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Callus Induction and Plant Regeneration of *Spilanthes acmella* M. an Ornamental and Medicinal Herb

Nasrin Nasr
Assistant Professor, Department of Biology,
Payame Noor University, Tehran, Iran.

(Corresponding author: Nasrin Nasr)
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ABSTRACT: *Spilanthes acmella* M is an important ornamental cum medicinal plant. In order to investigate the effects of different concentrations, different explants, and combinations of growth regulators on callus induction and plant regeneration of *Spilanthes acmella* M. Leaf explants were inoculated onto MS medium supplemented with different concentrations of α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxy acetic acid (2,4-D), indol acetic acid (IAA) and benzyl adenine (BA) alone and auxins in combinations with BA for callus induction. Callus initiation was observed in all media evaluated and the highest callus formation (0.29 ± 0.03 mg) was obtained from explants cultivated in the presence of IAA alone at 10 µM in nodal explant or 15 µM BA + 5 µM 2,4-D (0.33 ± 0.039 mg) in combination. Maximum shoot induction (81 %) and highest number of shoot per callus was obtained from callus induced on 15 µM BA + 5 µM 2,4-D three weeks after transferring the callus to a MS medium supplemented with 10 µM BA + 5 µM NAA. The regenerated plantlets were well rooted by exposing to auxins (NAA, IAA, and IBA) and auxin in combinations with increased level of sucrose. The success of plant tissue culture for in vitro culture of *S. acmella* was encouraged by acclimatization of the plantlets in the greenhouse conditions.

Keywords: Callus induction, plant regeneration, shoot induction, *Spilanthes acmella*.

Abbreviations: NAA = α-naphthalene acetic acid, 2,4-D = 2,4-dichlorophenoxy acetic acid, IAA = Indol acetic acid
BA = Benzyl adenine, IBA = Indole-3-butyric acid

INTRODUCTION

Spilanthes acmella Murr. (Asteraceae), commonly known as Toothache plant, is an important ornamental cum medicinal plant widely distributed to tropical and subtropical region of the world (Yadav and Singh, 2011). Spilanthes species is generally found in the tropical regions of the world like India. *Spilanthes acmella* is acutely threatened plant species. The flowers of this herb when chewed produce a numb sensation in the mouth and thus have been effectively used for the remedy of toothaches, sore mouth, itching, psoriasis, stammering problem and also to regulate the flow of saliva (Jayasinghe, 1994). Some of the efficacies of this herbal product have been scientifically proven. For example, larvicidal activity has been demonstrated in *S. acmella* against Culex quinquefasciatus (Pitasawat *et al* 1998). The flowers have also been shown to have diuretic activity in rats (Ratnasooriya *et al.,* 2004). It has been well documented for its uses as antibacterial, antifungal, and antimalarial activity. *S. acmella* is native to Brazil and is cultivated throughout the year as ornamental plant (Tiwari *et al.,* 2011). *Spilanthes acmella* is a flowering herb in many parts of the world. This fast growing plant is frost sensitive with bright green leaves accented by gold flowers with red inflorescences. This plant makes a most interesting deck plant and grows well as a container plant for the home or greenhouse, in addition to being a specimen plant.

The leaves are used as immunomodulatory, adaptogenic, diuretic, tooth paste, lithotriptic, antiscorbitic, sailagogine, antibacterial, tonic, digestive (Himada and Gomi, 1993) and insecticidal properties due to the presence of bioactive compounds, the isobutylamides (Ramsewak *et al.* 1999). The leaves are reported to contain alkaloids, carbohydrates, pungent amide tannins, steroids, carotenoids, essential oils, sesquiterpenes, amino acids, etc (Nagashima and Nakatani, 1992; Lemos *et al.,* 1991; Nagashima and Nobuji, 1991; Amal and Sudhendu, 1998). A few bioactive compounds have been isolated from S. acmella. Spilanthol has been isolated and is regarded as very highly bioactive due to its insecticidal activity. Another such compound, scopoletin (6-methoxy-7-hydroxycoumarin), has attracted the most attention because of its use in cardiovascular disease, and antitumor and antithyroid treatment (Abyari *et al.,* 2016).

Nowadays, plant tissue culture is a useful tool for the conservation and rapid propagation of endangered plants (Baskaran and Jayabalan, 2008).

