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RESEARCH ARTICLE

EFFICIENT PLANT REGENERATION VIA ORGANOGENESIS IN *Spilanthes acmella* M. AN IMPORTANT MEDICINAL PLANT

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ABSTRACT

Micropropagation and organogenesis successfully achieved in S*pilanthes acmella* M. is belongs to family *Asteraceae* and commonly known as Akarkara. Multiple shoots induced from nodal explants on Murashinge and Skoog (MS) medium supplement with various growth regulators BA and Kin alone. The highest shoot regeneration frequency was recorded on MS medium supplement with 5 µM BA (97.3 ± 1.2%), with highest number of multiple shoots (12.2 ± 0.9). *In vitro* raised shoots were excised and implanted on MS half strength medium supplement with various concentrations of auxins (NAA and IAA, 1.25 – 15 µM) to produce roots. The maximum frequency of roots obtained on MS medium fortified with 5 µM IAA (31.40 ± 3.2). The regenerated plantlets were successfully transferred into the pots containing sterilized soil and sand (3:1) combination.

INTRODUCTION

Spilanthes acmella Murr. (Asteraceae) commonly known as Akarkara or toothache plant is widely distributed in the tropical and sub-tropical regions including America, North Australia, Africa, Malaya, Borneo, India and Sri Lanka (Jansen, 1981). In India, it is confined to South India, Chhatisgarh and Jharkhand (Anonymous, 1989). The flowers and leaves of this plant have been used as traditional medicine for stammering, toothache, stomatitis and throat complaints. It has potent diuretic activity and the ability to dissolve urinary calculi. It exhibits antimalarial properties as well (Burkill, 1966; Singh, 1995; Ramsewak *et al.*, 1999; Pandey and Agarwal, 2009). A number of constituents had been isolated from the *S. acmella* M. for example, spilanthol, isobutylamides (Gokhale and Bhide, 1945; Ramsewak *et al*., 1999) phenolics (vanillic acid, *trans*-ferulic acid and *trans*isoferulic acid), coumarin (scopoletin, and triterpenoids like 3- acetylaleuritolic acid, β-sitostenone, stigmasterol and stigmasteryl-3-*O*-β-D-glucopyranosides, in addition to a mixture of stigmasteryl and β -sitosteryl-3-*O*-β-D-glucopyranosides) (Prachayasittikul *et al.*, 2009). Spilanthol, the most active antiseptic alkaloid extracted from this plant, is found effective at extremely low concentrations against blood parasites, and indeed is a poison to most invertebrates while remaining harmless to warm-blooded creatures. The leaves are used to treat bacterial and fungal skin diseases. Due to these medicinal values, the plant is being over-exploited in recent years. In addition, the efficiency of reproduction is also found to be less due to its low seed germination and viability and lack of vegetative propagation methods.

MATERIALS AND METHODS

Plant Material and Surface Sterilization

Seeds were surface sterilized by 1 % *Teepol* for 15 min followed by immersion in 70% ethanol for 1 min and in 0.1 % mercuric chloride for 10 min, and then rinsed thoroughly with sterile distilled water. Disinfected seeds were inoculated in the MS medium without growth hormones to raise aseptic seedlings (Fig.1- a).

Culture conditions

The leaf explants were cultured in conical flasks (Borosil Glass Works, Mumbai, India) containing 40 ml medium per flask. The pH of the media was adjusted to 5.8 before autoclaving. The nodal explants were inoculated in the MS medium fortified with different cytokinins (BA and Kin) in various combinations. The number of shoots formed and shoot regeneration frequency of the explant were recorded after two months of inoculation.

Rooting of Shoots

In vitro developed shoots measuring 2– 2.5 cm in length were excised, given an oblique cut at the base to increase the surface area of absorption for the nutrients from the medium and cultured on half strength MS medium either alone or supplemented with auxins, NAA, IAA or IBA (1.25-15 µM). Half strength MS basal medium served as the control. Twelve replicates were used per treatment and the experiment was repeated twice. Mean root length and root number were recorded on the 30th day of the culture.

