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Organogenesis in callus derived from leaf explants of *Spilanthes acmella* Linn: An endangered medicinal plant

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Abstract
Several experiments were conducted to test the effect of growth regulators with the different concentration and combination of (0.5-2.0 mg/l) 2, 4-D with cytokinins like 0.5 mg/l BAP and 0.5 mg/l KN on indirect organogenesis response from leaf segments of *Spilanthes acmella*. On the most media combinations, leaf segments either callused or differentiated into shoots. Maximum callus (70.5%) was induced on the media containing 1.5 mg/l 2, 4-D with 0.5 mg/l BAP within 30 days and the callus formed was dark green and fragile. After the callus formation the induced callus were sectioned into small pieces and transferred on MS media supplemented with BAP and KN separately or BAP and KN with NAA, IBA and IAA. The best response (75.0%) on shoot regeneration from callus was observed on MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l IBA with the formation of 6.5±0.41 number of shoots having length of 7.9±0.1 centimeters within 20 days. after shoot regeneration maximum (75.0%) of shoot multiplication rate was observed with an average of 7.5±0.41 adventitious shoots, directly from the explants, without any callus formation on medium supplemented with 1.0 mg/l BAP and 0.5 mg/l IBA. Shoots formed were remarkable healthy and achieved the length of 8.5±0.1centimetres within 20 days. While as maximum (70.0%) root induction was achieved directly from the base of the regenerated shoots on medium supplemented with 0.5 mg/l IBA of average length 6.0±0.6 centimetres within 20 days.

Keywords: Indirect organogenesis, *Spilanthes acmella* Linn, *In vitro* propagation, Endangered, Conservation, Plant growth regulators

1. Introduction
Spilanthes acmella belonging to family Asteraceae is a genus comprising of over 60 species is cultivated throughout the year as an ornamental or medicinal plant. It is an annual or short lived herb that is 40-60 centimeters tall. It grows in damp areas (Tiwari *et al;* 2011, Wongsawatkul *et al;* 2008) [36, 39] and has low rate of seed germination and pitiable vegetative propagation. Its flowers and leaves have pungent taste and when touched it is accompanied by tingling sensation and numbness but when cooked, the plants loses strong flavor and may be used as a green leafy vegetable. The plant species has been commonly used as a folk remedy since time immemorial for various ailments like toothache, rheumatism and fever (Wongsawatkul *et al;* 2008) [39], as fresh vegetable (Tiwari *et a1*; 2011) [36] as well as spice for Japanese appetizer (Leng *et a1;* 2011) [18]. An Indian tribe uses *Spilanthes acmella* to treat fungal skin conditions, such as athlete's foot, ringworm and nail infections.
Due to poor availability, possibilities of facing threats by the species, it is the need of time to increase the population size and ensure the greater biomass availability and conserve the species as well. But unfortunately, these medicinal plants are disappearing at an alarming rate because of destruction of their natural habitats due to urbanization, indiscriminate overexploitation and uncontrolled collection of plant materials for purposes other than medicinal use, thus making the plants endangered and threatened for extinction. It has been studied that due to low rate of seed germination, loss of viability and pitiable vegetative propagation of *Spilanthes acmella* there is need of the hour to conserve the important medicinal plant through alternative methods (Tiwari *et a1*; 2011) [36]. Due to these drawbacks, there is a great need for micropropagation of these plants along with genetic stock conservation. The significance of an efficient *in vitro* protocol would be to obtain maximum number of plantlets in minimum period of time with proper rooting along with acclimatization in the field.
In vitro micropropagation is a viable alternative for species which are difficult to regenerate by conventional methods where population have decreased due to over exploitation by destructive harvesting and can effectively be used to meet the growing demand for clonally uniform elite

plants. When species have been over collected by hobbyists for medicine, food or fragrance, *in vitro* propagation can provide an alternate source of plants and alleviate pressures on wild populations

The present study describes the indirect organogenesis strategies developed by using tissue culture technology for the species under the influence of different combinations and concentration of certain plant growth regulators.

Materials and methods

Collection of plant material

Young leaves of *Spilanthes acmella* from 7 months old plant were collected from the pot culture maintained in nursery and were used as explants.