Callus induction plays an important role in plant biotechnology for large scale production of medicinal compounds. In addition, callus cultures derived from plant tissues can sometimes produce higher amounts of secondary metabolites (Soorni and Kahrizi, 2015). Since *Spilanthes acmella* L. is an endangered plant species, due to over-exploitation and lack of vegetative propagation methods (Yadav and Singh, 2011), plant regeneration can be a useful tool for conservation of this plant. Formerly, several experiments have been made for in vitro plant regeneration of this plant through organogenesis (Saritha *et al.*, 2002; Haw and Keng 2003; Deka and Kalita 2005; Saritha and Naidu 2008). Also, Singh *et al.* (2009) established an efficient and improved in vitro propagation system for *S. acmella* L. using transverse thin cell layer (tTCL) culture system.

However, there is a serious constraint of recurrent availability of material in mass scale which in turn is needed for extraction of secondary metabolites. Therefore, the objective of the present study was to develop a procedure which allows to obtain a system for callus production giving cell masses that could be used in studies of secondary metabolites as well as to establish an efficient protocol to regenerate plants from callus of *S. acmella*. Present communication reports the callus induction from different parts of plant and plant regeneration of *S. acmella*, which can serve as an alternate effective in vitro technology for commercial production of these important natural compounds.

MATERIALS AND METHODS

A. Plant materials and culture conditions

The seeds of *Spilanthes acmella* (Fig. 1) were surface-sterilized with 0.1% $HgCl_2$ for 7-8 minutes. Following three rinses with autoclaved distilled water, the seeds were placed on half MS-based medium (Murashige and Skoog, 1962) in 250 ml flask with 30 seed in each and germinated under aseptic conditions and were incubated in the incubation room. When the seedlings were 5-10 cm high, leaves were excised from the seedling plants for callus induction.

Callus initiation. The seedlings were removed from the medium for callus induction. The leaf of seedling surface sterilized explants were cut with sterilized razor and cultured in test tubes and culture bottles containing solidified MS callus induction medium supplemented with different combinations of BA, IAA, NAA, 2,4-D and IBA alone and in combination (described in Table 1, 2 and 3). The test tubes or bottles consisting callus culture were incubated in the incubation room with controlled temperature (25±2 ºC) 8 hours light (35 µM.m-2.s-1) and 16 hours dark and humidity about 70%. After incubation period (28 days), the frequency of callus formation and callus fresh weight were determined. The growth of the callus i.e. the biomass increase was measure on the basis of dry weight.

Callus proliferation. In order to obtain and preserve the organogenic callus, the calli were subcultured on another MS callus subculture medium containing MS medium fortified with various concentration of cytokinin (BA) (10 and 15 µM) and auxins (IAA, NAA, 2,4-D) (1, 5, 10 and 15 µM) alone and BA in combinations with auxins. The 500 mg of callus induced from different explants were subcultured separately on nutrient medium supplemented with different cytokinin/auxin combination. In order to measure the increase in callus, weight was recorded before culturing on the proliferation medium. After 28 days, callus weights were again recorded and the difference was calculated.

Shoot induction and multiplication. White compact callus with green globular structures (approximately 300 mg each) growing on optimal growth regulator treatment was used for shoot differentiation. Shoot differentiation was studied using BA (10-15 µM) either alone or in combination with NAA (5 µM) incorporated into MS basal medium supplemented with 30 g/l sucrose and 8 g/l agar. The control treatment received no growth regulator. For all experiments, 12 calli of approximately 300 mg of each were used per treatment with three replications.

Rooting of shoots. Adventitious shoots, were rooted on MS basal medium containing sucrose and auxins (NAA, IAA and IBA) in various concentration. For evaluation of influence of the sucrose concentration along with auxins in the nutrient medium on rooting of *S. acmella* M. seven different rooting media were tested:

1. MS without plant growth regulator.
2. MS with 5µM IAA.
3. Half strength MS without plant growth regulator.
4. Half strength MS with 5µM IAA.
5. Half strength MS + 5µM IAA + 3, 4, 5 % sucrose without plant growth regulator.

Acclimatization of plantlets. The in vitro regenerated plantlets with sufficient roots were transferred to the plastic pots (5 cm) containing a mixture of autoclaved loam and garden soil (1:1). The plants were acclimatized by covering the pots with polythene bags to maintain high humidity for 1 week and irrigated with water in a day twice. After 7 days, 3-4 small holes were made in the bag. This helped the plants adapt to conditions. After 10 days, the acclimated plants were transferred to the greenhouse, and were maintained under natural conditions.

B. Statistical analysis

A completely randomized design with three replications was applied for each experiment using and data were analyzed using SPSS software. Mean comparisons were done by Duncan Multiple Range Test (DMRT) at 5% level of probability.

