoiding synthetic substances to maintain soil fertility and ecological balance thereby minimizing pollution and wastage. "Pesticides that kill insects also kill a tiny part of the living element in us" (Patakh *et al* 2007). Organic fertilizers in comparison of the chemical fertilizers have lower nutrient content and are slow release but they are as affective as chemical fertilizers over long period of use (Naguib 2011) [15]. The medicinal plants are the basic source of raw material for preparation of ayurvedic medicines. By implementing good agro technique and organic farming practices in medicinal plants cultivation, the safety and quality of plant materials and finished products could be assured. Furthermore, the application of vermicompost in the field enhances the quality of soils by increasing microbial activity and microbial biomass which are key components in nutrient cycling, production of plant growth regulators and protecting plants from soil-borne diseases and arthropod pest attack. The present study focused on the methods and benefits of organic farming of the medicinal plants prevalent nowadays, although more better and profitable techniques can be introduced by carrying out further research in this regard. Hence it concludes that application of organic and biofertilzers as substitute of inorganic fertilizers in order to grow the medicinal and aromatic plants, should not be considered as a simple objective and short term benefits, but as a mean to improve the environmental conditions and human health.

Therefore the present study was undertaken with the aim to prescribe the panacea by developing an effective method for rapid and large scale multiplication together with protocol for effective organic farming to boost the vigour and other quantitative traits of the species.

Materials and Methods

Nodal segments were excised from the plants of *Spilanthes acmella* growing in pots in CSIR IIIM Canal Road Jammu. The explants were washed under running tap water for 30 mts and then washed thoroughly in sterile double-distilled water (DDW). These explants were then kept in 1% (w/v) Bavistin (Carbendazim Powder, BASF India Limited), a broad spectrum fungicide for 10 min, followed by 5% (v/v) Teepol (Qualigens Fine Chemicals, India), a liquid detergent for 5 mts by continuous shaking method. The treated explants were washed in sterile DDW 4-5 times to remove the chemical inhibitors. The explants were then surface sterilized by immersing in a freshly prepared solution of 0.5 % (w/v) NaOCl (Qualigens Fine Chemicals, India), for 1 m. under laminar flow. Finally, the explants were washed 5-6 times with sterile DDW for 5 mts to remove all traces of sterilizing agent used (Shahid *et al* 2007) [28]. These explants were then inoculated in MS media with various concentrations of BAP, Kn, NAA, IAA and IBA (Table 1-3) either singly or in various combinations for shoot induction, multiplication and root formation. The media was prepared in culture tubes (25×150 mm, Borosil) and pH was adjusted at 5.8±0.2 and was solidified by adding 1% agar (HiMedia Lab. Ltd., India) and sterilized by autoclaving at 15 lb pressure per square inch, 121°C temperature for 15 mts. The sterilized explants were then inoculated aseptically into the medium and incubated at 25 ± 2°C with relative humidity of 55 ± 5% and exposed to photocycle of 2,500 Lux intensity for 16 hrs (Sharma *et al* 2012) [29]. Visual observations like, number of days taken for bud break, percentage of bud break and number, length of shoots regenerated per explants and length of roots per regenerated shoots were recorded after 2 weeks. The *in vitro* developed single/multiple shoots (2.5 -3.0 cm) were excised and implanted in culture tubes containing MS medium fortified with IBA, IAA and NAA under aseptic conditions for rooting (Table 3).

Effect of the organic farming on tissue culture raised plants of *Spilanthes acmella*

After the micropropagation of *Spilanthes acmella* through tissue culture technique impact of organic fertilizers on the growth of plants under field condition was studied and were compared with control i.e. without any organic fertilizer. To study the effect of organic farming on tissue culture raised plants of *Spilanthes acmella* three organic fertilizer combinations were used. i.e. soil, sand and vermiculite (1:2:1), soil, sand and solurite (1:2:1) and soil, sand and dry cow dung powder (1:2:1) and were compared with the effect of control i.e. soil and sand only in the ratio of (1: 2) under green-house conditions. The pots were maintained in hardening unit i.e. green house (micropropagation unit) for observation. (Table 4).