Acclimatization and Transfer of Plantlets to Soil

Healthy plantlets with well-developed roots were removed from the culture medium and were washed gently under running tap water. They were treated with 1% Bavistin (BASF, Mumbai, India) solution to prevent any fungal infection before being transferred to plastic pots (5-cm diameter). Subsequently, they were covered with polythene bags to maintain the humidity. The plantlets were irrigated with distilled water. The potted plantlets were maintained inside the culture room (Fig.1-f).

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Fig. 1. Shoot formation from nodal explants and micro propagation of *Spilanthes acmella*: a-Seed germination on half MS medium after 40 days. b- Shoot formation on MS + 5 µM BA after 30 days. c- Shoot formation on MS +7.5 µM BA after 30 days. d- Shoot induction on MS + 10 µM Kin after 45days. e- Root induction from the *in vitro* regenerated shoots of *S. acmella* M. f- A hardened micropropagated plant, 2 weeks after transfer to soil.

RESULTS

The morphogenetic response of the nodal explant of *Spilanthes acmella* M. to various concentrations of Kin and BA is summarized in Table. 1 and Fig.1 b,c,d. In the MS medium without any growth regulator, the nodal explants remained creamish green with development of single axillary shoot but the caulogenic response was in the form of elongation of shoot arising from the axillary bud in the nodal segments. The percentage frequency of nodal explant producing shoot on MS growth regulator free medium was 20.0 ± 1.3%. The rest of the nodal explant survived for two to three weeks and eventually died in the fourth week of culture. Nodal explants cultured on MS media fortified with cytokinins alone induced multiple shoots at a more frequency (Table. 1). Addition of BA in the nutrient medium improved the shoot regeneration potential in nodal explants, but shoot

Table 1. Effect of cytokinins alone on shoot multiplication in nodal and root explants of *Spilanthes acmella* M.

MS+PGRs (µM)		Root	Nodal	
		Shoot Regeneration (%)	Shoot Regeneration (%)	No. of Shoot / explants (Mean ± SE)
	0.0	0 C	20.0 ± 1.3^{i}	1.11 ± 0.11^{ef}
BA	2.5	0 C	76.0 ± 1.6^{e}	5.40 ± 0.50^{c}
	5.0	0 C	97.3 ± 1.2^{a}	12.20 ± 0.90^{a}
	7.5	0 C	90.2 ± 1.3^{d}	10.40 ± 0.50^{b}
	10.0	0 C	94.8 ± 2.2^{b}	4.07 ± 0.60^{d}
	12.5	0 C	90.4 ± 1.2^{d}	6.00 ± 0.80^{c}
	2.5	0 C	92.2 ± 1.7^{c}	2.05 ± 0.14^{e}
Kin	5.0	0 C	96.0 ±1.4^{ab}	2.05 ± 0.50^{e}
	7.5	0 C	76.8 ± 4.9^{e}	2.00 ± 0.00^{e}
	10.0	0 C	66.2 ± 2.8^{f}	2.17 ± 0.20^{e}
	12.5	0 C	92.6 ± 1.2^{c}	2.16 ± 0.70^{e}

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) C: Callus formation

Table 2. Influence of auxins on the root induction in the *in vitro* regenerated shoots of *Spilanthes acmella* M.