Fig. 1. *Spilanthes acmella* L.

RESULTS and DISCUSSION

Callus production was induced by all growth regulator treatments and a wide range of variations in callus dry weight depending on culture media formulations were observed between cultures (Table 1, 2 and 3). As depicted in Table 1, among all the growth regulators when used alone, the highest callus fresh weight was obtained when leaf segments were cultivated on media containing BA alone. High concentration of BA (15 µM) resulted in significantly higher dry weight compared to low concentration (10 µM) of BA (0.17 ± 0.3). The callus produced on the said media was dark green and compact.

Within the different auxins when used alone, the highest callus dry weight and extensive callus formation from the leaf explant was recorded in the MS medium supplemented with 10 µM IBA (Table 1). The dry weight of callus induced from leaf explants cultured on MS medium +10 µM IBA was 0.16 ± 0.5 g. The MS medium supplement with IAA, NAA, 2,4-D alone for proliferation and growth of callus respectively was inferior to the effect of IBA alone.

Table 1: Influence of auxins and cytokinin alone or in combination with together on the growth in callus derived from leaf explants of *Spilanthes acmella* M.

MS + PGRs	Moisture (%)	D.W (mg) (Mean ± SE)
10 µM BA	85.05 ±0.62^{a}	0.13 ± 0.017^{bc}
15 µM BA	91.78 ± 0.14 ^{a}	0.20 ± 0.034^{a}
10 µM 2,4-D	91.72 ± 0.08 ^{a}	0.11 ± 0.022^{bc}
15 µM 2,4-D	92.68 ± 0.40 ^{a}	0.06 ± 0. 22^{c}
10 µM NAA	89.55 ± 0.07 ^{a}	0.15 ± 0.012^{ab}
15 µM NAA	90.18 ± 0.09 ^{a}	0.13 ± 0.011^{bc}
10 µM IAA	89.12 ± 0.15 ^{a}	0.16 ± 0.^{015ab}
15 µM IAA	90.44 ± 1.08 ^{a}	0.12 ± 0.034^{bc}
10 µM IBA	94.95 ± 0.25 ^{a}	0.16 ± 0.029^{ab}
15 µM IBA	95.51 ± 0.12 ^{a}	0.17 ± 0.027^{ab}
10 µM BA+1 µM 2,4-D	92.47 ± 0.22 ^{a}	0.19 ± 0.014^{de}
10 µM BA +5 µM 2,4-D	94.94 ± 0.31 ^{a}	0.15 ± 0.005^{ef}
10 µM BA +1 µM NAA	89.29 ± 0.34 ^{a}	0.10 ± 0.006^{fg}
10 µM BA + 5 µM NAA	86.56 ± 0.69 ^{a}	0.27 ± 0.034^{c}
10 µM BA + 1 µM IAA	89.45 ± 0.10 ^{a}	0.19 ± 0.010^{de}
10 µM BA + 5 µM IAA	86.30 ± 0.35 ^{a}	0.27 ± 0.007^{c}
10 µM BA + 1 µM IBA	90.15 ± 1.08 ^{a}	0.05 ± 0.005^{h}
10 µM BA + 5 µM IBA	93.97 ± 0.64 ^{a}	0.15 ± 0.020^{ef}
15 µM BA + 1 µM 2,4-D	92.11 ± 0.65 ^{a}	0.18 ± 0.009^{de}
15 µM BA + 5 µM 2,4-D	93.80 ± 0.31 ^{a}	0.33 ± 0.039^{a}
15 µM BA + 1 µM NAA	88.09 ± 0.44 ^{a}	0.09 ± 0.010^{gh}
15 µMBA + 5 µM NAA	93.16 ± 0.10 ^{a}	0.29 ± 0.030^{bc}
15 µM BA +1 µM IAA	90.92 ± 0.03 ^{a}	0.21 ± 0.008^{d}
15 µM BA + 5 µM IAA	90.78 ± 0.16 ^{a}	0.32 ± 0.008^{ab}
15 µM BA +1 µM IBA	93.32 ± 0.06 ^{a}	0.11 ± 0.006^{fg}
15 µMBA + 5 µM IBA	92.60 ± 0.73 ^{a}	0.18 ± 0.008^{de}

The values represented the mean ± SE calculated on three independent experiments, each based on minimum of 15 replicates. Values followed by the same letter were not significantly different at 5 % level (DMRT).