Growth parameters of tissue culture raised plants supplemented with organic fertilizers were compared with the control (without organic fertilizers). Visual plant growth observations like, height of plants and number, length of shoots and length of roots, morphology of leaves, number of days taken for flower formation, number of days required for seed formation, were recorded and finally data obtained was analyzed statiscally.

Results and Discussion

Establishment of Cultures

Shoot Induction and Multiplication

Nodal segments taken from field were used as explants in which 3 explants were used per replicate. MS basal medium without any growth regulator act as the control. This medium could not produce even single shoot per explant. A combined effect of different concentrations of BAP and auxins was explored. BAP (0.5 mg/l) with IBA (0.5mg/l) showed maximum (90.5 %) bud break after 15 days of inoculation. The medium supplemented with BAP (0.5mg/l) + IBA (0.5 mg/l) induced maximum number of shoots (3-4) per explant of length 5.8 cm and responded best among all combinations used shown in (Table 1). In case of BAP supplemented media, the medium with BAP (1.0 mg/l) showed 70% bud break after 25 days of inoculation with 2-3 shoots per explants. (Table 1).Figure.1 (a-b).

For multiplication induced shoots were sub cultured on MS basal medium supplemented with different concentrations of BAP either alone or with auxins. Among the various concentrations, BAP (0.5mg/l) with IBA (0.5mg/l) was found

to be best for shoot multiplication produces 5-6 shoots of length 8.0 cm. (Table 2) Figure.1 (c-e).Increasing the concentration of BAP more than (2.0 mg/l) produced stunted shoots. Cytokinins have been shown to be most critical growth regulator for shoot multiplication so in present study MS medium with 0.5 mg/l BAP acted as trigger for initiating multiplication of shoot bud meristem shown in (Table 2).

The stimulating effect of BAP on bud break and multiple shoot formation has been earlier reported for several medicinal plant species including *Peganum harmala* L. (Rugini E. *et al* 1983) [24]. Similar results were also observed in *Spilanthes acmella* Joshi V. *et al* (2015) [9], (Pandey V. *et al* (2009) [19], Saritha K.V. *et al* (2002), Earlier reports also showed the effectiveness of BAP has been reported by (Thakur *et al* 2001) [32] in *Alnus nepalensis*, (Johonson *et al* 2002) in *Rhinacanthus nasutus* and (Thind *et al* 2008) [33] in *Aloe vera.*

Rooting of *in vitro* Regenerated Shoots

Elongated and well developed regenerated shoots via nodal segments were aseptically excised and implanted on MS medium supplemented with different concentrations of auxins (IAA, NAA and IBA). For rooting. MS medium supplemented with (0.5 mg/l) IBA was found the most suitable for rooting of shoots. About (85 %) root induction of length (7.0 cm) were observed within least time period (15 days). However IAA (0.5 mg/l) and NAA (0.5 mg/l) failed to develop efficient root system in explants (Table 3) Figure 1(f). Supplementation of IBA was also found to be effective on root development in *Artemisia judaica* (Liu *et al*., 2003) [13] and *Eclipta alba* (Bhaskaran and Jayabalan, 2005) [4]. Similar results were reported in *Spilanthes acmella* by Joshi V. *et al* (2015) [9], Yadav K. *et al* (2011) [11] in *Psoralea corylifolia by* Jeyakumar M. *et al* (2002) [7], Anis M. *et al* (2005) [14], and Pandey P. *et al* (2013) [21].