Sterilization of explants and preparation of culture

The leaves were thoroughly washed with running tap water to remove all the dust particles adhere with 1% bavistin for 10 minutes, followed by three rinses with sterile double distilled water. To eliminate other contamination explants were rinsed with 70% alcohol for 1 minute followed by distilled water washing twice. The explants were then surface sterilized by immersing in a freshly prepared solution of 3.0% (w/v) NaOCl (Qualigens Fine Chemicals, India) for 5 to 6 minutes under laminar flow. Finally the explants were washed 5 to 6 times with sterile double distilled water for 5 minutes to remove all traces of sterilizing agents used Shahid M. *et al*. (2007) [27].

Callus induction medium

For callus initiation experiment the sterilized leaf segments of 5 to 10 centimetre length were taken and were inoculated on MS media supplemented with auxins either alone in the concentration of (0.5-2.0 mg/l) 2, 4- D or with varied concentration and combinations of cytokinins like (0.5 mg/l) BAP and (0.5 mg/l) KN for callus induction. The cultures were incubated at a temperature of 25±2 ºC and a photoperiod of 8 hrs light (intensity of 2000 lux) and 16 hrs of dark). (Table. 1) Visual observations like callus induction, growth of callus, number of days taken for callus induction were recorded regularly.

Shoot regeneration medium

For the regeneration of shoots the induced callus were sectioned into small pieces and transferred on MS media supplemented with cytokinins either alone 0.5-1.0 mg/l BAP (6-benzylaminopurine) and 0.5-1.0 mg/l KN (Kinetin) or different higher concentrations of cytokinins like 0.5-2.0 mg/l BAP (6-benzylaminopurine and 0.5-2.0 mg/l KN (Kinetin) with auxins i.e. 0.5 mg/l IAA (indole-3-aceticacid),0.5 mg/l NAA (α naphthalene acetic acid) and 0.5 mg/l IBA (Indole-3-butyric acid). (Table. 2). The cultures were incubated at a temperature of 25±2 ºC and a photoperiod of 16 hrs light (intensity of 2000 lux) and 8 hrs of dark. Visual observations like number of days taken for shoot regeneration and number of shoots regenerated per explants were recorded regularly. A mean of 10 replicates was taken per treatments.

Shoot multiplication medium

Clonal propagation by shoot multiplication is the main phase of the *in vitro* micropropagation of an endangered medicinal plants (Saini R. *et al;* 2002) [25]. Different methods and experiments were tried to maximize the number of multiple shoots. The number of propagules are multiplied by repeated sub culturing of the shoot cultures until the desired (or planned) number of plants are attained.

For shoot multiplication the regenerated shoots from callus were separated and transferred on MS media supplemented with different concentration of cytokinins like (0.5-2.0 mg/l) BAP and (0.5-2.0 mg/l) KN either alone or with different combination of auxins like (0.5 mg/l) IBA, NAA and IAA. The growth response of explants were studied at weekly interval. The parameters were taken as the average number of shoots initiated and multiplied, and the length of regenerated and multiplied shoots were recorded). (Table.3).

Rooting of elongated shoots

After proper shoot elongation, the plantlets were properly removed from medium and were transferred into full strength MS medium supplemented with different concentrations of auxins like NAA, IAA and IBA for rooting). (Table.4). After one month the complete plantlets were transferred in field for hardening.

Hardening of *in vitro* raised plantlets

The rooted plantlets were washed with 1% bavistin and transferred to humus rich soil: coco-pit filled plastic cups in the ratio 3:1 and were kept under moist chamber in green house for 25days (35-38 ^{0}C temperature and humidity at 90% RH). After 25 days the rooted plantlets were transplanted to single net shade house in plastic cups of traditional potting mixture containing soil: compost: coco pit in the ratio 1:1:1 for the secondary hardening.

Results and discussion

Callus induction

Several experiments were conducted to test the effect of growth regulators with the different concentration and combination of (0.5 -2.0 mg/l) 2,4-D with cytokinins like 0.5 mg/l BAP and 0.5 mg/l KN on callus induction response from leaf segments. On the most media combinations, leaf segments either callused or differentiated into shoots. Maximum callus (70.5%) was induced on the media containing 1.5 mg/l 2, 4-D with 0.5 mg/l BAP within 30 days and the callus formed was dark green and fragile. (Table. 1, Figure 1 (a-b). Whereas (60.8%) callus was induced from leaf segments on MS medium supplemented with 2.0 mg/l 2, 4-D and 0.5 mg/l BAP within 30 days which was Brownish and compact in morphology.