Inclusion of high concentration of auxins in culture medium resulted in decreasing dry weight of leaf callus. Leaf, nodal and root calli when subcultured on the medium containing NAA alone, were produced root from the surface along with extensive proliferation. On media containing 10 µM IAA, 0.29 ± 0.03 dry callus was achieved with nodal explant which was more than the callus biomass obtained on media fortified with another plant growth regulators. The nodal callus obtained with 2,4-D showed less dry weigh as compare with other auxins. In the study the effect of plant growth regulators on root explants of *S. acmella* for callus induction the medium fortified with 15 µM IBA resulted in maximum callus dry weight (0.13 ± 0.02 g) as compare with other plant growth regulators. Concentration of 10 µM IAA also resulted in considerable growth of callus (0.12 ± 0.02 g) by root explants. Among the callus produced from root, nodal and leaf explant with plant growth regulator alone, the maximum fresh weight and dry weight of the callus was obtained with leaf explant on MS medium fortified with 10 µM IAA.

Table 2: Influence of auxins and cytokinins alone or in combination with together on the growth and callus derived from nodal explants of *Spilanthes acmella* M.

MS + PGRs	Moisture (%)	D.W (mg) (Mean ± SE)
10 µM BA	88.51 ± 0.83^{a}	0.19 ± 0.02^{bc}
15 µM BA	93.40 ± 1.19^{a}	0.11 ± 0.05^{e}
10 µM 2,4-D	92.93 ± 0.02^{a}	0.20 ± 0.02^{bc}
15 µM 2,4-D	92.89 ± 1.12^{a}	0.17 ± 0.01^{d}
10 µM NAA	89.73 ± 0.71^{a}	0.25 ± 0.01^{ab}
15 µM NAA	89.38 ± 0.20^{a}	0.12 ± 0.01^{e}
10 µM IAA	90.60 ± 0.01^{a}	0.29 ± 0.03^{a}
15 µM IAA	90.81 ± 0.23^{a}	0.27 ± 0.00^{a}
10 µM IBA	93.39 ± 0.97^{a}	0.20 ± 0.03^{bc}
15 µM IBA	95.02 ± 0.78^{a}	0.21 ± 0.01^{bc}
10 µM BA+1 µM 2,4-D	93.97 ± 0.20^{a}	0.09 ± 0.012^{ef}
10 µM BA +5 µM 2,4-D	93.20 ± 1.25^{a}	0.07 ± 0.014^{f}
10 µM BA +1 µM NAA	93.01 ± 0.73^{a}	0.11 ± 0.022^{ef}
10 µM BA + 5 µM NAA	92.29 ± 1.63^{a}	0.19 ± 0.030^{bcd}
10 µM BA + 1 µM IAA	90.11 ± 0.08^{a}	0.20 ± 0.013^{bcd}
10 µM BA + 5 µM IAA	90.47 ± 0.02^{a}	0.29 ± 0.021^{a}
10 µM BA + 1 µM IBA	81.03 ± 0.03^{a}	0.14 ± 0.014^{de}
10 µM BA + 5 µM IBA	75.00 ± 0.89^{a}	0.14 ± 0.008^{de}
15 µM BA + 1 µM 2,4-D	94.10 ± 0.64^{a}	0.06 ± 0.012^{f}
15 µM BA + 5 µM 2,4-D	96.33 ± 0.39^{a}	0.18 ± 0.022^{bcde}
15 µM BA + 1 µM NAA	88.57 ± 0.99^{a}	0.10 ± 0.019^{ef}
15 µMBA + 5 µM NAA	88.74 ± 0.68^{a}	0.09 ± 0.006^{ef}
15 µM BA +1 µM IAA	90.62 ± 0.23^{a}	0.18 ± 0.012^{bcde}
15 µM BA + 5 µM IAA	90.40 ± 0.04^{a}	0.25 ± 0.027^{ab}
15 µM BA +1 µM IBA	85.01 ± 0.02^{a}	0.26 ± 0.027^{ab}
15 µMBA + 5 µM IBA	87.18 ± 0.37^{a}	0.23 ± 0.007^{abc}

The values represented the mean ± SE calculated on three independent experiments, each based on minimum of 15 replicates. Values followed by the same letter were not significantly different at 5 % level (DMRT).