Identification of Suitable Hardening Medium for Better Establishment

The transfer of plants from the culture flasks to the soil requires a careful, stepwise procedure. After 15 to 20 days of culture on rooting media, the rooted plantlets were transplanted to pots for hardening prior to their final transfer to soil. Rooted plantlets were taken out of the culture bottles with the help of forceps and washed thoroughly with water to remove any remaining of the medium**.** A minimal survival rate of 40-50% was recorded during the months of May, June, July and August. However, the plants taken out after September showed a substantial increase in survival percentage. All hardened plants survived on transfer to pots in greenhouse containing soil, sand and farmyard in ratio of (1:2:1) mixture gave the maximum survival percentage with better plant growth resulting as a suitable medium for hardening. Figure 1(g-h).Similarly, *in vitro* raised rooted shoots of tomato plants were more effectively when transferred to garden soil, farmyard soil and sand 2:1:1 ratio mixture and the plants were successfully acclimatized. (Sherkar H. D. and Chavan A. M. 2014) [30].

Impact of organic farming on tissue culture raised plants of *Spilanthes acmella*

Hardened *in vitro* raised plants of 15 to 20 centimeters height with healthy roots were transferred to different organic compost to study the impact of organic farming of tissue culture raised plants of *Spilanthes acmella.* In the present study three organic fertilizer substance, combinations were used. i.e. soil, sand and vermiculite (1:2:1), soil, sand and solurite (1:2:1) and soil, sand and dry cow dung powder (1:2:1) and were compared with the effect of control i.e. soil and sand only in the ratio of (1: 2) under green house condition. It is observed that the application of cow dung, compost and vermicompost increased the biomass yield. On application of all three compost, production of *Spilanthes acmella* increased than control i.e. shows minimum growth (35 %) when cultivated only on soil and sand (1:2) mixture. Poor growth and branching (3.0±0.7) is recorded having about 4.4±0.1centimeters height and low flowering after 50 days. (Table.4), Figure 2. (l). The results indicated that soil, sand and vermiculite combination in the ratio of 1:2:1 shows best growth (90%). By the application of this compost the plants improved the height of 13.3 ±0.2 centimeters, early flowering, and about 10.0 ±0.1 number of branches and diameter significantly within 35 days. (Table.4), Figure 2.(i).The organic matter after decomposition release both macro and micro nutrients to soil solution, which becomes available to the plant resulting in higher uptake. The increase of leaf yield in manure application may be due to increase in plant photosynthesis. While as soil, sand and solurite (1:2:1) compost shows better and satisfactory growth rate (80%) good branching (7.0±0.3) of about 9.5±0.7 centimeters height, and flowering within 40 days. (Table. 4 and Figure.2 (j), and 70.0 % growth were observed in soil, sand and dry cow dung mixture compost (1:2:1). Figure. 27(k).

The results indicated that soil, sand and vermiculite combination in the ratio of 1:2:1 shows best growth (90%). In this compost the increased biomass specially leaf yield is recorded, may be due to increase in plant photosynthesis. Plants in the vermiculite treated soil yield higher leaf biomass. This may be due to higher nitrogen content in vermiculite treated soil. (Table. 4). Nitrogen rich organic manure generally helps in increasing vegetative growth. The practices adopted for organic cultivation involve mainly use of organic manures viz. cow dung, compost and vermicompost.

Although vermicompost is considered one of the best organic manure for organic cultivation, it is also advisable in case of many other medicinal plants to take the practice as far as more integrative manner along with some other cash crops so that the total earning from all the sources becomes high. Arya and Arya (1996) reported that the mixture of soil, sand, organic manure and vermiculite in ratio of (1:1:1:1) were used in micropropagated plants for hardening. The harden plants survive well and were ready for transfer after 30 days. The use of vermicompost extract results in the control of some soil borne pathogens on three ornamental plant species significantly reduced sporulation of the pathogen *Phytophthora cryptogea* (Orlikowski 1999) [18]. While aqueous extracts of organic matter (vermicompost) were capable of reducing the growth of pathogenic fungi such as *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Corticium rolfsii*,

Rhizoctonia solani and *Fusarium oxysporum* (Nakasone *et al;* 1999) [16]. Similarly in *Gloral* (Azizi *et al* 2008) [3], in *Tea* (Edwards *et al* 2006) [5] and in *Thymus Vulgaris* L (Ateia *et al* 2009) [2]. It was reported that antioxidant activity, phenol and flavonoid content increases by using vermicompost in *Asparagus recemosus* (Saikia and Upadhyaya 2011) [26]. The production of natural substances by plants is affected by genotype and environmental conditions. Adopting organic farming in medicinal plants cultivation is the need of the day. Continuous usage of inorganic fertilizer affects soil structure. Hence plant and animal manures, compost and vermicompost can serve as alternative mineral fertilizers for improving soil structure and microbial biomass.