The callus produced in 2, 4-D alone was green and compact whereas the callus formed in the medium containing 2, 4- D with BAP was green and fragile. Reduced callus (50.5%) was observed when 2, 4-D was increased more than 2.5 mg/l in combination with BAP as observed on MS medium supplemented with 2.5 mg/l 2, 4-D with 0.5 mg/l BAP after 35days which was brownish and compact. (Table.1).

The use of sterile leaves to establish the callus cultures would be very convenient for clone elite individuals and for enhancing the secondary metabolite production. This is due to the fact that the leaves are easy to obtain and do not require to sacrifice the mother plant.

It was observed that the present investigation revealed the direct contrast to the reports of**,** Pandey and Agrawal (2009) [38] and Sing and Chaturvedi (2012) [31]. Till date only three reports are available on leaf segment culture of *Spilanthes acmella*, it was also found that a balanced ratio of the two growth regulators controlled the induction of callus tissue *in vitro*. A high auxin to cytokinin content results maximum callus formation, Harsh P. V. (2002) [7] recorded similar observations in *Spilanthes mauritiana* a sister species of

Spilanthes. Pandey and Agrawal (2009) [38] reasoned that the ratios of the hormones playing such an important role in application experiments may be due to the tendency of either hormone to depress the synthesis and/or transport of the other, causing its absolute level to fall below specific threshold levels. Similar results were also observed in *Jojoba* (Sunil kumar *et al;* 2013) [35] in *Potato Cultivars* (AL-Hussaini Z.A. *et al;* 2015) [1] in *Tecomella undulata* (Manisha B. Patel *et al*; 2013) [19] in *Acacia sinuata* (Vengadesan G. *et al*; 2000) [37], (Ana Hortencia Fonseca Castro *et al;* 2016) [2] in *Byrsonima verbascifolia* and in *Hypericum perforatum* (Pretto and Santarem, 2000) [23].

Callus regeneration

To regenerate shoots from callus, the initiated callus was transferred on fresh medium containing different concentration and combination of auxins/ cytokinins ranging from 0.5-2.0 mg/l BAP, 0.5-2.0 mg/l KN and 0.5 mg/l of IAA, NAA and IBA. Different types and concentrations of auxins and cytokinins used in the medium and a possible interaction between exogenous and endogenous concentrations of plant growth regulators have a marked effect on the *in vitro* culture responses (Skoog and Miller, 1957) [32].

A combined effect of cytokinins with auxins was evaluated on shoot regeneration experiment. Incorporation of (0.5 mg/l), NAA, IAA IBA, in the BAP and KN ranges from (0.5-2.0 mg/l) containing medium show significant effect on number of shoots regenerated. Although 100% cultures showed regeneration in cytokinin /auxin containing medium. The best response (75.0%) on shoot regeneration from callus was observed on MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l IBA with the formation of 6.5±0.41 number of shoots having length of 7.9±0.1centimeters within 20 days. (Table. 2, Figure.1 (c-d). It is observed that the callus shows very regenerative effect on the medium containing BAP with IBA and IAA. The addition of IAA to the medium also enhanced the number of shoot regenerants per culture significantly, which promoted differentiation of shoot formation directly, in the average of number 4.0±0.3 shoots having 5.5±0.4 centimetre length within 30 days. (Table. 2).

The callus enlarged and adventitious shoot-buds appeared to arise directly from the cut ends. Though, few shoot-buds were differentiated from callus derived from leaf segment explants on medium supplemented with 2.0 mg/l of KN and 0.5 mg/l of NAA. In this combination about 2.0±0.1 average number of shoots having length of 3.0±0.3 centimetres were observed within 25 days. Hence results showed that cytokinins particularly 0.5-2.0 mg/l BAP acts as trigger for shoot regeneration from callus and the MS medium supplemented with the 1.5 mg/l BAP with 0.5 mg/l IBA was however more significant than other concentrations and combinations for shoot regeneration from callus.