Addition of 2,4-D alone in MS medium did not promote growth of callus as compared to combination of BA with 2,4-D. Incorporation of BA with 2,4-D enhanced the proliferation and growth of leaf callus. The maximum dry weight of the callus was obtained on MS medium containing 15 BA + 5µM 2,4-D. The growth of leaf callus in terms of dry weight was low on MS medium enriched with 2, 4 -D alone compare to that of medium supplement with BA+ 2,4-D. The results showed that high level of 2,4-D in conjugation with BA increased the callogenesis potential of leaf explant as compared to low concentration of 2,4-D in conjugation with BA. The dry weight of callus derived from leaf was 0.33 ± 0.04 g, nodal was 0.18 ± 0.02 g and the root derived callus was 0.09 ± 0.02 g.

Presence of 5 µM IAA with 15 µM BA in MS medium (0.32 ± 0.04) was effective for obtaining considerable amount of callus. The callus induced from root, nodal and leaf explants on MS supplemented with IAA (5 µM) in combination with BA showed more growth as compared to those obtained on MS medium supplemented with IAA, NAA, BA and 2,4-D individually. In the case of NAA, the results showed addition of 5 µM NAA to BA containing medium was effective for proliferation of leaf callus but it wasn't effective for growth of nodal and root callus.

As results, inclusion of IBA with BA did not promote proliferation and growth of callus and addition of IBA in combination with BA wasn't effective for callus growth and increasing callus biomass in all explants.

The calli obtained on MS fortified with 15 µM BA in combination with 5 µM NAA were more to that obtained with NAA alone containing media.

The results showed that proliferation of callus in media with different growth regulator with different concentration of phytohormones was varied and was mainly depended on growth regulator type and present of cytokinin in media containing auxin. BA when supplemented with the various auxins induced extensive proliferation, whereas the growth of callus was slower on MS supplemented with BA or auxins individually. Among the auxins used IBA was superior to that of 2,4-D, NAA and IAA for callus induction and proliferation. 2,4-D showed less callus proliferation as compare to that of other tested auxins. On the results, the growth and proliferation of leaf explant callus was comparatively superior to nodal and root explants. Based on the results obtained in the various experiments, the MS medium fortified with 15µM BA+ 5µM 2,4-D were selected for further growth and maintenance of the callus.

Table 3: Influence of auxins and cytokinins alone or in combination with together on the growth of callus derived from root explants of *Spilanthes acmella* M.

MS + PGRs	Moisture (%)	D.W (mg) (Mean ± SE)
10 µM BA	85.47 ± 0.85^{a}	0.11 ± 0.022^{b}
15 µM BA	90.68 ± 0.84 ^{a}	0.08 ± 0.021^{c}
10 µM 2,4-D	91.67 ± 0.80 ^{a}	0.09 ± 0.015^{c}
15 µM 2,4-D	89.57 ± 1.49 ^{a}	0.08 ± 0.026^{c}
10 µM NAA	86.81 ± 1.36 ^{a}	0.08 ± 0.023^{c}
15 µM NAA	88.69 ± 0.93 ^{a}	0.05 ± 0.013^{d}
10 µM IAA	87.81 ± 0.88 ^{a}	0.12 ± 0.021^{b}
15 µM IAA	89.58 ± 0.91 ^{a}	0.09 ± 0.017^{c}
10 µM IBA	94.08 ± 1.09 ^{a}	0.11 ± 0.019^{b}
15 µM IBA	94.64 ± 0.94 ^{a}	0.13 ± 0.027^{a}
10 µM BA+1 µM 2,4-D	95.45 ± 0.08 ^{a}	0.04 ± 0.008^{gh}
10 µM BA +5 µM 2,4-D	96.25 ± 0.46 ^{a}	0.03 ± 0.009^{h}
10 µM BA +1 µM NAA	92.56 ± 0.27 ^{a}	0.11 ± 0.013^{def}
10 µM BA + 5 µM NAA	89.79 ± 0.30 ^{a}	0.15 ± 0.018^{cd}
10 µM BA + 1 µM IAA	92.23 ± 0.15 ^{a}	0.09 ± 0.020^{efg}
10 µM BA + 5 µM IAA	90.53 ± 0.00 ^{a}	0.12 ± 0.015^{def}
10 µM BA + 1 µM IBA	92.56 ± 0.96 ^{a}	0.24 ± 0.021^{a}
10 µM BA + 5 µM IBA	92.38 ± 0.57 ^{a}	0.13 ± 0.011^{def}
15 µM BA + 1 µM 2,4-D	94.1 ± 0.19 ^{a}	0.05 ± 0.014^{gh}
15 µM BA + 5 µM 2,4-D	95.48 ± 0.26 ^{a}	0.09 ± 0.020^{efg}
15 µM BA + 1 µM NAA	90.54 ± 0.85 ^{a}	0.21 ± 0.016^{ab}
15 µMBA + 5 µM NAA	91.01 ± 0.00 ^{a}	0.08 ± 0.016^{fgh}
15 µM BA +1 µM IAA	90.95 ± 0.30 ^{a}	0.14 ± 0.004^{de}
15 µM BA + 5 µM IAA	88.80 ± 0.55 ^{a}	0.21 ± 0.005^{ab}
15 µM BA +1 µM IBA	92.63 ± 0.30 ^{a}	0.20 ± 0.013^{ab}
15 µMBA + 5 µM IBA	92.75 ± 0.75 ^{a}	0.19 ± 0.011^{bc}