Table 1: Effect of MS media and growth regulators either alone or in combination on shoot induction of *Spilanthes acmella*

S. No	MS+Auxin/ cytokinin (mg/l).	%age of bud break.	No. of days required for bud break.	Mean No. of shoots produced ± SE.	Mean shoot length in cm.±SE.
01	Control	0	20	0	0
02	0.5 BAP+0.5 NAA	20.2	25	1.5±0.7	2.1±0.0
03	1.0 BAP+1.0 NAA	45.4	20	3.6±0.7	3.2±0.5
04	0.5BAP.	30.6	10	3.8±0.8	2.8±0.7
05	1.0 BAP	70.1	25	3.8±0.7	4.1±0.4
06	0.5BAP+0.5 IBA	90.5	15	4.0±0.2	5.8±2.7
07	1.0 BA+0.5 IBA	60.8	30	3.4±0.6	2.4±0.1
08	0.5 BAP+0.5 IAA	50.5	20	3.7±0.7	3.6±0.7
09	1.0BAP+1.0 NAA	50.6	17	3.6±0.1	3.9±0.8
10	2.0 BAP+0.5 IBA	45.7	25	3.1±0.4	3.3±0.0

Table 2: Effect of MS media and growth regulators either alone or in combination on shoot multiplication of *Spilanthes acmella*

Media code.	MS+Auxin/cytokinin (mg/l).	%age of shoot multiplication.	No. of days required.	Mean No. of shoots.	Shoot length in cm.±SE.
S.M.1	0.5 mg/l BAP.	55.8	20	2.6±0.5	4.2±0.9
S.M.2	0.5 mg/l KN.	40.0	25	`1.0±0.08	6.3±0.9
S.M.3	0.5 mg/l BAP+ 0.5mg/l IBA.	90.0	15	6.3±0.9	8.0±0.2
S.M.4	0.5 mg/l KN + 0.5mg/l 0.5 IBA.	50.5	20	2.3±0.1	4.3±0.9
S.M.5	0.5 mg/l BAP + 0.5 mg/l NAA.	75.5	25	4.0±0.7	6.0±0.5
S.M.6	0.5mg/l KN + 0.5mg/l NAA.	45.4	30	4.5.±0.5	3.0.±0.1
S.M.7	1.0 mg/l BAP + 0.5mg/l IAA.	70.0	30	4.0±0.9	5.7±0.6
S.M.8	1.0 mg/l KN + 0.5 mg/l IAA.	40.0	30	3.8±0.5	3.5±0.0
S.M.9	2.0 mg/l BAP + 0.5 mg/l IBA.	60.0	30	3.5±0.9	5.5±0.4
S.M.10	2.0 mg/l BAP + 0.5mg/l NAA.	30.5	35	2.0±0.1	3.2±0.8

Table 3: Effect of MS media and growth regulators on root induction of *Spilanthes acmella*