MS+PGRs (µM)		Root Regeneration (%)	No. of Root / explants (Mean ± SE)
NAA	1.25	100 ± 00^{a}	18.50 ± 1.8^{cde}
	2.5	100 ± 00^{a}	19.16 ± 2.3^{cde}
	5	100 ± 00^{a}	30.50 ± 2.8^{ab}
	10	100 ± 00^{a}	24.28 ± 2.3^{abcd}
	15	100 ± 00^{a}	22.37 ± 2.2^{abcde}
IAA	1.25	100 ± 00^{a}	29.50 ± 1.9^{ab}
	2.5	100 ± 00^{a}	26.50 ± 2.9^{abc}
	5	100 ± 00^{a}	31.40 ± 3.2^{a}
	10	100 ± 00^{a}	27.55 ± 3.6^{abc}
	15	100 ± 00^{a}	29.33 ± 1.6^{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). *The root formation was associated with callus formation

regeneration and the number of shoot per explant varied with the concentration of BA. At the low concentration of BA (2.5 µM) 5.40 ± 0.50 shoots bud were produced in 76.0 ± 1.6% of explants, which grew slowly and attained maximum length of 3.5-4.0 cm within four weeks after initiation of culture. With addition increased level of BA up to 5 µM, the shoot regeneration frequency of the explant and number of shoots per explant were increased. The maximum shoot regeneration frequency of the explants was 97.3 ± 1.2% and mean number of shoots per explant were 12.20 ± 0.90 on medium supplemented with 5 µM BA. The regenerated shoots were healthier, grew vigorously and attained height slightly more than that of the shoots produced at low concentration. Increasing the level of BA induced multiple shoot formation directly from nodal explant. Higher level of BA (7.5-12.5 µM) caused slight callusing from the cut end of the explant in first two weeks of culture. The average number of shoot was in the range of 6.82±0.1 at these concentrations of BA. Therefore, at high concentration of BA the frequency of explants producing shoots and number of shoots per explant was decreased.

Addition of 2.5 µM Kin to the MS medium induced shoots regeneration from nodal explants within two weeks. Shoot formations were induced after two weeks of culture in about 92.2 ± 1.7 to 96.0 ±1.4 of nodal explant on MS fortified with low levels of Kin (2.5-5.0 µM). Usually 1-2 shoots were developed per explant without callusing at the cut end and on surface of explants. Increase in the level of Kin (7.5 and 12.5 µM) decreased the percentage frequency of the explant producing shoots. The maximum number of shoots per explant (2.17 ± 0.20) was observed on MS + 10.0 µM Kin. From these experiments it was evident that the MS medium with 5µM BA could be the best for the production of shoots from nodal explant of *S. acmella* M.

Rhizogenesis

The data on the effect of various concentration of auxins (NAA and IBA, 1.25 – 15 µM) on root induction from the *in vitro* regenerated shoots of *S. acmella* M. are recorded in Table. 2, Fig. 1.e. Rooting was induced in the excised *in vitro* shoots on half strength MS basal medium with different auxins in various concentrations (IBA or NAA). Of all the combinations of auxin tried, MS (1/2) + 5.0 µM IAA proved best for differentiating an average of 31.40 ± 3.2 roots per shoot in 100 % shoots Table. 2. At higher concentrations of IAA (10 or 15 µM), though large number of roots were induced, there was extensive callusing at basal end of shoots which hindered its further growth. On NAA supplemented MS (1/2) medium, roots formed were very slender and thin. Thus, MS (1/2) + 5.0 µM IAA was selected as the optimum medium for rhizogenesis.

DISCUSSION

In the present study, caulogenesis was achieved from nodal explant of *S. acmella* M. on MS medium supplemented with various concentrations of BA and Kin. In this study addition of 5 µM BA in MS medium indicated highest shoot regeneration (97.3 ± 1.2%) with mean number of 12.20 ± 0.90 shoots per explant. Nodal segment explants remained green and fresh and the frequency of bud break was low (20 ± 1.3%), single shoot regeneration was observed on growth regulator free MS medium (control). Similar observation was noted in seedling explant of *Peganum harmala* L. a member of Zygophyllaceae (Saini and Jaiwal, 2000; and Khawar *et al.*, 2005). All the concentrations of BA and Kin facilitated shoot bud differentiation. The results indicated that BA was more efficient than Kin in terms of percent regeneration, number of shoots and shoot length. Among the various concentrations of BA and Kin tested, 5.0 µM BA showed the highest shoot regeneration frequency (97.3 ± 1.2 %), number of shoots (12.20 ± 0.90). At the same concentration Kin produced 2.05 ± 0.5 shoots in 96.0 ±1.4 % cultures (Table. 1). Similar observations have also been reported by Yadav and Singh (2010). The different effects of BA and Kin on shoot growth might be due to different mode of action of BA and Kin in shoot development. Like other cytokinins, both BA and Kin are known to promote cell division and cell expansion in plant development. However, suitable cytokinin types and concentrations to stimulate shoot formation and growth vary depending on plant species. Differences in the activity of cytokinins can be explained by their various translocation rates to meristematic