The values represented the mean ± SE calculated on three independent experiments, each based on minimum of 15 replicates. Values followed by the same letter were not significantly different at 5 % level (DMRT).

White compact calli with green globular structures obtained from leaf explants growing on optimal growth regulator treatment were selected for plant regeneration. Plant regeneration of S. acmella was achieved in two steps. The compact calli were first cultured on MS regeneration medium I (MS medium containing different concentrations of BA alone or in combination with 5 µM NAA, Table 3). MS basal medium alone failed to induce shoot differentiation from the callus and also did not promote further growth. Addition of BA markedly increased the growth rate and the number of regenerated plants. However, green globular structures of whitish green compact leaf callus differentiated to form complete shoots within 20 days on medium supplemented with BA (5.0-20.0 µM) either alone or in combination with NAA (5.0 µM). When BA alone was supplemented in the culture medium, there was low frequency of adventitious shoot bud differentiation. Within different concentrations of BA, maximum number of adventitious shoot-bud differentiation (2-3 shoots per explant) was obtained on MS medium containing 15 µM BAP after 28 days of culture. Both shoot regeneration frequency and regenerated shoot number increased with the increase in BA concentration from 5 to 15 µM and it was effective in inducing shoot regeneration, with 48.55% of the calli forming shoots. The percentage of calli forming shoots, the number of regenerated shoots per callus, varied according to the growth regulator treatment (Table 4).

MS medium containing BA alone (5-20 µM) resulted in shoot differentiation, but lower frequency as well as shoot-forming index than with the addition of NAA (5 µM), suggesting the role of cytokinin to auxin ratio in shoot differentiation in S. acmella. The optimum growth regulator combination for shoot differentiation from white compact leaf callus was found to be 15 M BA and 5 µM NAA. In this medium, the highest percentage of callus forming shoots (66.66 %) and highest number of shoots per callus (5.20 ± 0.52) was also noted (Table 4).

Table 4: Effect of BAP alone or in combination with NAA on organogenesis from leaf derived callus of *Spilanthes acmella* M.

MS+PGRs (µM)		Organogenic callus (%)	No. of shoot / explant (Mean ± SE)
BA	NAA		
0	0	0.0	0.0
5	0	16.68^{e}	1.68 ± 1.33^{c}
10	0	29.16^{d}	2.10 ± 0.91^{c}
15	0	48.55^{b}	3.20 ± 0.40^{bc}
20	0	38.66^{c}	2.80 ± 1.00^{bc}
5	5	19.16^{d}	2.10 ± 0.86^{c}
10	5	45.83^{b}	3.96 ± 0.37^{ab}
15	5	66.66^{a}	5.20 ± 0.52^{a}
20	5	20.83^{e}	4.15 ± 0.97^{ab}

The values represent the mean±SE calculated on three independent experiments, each based on minimum of 15 replicates. Values followed by the same letter were not significantly different at 5% level (DMRT).

In the second step of plant regeneration, the differentiated calli were transferred from MS regeneration medium I to MS regeneration medium II (MS medium containing BA alone in the concentrations of 5-20 ?M for elongation and multiplication). This medium was found to be essential for successful plant regeneration, whereas shoot elongation were less at all without the medium replacement.

The highest plant regeneration after transferring to second medium achieved with 10 µM BA. The effects of various concentration of sucrose in the nutrient medium on rooting of *S. acmella* M. are depicted in Table 5. In present study five different rooting media were evaluated for root induction from shoots of *S. acmella* M. Rooting was observed in all tested media within 3-5days of culture.

Table 5: The effects of various concentration of sucrose in the nutrient medium on rooting of *Spilanthes acmella* M.