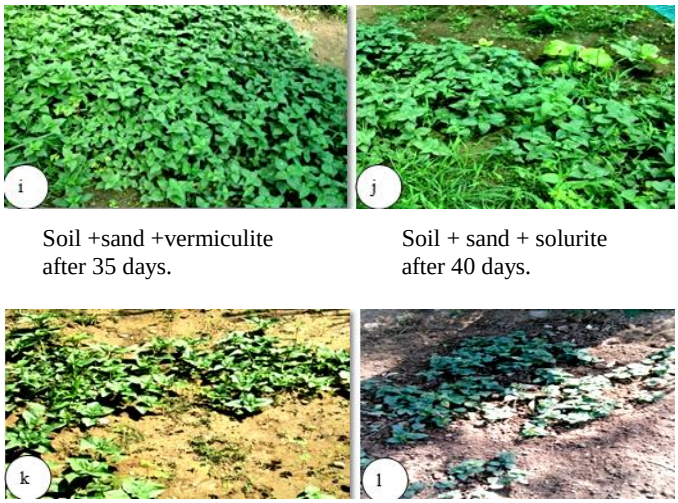
Media code.	Media composition. (mg/ l).	% age of root induction.	No. of days required.	Mean root length in cm.±SE.
S.R.1	MS +0.5 IAA.	60.0	20	4.0±0.4
S.R.2	MS +0.5 IBA.	85.0	15	7.0±0.6
S.R.3	MS +0.5 NAA.	68.2	20	5.3±0.8
S.R.4	MS +1.0 IAA.	50.0	25	3.5±0.0
S.R.5	MS +1.0 IBA.	75.0	20	6.5±0.6
S.R.6	MS +1.0 NAA.	55.0	25	3.8±0.2
S.R.7	MS +1.5 IAA.	40.5	30	2.5 ±0.7
S.R.8	MS +1.5 IBA.	65.0	30	5.0±0.1
S.R.9	MS +1.5 NAA.	53.0	25	4.0±0.2
S.R.10	MS +2.0 IBA.	30.0	30	2.0±0.2

Table 4: Effect of different organic fertilizers and control on tissue culture raised plants of *Spilanthes acmella*

S. No	Organic fertilizers.	%age of plant growth.	No. of Days required.	No. of branches.	Shoot length cm±SE.
S.O.1	Soil + sand(control)	35	50	3.0±0.7	4.4±0.1
S.O.2	Soil+sand+vermiculite	90	35	10.0±0.1	13.3±0.2
S.O.3	Soil + sand + solurite	80.5	40	7.0±0.3	9.5±0.7
S.O.4	Soil + sand + dry cow dung powder	70	45	5.5±0.1	7.8±0.3

a-b Shoot regeneration from nodal explant on MS medium + BAP. (0.5mg/l)+IBA (0.5mg/l)
c-e. Regeneration of multiple shoots from nodal explant on MS medium + BAP (0.5 mg/l)
f. Differentiation of roots from induced shoots on MS medium +IBA (0.5mg/l)
g-h. Hardening of Tissue culture raised plants of *Spilanthes acmella*.

Fig (a-h) *In vitro* regeneration of *Spilanthes acmella* Linn. via Nodal Segments,



Soil +sand +vermiculite after 35 days.

Soil + sand + solurite after 40 days.

Soil + sand +dry cow dung powder after 45 days.

Soil + sand after 50 days

Fig 2: (i-l) Tissue culture raised plants of *Spilanthes acmell* in field supplemented with different organic fertilizers and control (without organic fertilizers).

Conclusion

Therefore, the present work demonstrated that *in vitro* propagation of *Spilanthes acmella* was successfully done by using different explants and different combinations of growth regulators in MS media to promote shoot induction and root formation. The present research also focused on the methods and benefits of organic farming on the i*n vitro* raised medicinal plants of *Spilanthes acmella*. Hence it concludes that application of organic and biofertilzers acts as substitute of inorganic fertilizers in order to grow the medicinal and aromatic plants, should not be considered as a simple objective and short term benefits, but as a mean to improve the environmental conditions and human health. Therefore the present investigation was undertaken with the aim to prescribe the panacea by developing an effective method for rapid and large scale multiplication with protocol for effective organic farming to boost the vigour and other quantitative traits of the plant. Therefore the protocol developed could be used for conservation of elite germplasm and true to type mass propagation of *Spilanthes acmella* of immense pharmaceutical relevance. This is highly advantageous for the production of uniform source of *Spilanthes acmella* plants for a range of further biotechnological applications and will also help in the production of improved plants.

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