The synergetic effect of BAP with IBA for shoot regeneration from callus cultures has been also reported in *Amygdalus communis* L (Songul Gurel and Yucel Gulsen, 1998) [33]. The requirement of auxin along with cytokinin for obtaining optimal response of shoot-bud differentiation is well documented in a number of plants including *Aegle marmelos* (Hossain *et al*; 1995) [10], *Emblica officinalis* (Nayak *et al*; 2010) [22], *Musa acuminate* (Jafari *et al;* 2011) [11]. In this study, shoot proliferation from leaf-disc cultures occurred without an intervening callus formation.

The present investigations also corroborate with those of Pandey and Agrawal (2009) [38] and Sing and Chaturvedi (2012) [31] in *Spilanthes acmella*. It was found that a balanced combination of the two growth regulators controlled the induction of shoot buds from callus tissue. A high auxin to cytokinin content resulted in root formation, whereas the reverse promoted shoot bud development. Koroch *et al*; (2002) [17] recorded similar observations in *Echinaceae purpurea*, a sister species of *Spilanthes acmella.* Eklof *et al;* (1997) [6] reasoned that the ratios of the hormones playing such an important role in application experiments may be due to the tendency of either hormone to depress the synthesis and/or transport of the other, causing its absolute level to fall below specific threshold levels.

Shoot multiplication

In order to get profuse rapid shoot multiplication medium supplemented with cytokinin and auxin combinations were used. Auxins, like IBA, IAA, and NAA were added and tested along with varied concentration of BAP and KN to study their effect and to maximize shoot multiplication and elongation. Nevertheless, addition of auxins to the medium did not have a positive effect on multiplication fold and shoot elongation. It was observed that every medium having various combinations of cytokinin with auxin gives 100% response. Cultures showed highest percentage (75.0%) of shoot formation with an average of 7.5±0.41 adventitious shoots, directly from the explants, without any callus formation on medium supplemented with 1.0 mg/l BAP and 0.5 mg/l IBA. Shoots formed were remarkable healthy and achieved the length of 8.5±0.1centimetres within 20 days. (Table. 3, Figure.1 (e -f).

None of the KN supplemented medium helped in the elongation of shoots, thus higher supplement of (2.0 mg/l) BAP minimizes the shoot multiplication and elongation. Only 30.5% of cultures were differentiated, an average of 2.0±0.1 into shoots with an average length of 3.2±0.8 after 35 days. Shoots formed were stunted and turned yellow after some days. High frequency of shoot regeneration as well as the number of shoots per regenerating explant were obtained from callus are more responding than mature and primary meristems.

Therefore, 1.0 mg/l BAP with 0.5 mg/l IBA produced the most desirable results both in terms of multiplication fold and cluster elongation, Thus, as usual BAP was found to be the most effective indicating, the cytokinin specificity of any nodal explants of *Spilanthes acmella* for shoot formation. In contrast to the synergistic effect of BAP in combination with an auxin has been previously reported in *C. candelabrum* (Beena *et al*; 2003) [4] and also in plant species like *Holostemma annulare* (Sudha *et al*; 1998) [5] in *Hemidesmus indicus* (Sreekumar *et al*; 2000) [34]. in *Amygdalus communis* L. (Songul Gurel and Y. Gulsen, 1998) [33] in *Hypericum retusum Aucher Sureyya* (Namli *et al*; 2010) [21]. Yadav K. *et al;* (2011) [40] revealed the same findings of *Spilanthes acmella* also showed the same protocol as described by Singh and Chaturvedi (2010) [30].

Rooting of *in vitro* regenerated shoots:

Various experiments were designed for root induction on MS medium with and without (control) growth regulators. In the present study, the role of different auxins like IAA, NAA and IBA in the root induction were tested. The induction of roots has been observed in every medium tried. Following the protocol of Singh and Chaturvedi (2010) [30] medium with root inducing growth regulators at the concentration of 0.5 to 1.5 mg/l IAA, 0.5 to 1.5 mg/l NAA and 0.5 to 2.0 mg/l IBA were tested from 1-2 month old shoots. Maximum (70.0%) root