Nutrient medium type	Shoot producing Roots (%)	No. of roots / shoot (Mean ± SE)
MS	92 ± 0.0	16.66 ± 1.5
1/2 MS	100 ± 0.0	21.12 ± 1.1
MS + 5µM IAA	100 ± 0.0	31.40 ± 3.2
1/2 MS + 5µM IAA	100 ± 0.0	37.44 ± 1.2
1/2 MS + 5µM IAA + 3% Sucrose	100 ± 0.0	42.00 ± 1.9
1/2 MS + 5µM IAA + 4% Sucrose	100 ± 0.0	47.50 ± 1.4
1/2 MS + 5µM IAA + 5% Sucrose	100 ± 0.0	51.85 ± 1.8

As the results, response of shoot explants for rooting was varied with composition of basal media and increasing of sucrose concentration in the basal medium. The percent frequency of shoots producing roots (100%) from stem explant and number of roots per shoot (22.12 ± 1.2) on half strength MS medium without plant growth regulator were superior as compare with percent frequency of shoots producing roots (92 ± 0.0%) from shoot explant and number of roots per shoot (16.66 ± 1.5) on MS medium without plant growth regulator. The roots obtained on the half MS medium more branches as compare with those achieved on full MS medium. Since half MS medium indicated good results as compare to MS medium for rooting of shoot explants, for next experiments half MS medium was used as rooting medium for study the effects of sucrose concentration on rooting of shoot explants of *S. acmella* M. Addition of 5 µM IAA enhanced formation of root in both media containing half strength MS and full strength MS. Full strength MS media containing 5 µM IAA induced 31.40 ± 3.2 means number of roots in 100% of shoots, while the media supplemented with half strength MS + 5 µM IAA induced 37.44 ± 1.2 means number of roots in 100% of shoots. Increasing of sucrose concentration improved the response of shoots for rooting on half MS medium during 4 weeks of inoculation. Among the various concentration of sucrose added to half MS medium, half MS medium containing 5% sucrose showed the maximum number of roots (51.85 ± 1.8) in 100 ± 0.0 of explants.

The plantlet obtained on half strength MS + 5 µM IAA + 4% sucrose with 47.50 ± 1.4 mean number of root per explant was also showed a considerable rooting but there was significant difference in number of roots per shoot between this treatment and full strength MS + 5µM IAA + 5% sucrose.

Among the different concentration of sucrose in half strength MS medium, ½ MS + 5µM IAA + 5% sucrose was found to be effective in induction of rooting in the shoots of *S. acmella* M. After acclimatization for one month, the plantlets derived from mature explants were transferred to pots. About 95% of survival rate was observed in in vitro produced plants. 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. The plants showed normal flowering within eight weeks without any morphological variations.

The success of in vitro techniques largely depends on the availability of efficient and robust tissue culture protocols. In order to obtain high percentage of organogenic calli in the tissue culture system, timely and frequent subculture (i.e., medium innovation) with the selection of various growth regulators and their proper combinations are necessary (Tang *et al.* 2003). This work established a step-wise protocol for in vitro multiplication of *S. acmella*.

Leaf, nodal and root explants of *S. acmella* were cultured on MS media containing different concentrations of 2,4-D, NAA, IAA and BA alone and auxins in combination with BA. Data were analyzed after four weeks of culture and the results showed that there was a wide range of variations in callus dry weight and degree of callus formation depending on culture media formulations (Table 1, 2, 3). Callus initiation on cut ends of in vitro cultured explants could be observed in all hormone combinations after 7-20 days. However, the explants cultured on MS medium without growth regulators did not produce any callus. Within the different plant growth regulators when used alone, the highest degree (0.13 ± 0.017 g) for compact green callus formation from the leaf explant was recorded in the MS medium supplemented with 15 µBA (Table 1). Similarly, dos Santos *et al.,* (1990) reported callus formation of D. insignis in all tested concentration of 0.01 to 2.0 mg/l BA.

As is well known, 2,4-D promotes cell division and is mostly employed for callus induction in tissue culture studies (Borkird *et al.,* 1986). All explants cultured on medium fortified with 2,4-D alone, produced optimum amount of callus. According to the results, the medium fortified with 10µ IAA and 15µIBA showed highest callus formation in nodal and root explants respectively. In order to evaluate the effect of BA in combination with auxins (2,4-D, NAA, IAA and IBA) on callus induction, the results were recorded for leaf, nodal and root explants cultured on MS medium supplemented with different combinations of BA and auxins (Table 1, 2, 3). Callus initiation was observed between 7-15 days depending on concentrations and combinations. The best result for callus formation in leaf explant was obtained when 15µM BA was used in combination with 2,4-D at 5 µM (Table 1). This combination was not suitable for callus proliferation of root explant. This difference might be due to sensitivity of the root cells to 2,4-D. In leaf explants among the auxins 2,4-D was superior for callus induction in combination with BA. IAA and NAA were more effective for callus induction than IBA when used in combination with cytokinin. In combination of cytokinin with auxins inclusion of high concentration of BA (15 µM) was more effective compare to that of 10µM BA. The medium supplemented with 15 µM BA + 5 µM IAA and 10 µM BA + 5 µM IAA induced more callus in terms of fresh weight and dry weight in nodal and root explants respectively. The effective use of IAA (0.5 mg/L): BA (1.0 mg/L) combination for induction and growth of callus in stem explant of *D. innoxia*, was reported by Zayed *et al.* (2006). The results of present investigation revealed that the exogenous addition of auxins together with BA in medium was effective and promoter for callus induction and proliferation of *S. acmella* callus. The earlier reports (Saritha *et al.* 2002, Bais *et al.* 2002) have conformed the effect of combination of BA and NAA for inducing morphogenic callus in *Spilanthes* spp. Thus, it is approved that in *Spilanthes* spp., the combination of the aforementioned two growth regulators is absolutely necessary for in vitro regeneration. The results of present investigations also is in agreement with those of Agrawal and Sardar (2006, 2007) in *Cassia angustifolia*, Dhar and Joshi (2005) in *Saussuraea* spp., Azad *et al.* (2005) in *Phellodendron* spp. and Echeverrigaray *et al.* (2000) in *Chaememelum* spp., where the combination of NAA+BA proved optimum for shoot differentiation from leaf explants.

It was found that a balanced ratio of the two growth regulators controlled the induction of shoot buds from callus tissue in vitro. A high auxin to cytokinin content resulted in root formation, whereas the reverse promoted shoot bud development. The shoot buds obtained from leaf explants on MS+ 1 µM NAA+10 µM BA did not elongate on the same medium even after subculture. Such shoot buds, however, elongated on BA alone. This proves that NAA is essential for initial induction of shoot buds, but its continuous presence is inhibitory for elongation.

Among different concentration of BA, 10 µM BA alone proved effective for elongation of shoot buds and formation of multiple shoots. The efficiency of BA in in vitro regeneration studies has been reported by several earlier workers (Agrawal *et al.* 2002, Zhang *et al.* 2006, Agrawal and Sardar 2006, 2007, Kumar *et al.* 2005).

It has been suggested that the shoot bud differentiation is the results of relationship between the exogenous and endogenous auxins and cytokinin proportion and influence of other factors. In this investigation the callus derived from root and nodal explant cultured on medium containing various concentration of growth regulators failed to shoot formation and only leaf explant indicated shoot formation on medium fortified with BA. The results of the present investigation and earlier reports on other medicinal plants revealed that the explant type, type and concentration of plant growth regulators are effective in morphogenetic response of the callus of *S. acmella*.

Hardening is a very essential and crucial step for micropropagation. Plantlets with 4-6 fully expanded leaflets and well developed roots were successfully acclimatized inside the culture room and eventually established in soil. The survival of the plantlets after transfer to vermicompost: soilrite mix (1:1) was high, and 95% of the plants transferred to garden soil survived. The regenerated plants did not show detectable variation in morphology and growth characteristics when compared with that of donor plant. Under field conditions also, the plants registered normal growth with straight and sturdy stem. All plants grew to maturity producing flowers.

CONCLUSION

In this study, a protocol was established for callus induction and plant regeneration from the organogenic calli. In conclusion, calli were successfully induced from all three explants and the procedure for plant regeneration from induced calli has been developed. The analysis of data indicates that proliferation of callus in media with different growth regulator with different concentration of phytohormones was varied and the callus induction was mainly depended on growth regulator type and present of cytokinin in media containing auxin. However if multiplication of selected clones for further studies is required, leaf explants could be cultured on MS medium supplemented 15 µM BA + 5 µM 2,4-D. Organogenesis in *S. acmella* M. proved to be controlled by the ratio of cytokinin /auxin in the medium. Our result showed that the medium supplemented with only BA induced shoots from compact callus in less frequency whereas higher percentage of shoot proliferation obtained with callus explants on 10 µM BA+1 µM NAA containing medium. Probably presence of auxin in less quantity played a certain role in enhancing the shoot formation process. As the results, response of shoot explants for rooting was varied with composition of basal media and increasing of sucrose concentration in the basal medium. Increasing of sucrose concentration improved the response of shoots for rooting on half MS medium during 4 weeks of inoculation.

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