induction was achieved directly from the base of the shoots on medium supplemented with 0.5 mg/l IBA of average length 6.0±0.6 centimetres within 20 days. (Table. 4, Figure. (g). On the contrary, by increasing the concentration and supplementation of other auxins shoots decreases inducting root. However 1.0 mg/l NAA and 1.0 mg/l IAA failed to develop an efficient root system from in *vitro* raised shoots. Minimum (30%) percentage of root induction was observed on higher (2.0 mg/l) concentration of IBA. At these concentrations though roots were induced but there was extensive callusing at the basal end of shoots which hindered its further growth. The roots formed were very slender and thin in the medium containing NAA. Among the various concentration and combinations full strength of MS basal medium supplemented with 0.5 mg/l IBA shows best root induction protocol for healthy roots within minimum time period. Similar results were reported in *Spilanthes acmella* by Joshi V. *et al*; (2015) [13], Yadav K. *et al*; (2011) [40] in *Psoralea corylifolia by* Jeyakumar M. *et al*; (2002) [12], Anis M. *et al;* (2005) [20], and Pandey P. *et al*; (2013) [24]. The effectiveness of IBA over other auxins for root induction has been already reported in several other plant species such as in *Glinus lotoides* (L) (Teshome S. *et al*; 2015) [29], *Eclipta alba* L. (Archana Sharma *et al*; 2013) [3] and in *chrysanthemum* (Kashif Waseem *et al*; 2011) [15], Hoque and Fatema (1995) [8], Hoque *et al; (*1998) [9], Sarker and Shaheen (2001) [26], Khan *et al;* (1994) [16], Karim *et al;* (2002).

Identification of suitable hardening medium for better establishment

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. (Figure 1. (h-i). 0.1% Bavistin (BVN) treatment was given to the plants in order to protect them from the fungal attack in the near future. The most essential requirement for the successful transplantation is to maintain the plants under a very high humidity (90-100%). For the first 10 to 15 days by keeping them under mist or covering them with clear plastic. Some small holes may be poked in the plastic for air circulation. Inside the culture vessel the humidity is high and thus, the natural protective covering of cuticle is not fully developed. During this time plants attain ability to synthesize more food and develop cuticular covering. Plants are maintained under shade and are then ready to be used in open nursery. After this the plants were carefully planted in the containers containing soil sand and farmyard in the ratio of (1:2:1). From the 30 plants transferred to mixture of soil, sand and farmyard in the ratio of (1:2:1), 20-25 plants survived and hardened in 3 weeks. 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. Hence soil sand and farmyard in the ratio of (1:2:1) mixture gave the maximum survival percentage with better plant growth resulting as a suitable medium for hardening. (Fig 1. (h-i). Similarly Sherkar H. D. and Chavan A. M. (2014) [28] reported that *in vitro* raised rooted shoots of tomato plants were effectively transferred to soil sand and farmyard in the ratio of (1:2:1) mixture and the plants were successfully acclimatized.

Callus growth measurements

The growth of the shoot callus cultures was measured in terms of fresh weights (FW) as well as dry weight (DW). The explants were placed between the folds of blotting paper to remove excess moisture and the fresh weight was then determined. Dry weight was measured after drying the fresh materials in an oven at 60 ± 1°C for 48 hrs.

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

Media Code.	MS+ Auxin/cytokinin (mg/l).	% age of callus induction.	No. of days required.	Morphology of callus.
S.C.1	0.5 2,4-D.	40.3	35	Brown compact.
S.C.2	1.0 2,4-D.	40.5	35	Yellow green.
S.C.3	1.5 2,4-D.	50.5	35	Green compact.
S.C.4	2.0 mg/l 2,4-D.	40.0	35	Dark Brown.
S.C.5	0.5 mg/l 2,4-D + 0.5 mg/lBAP.	60.5	30	Light green compact.
S.C.6	0.5mg/l 2, 4-D+0.5 mg/l KN.	40.0	25	Greenish white.
S.C.7	1.5 mg/l 2,4-D + 0.5 mg/l BAP	70.5	30	Dark Greenish fragile.
S.C.8	1.0 mg/l 2,4-D + 0.5mg/KN.	50.0	30	Brown watery calli.
S.C.9	2.0 mg/l 2,4-D + 0.5 mg/l BAP.	60.8	30	Brownish and compact.
S.C.10	2.5 mg/l 2, 4-D + 0.5 mg/l BAP.	50.5	35	Brownish and compact.

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

Media code.	MS+Auxin/cytokinin (mg/l).	%age of shoot regeneration.	No. of days required.	No. of shoots ±SE.	Shoot length in cm.±SE.
S.C.R.1	0.5 mg/l BAP.	55.0	25	4.0±0.5	5.3±0.3
S.C.R.2	0.5mg/l KN.	40.0	25	3.0±0.5	4.0±0.3
S.C.R.3	1.0 mg/l BAP.	60.7	25	4.9±0.17	4.1±0.24
S.C.R.4	1.0mg/l KN.	45.5	30	3.5±0.9	4.5±0.9
S.C.R.5	0.5 mg/l BAP+0.5mg/l IAA.	40.0	30	4.0±0.9	4.0±0.3
S.C.R.6	0.5 mg/l KN+ 0.5mg/l IAA.	40.0	25	3.0±0.4	4.0±0.0
S.C.R.7	1.5 mg/l BAP+ 0.5mg/l IBA.	75.0	20	6.5±0.41	7.9±0.1
S.C.R.8	1.0 mg/l KN+0.5 mg/l IBA.	55.0	25	3.8±0.5	5.0±0.9
S.C.R.9	2.0 mg/l BAP+ 0.5 mg/l IAA.	50.0	30	4.0±0.3	5.5±0.4
S.C.R.10	2.0mg/l KN+ 0.5 mg/l NAA.	35.5	25	2.0±0.1	3.0±0.3

Table 3: Effect of MS media and growth regulators either alone or in combination on shoot multiplication from regenerated shoots 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	4.6±0.5	6.2±0.9
S.M.2	0.5 mg/l KN.	40.0	25	3.0±0.08	6.3±0.9
S.M.3	1.0 mg/l BAP+ 0.5mg/l IBA.	75.0	20	7.5±0.41	8.5±0.1
S.M.4	0.5 mg/l KN + 0.5mg/l 0.5 IBA.	50.5	30	4.3±0.1	6.3±0.9
S.M.5	0.5 mg/l BAP + 0.5 mg/l NAA.	65.5	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	5.0.±0.1
S.M.7	1.0 mg/l BAP + 0.5mg/l IAA.	70.0	30	5.0±0.9	6.7±0.6
S.M.8	1.0 mg/l KN + 0.5 mg/l IAA.	40.0	30	3.8±0.5	4.5±0.0
S.M.9	2.0 mg/l BAP + 0.5 mg/l IBA.	60.0	30	3.5±0.9	6.5±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 4: 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.	70.0	20	6.0±0.6
S.R.3	MS +0.5 NAA.	60.2	20	4.3±0.8
S.R.4	MS +1.0 IAA.	50.0	25	3.5±0.0
S.R.5	MS +1.0 IBA.	60.0	20	4.5±0.6
S.R.6	MS +1.0 NAA.	50.0	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.	50.0	30	4.5±0.1
S.R.9	MS +1.5 NAA.	50.0	25	4.0±0.2
S.R.10	MS +2.0 IBA.	30.0	30	2.0±0.2

Fig 1: (a-i) Callus organogenesis.
(a - b) Callus induction from leaf segments of *Spilanthes acmella* on MS+1.5 mg/l 2, 4-D+0.5 BAP mg/l after 30 days.
(c - d) Shoot regeneration from callus of *Spilanthes acmella* on MS +1.5 mg/l BAP +0.5 mg/l NAA after 20 days.
(e - f) Shoot multiplication from regenerated shoots of *Spilanthes acmella* on MS+1.0 mg/l BAP+0.5 mg/l NAA after 20 day.
(g) Root organogenesis from shoots of *Spilanthes acmella* on MS +0.5 mg/l 1BA after 20 days
(h - i) Hardening and Acclimatization of *Spilanthes acmella* plantlets.

Conclusion

In the present study, a fruitful indirect organogenesis protocol was set up for *Spilanthes acmella* through callus formation, shoot regeneration and successful regeneration of new plantlets. The result of this study shows that tissue culture techniques can play an important role in clonal propagation of elite genotypes of *Spilanthes acmella* which has diverse medicinal applications and eventually due to over exploitation and irregular concern this plant is facing local extinction.

In conclusion we demonstrate that protocol developed could be used for conservation of elite germplasm and true to type mass propagation of this herb 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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