regions and metabolic processes, in which the cytokinins may be degraded and conjugated with physiologically inert compounds, like sugars or amino acids (Kaminek, 1992). Many studies show that kinetin is effective in stimulating shoot elongation but ineffective for shoot multiplication. In contrary, BA is known to be very effective in stimulating shoot multiplication rather than inducing shoot elongation (Bon *et al.*, 1998; Sinha *et al*., 2000; Rajeswari and Paliwal, 2006). The promotive role of BA on shoot proliferation of nodal cuttings has been previously reported in many plant species, such as in various species of *Albizia* (Bon *et al.*, 1998; Sinha *et al*., 2000; Rajeswari and Paliwal, 2006), in *Acacia mangium* (Bon *et al.*, 1998), in *Bacopa monniera* (Tiwari *et al.*, 2001) and in pear (Kadota and Niimi, 2003). Also the effectiveness of BA on shoot proliferation of nodal explant has been well documented in *Schinopsis balansae* (Sansberro *et al.*, 2003), *Holarrhena antidysenterica* (Kumar *et al*., 2005), *Eclipta alba* (Ray and Bhattacharya 2008), *Nelumbo nucifera* (Shou *et al*. 2008), and *Centaurea ultreiae* (Mallo´n *et al.* 2010). At the lower concentrations of BA and Kin, growth was normal while at higher concentration callus formation was induced as recorded in *Dioscorea bulbifera* (Uduebo, 1971), *Dioscorea alata* and *Dioscorea rotundata* (Mantell *et al*., 1978), *Dioscorea oppositifolia* and *Dioscorea pentaphylla* (Poornima and Ravishankar, 2007). Our experimental results showed that the addition of BA and kinetin affected the formation of shoot per explant. The addition of kinetin alone induced a low average numbers of shoot per explant and decreased the average numbers of shoot per explant. As results among different concentration of BA, low concentration was optimal for shoot multiple from nodal segment of *S. acmella* M.

Conclusion

Regeneration potential of nodal segments was explored on MS medium supplemented with various concentrations of BA and Kin (0.0 – 12.5 µM) alone. All the concentrations of BA facilitated shoot bud differentiation. Among the various concentrations of BA and Kin tested, 5.0 µM BA showed the highest shoot regeneration frequency (97.3 ± 1.2%), number of shoots (12.2 ± 0.9) and shoot length (4.1 ± 0.27). At the same concentration Kin produced 2.05 ± 0.5 shoots in 75% cultures respectively. It was found that MS containing 5.0 µM of Kin induced maximum shoot proliferation in 96.6% nodal explants within 8–9 days. Of the two different cytokinins tested (BA and Kin), BA at 5.0 µM was the most effective. The results indicated that the shoot regeneration of nodal explants occur at low concentration of cytokinins (BA and Kin) while high concentration of tested cytokinins (BA and Kin) induced callus formation at cut end of nodal explants. On the medium supplemented with different concentration of auxins IAA and NAA (1.25 - 15.0 µM), all shoot explants responded for rhizogenesis. Of the two auxins supplement used to induce root formulation from nodal explants (IAA and NAA), IAA was most effective when added to MS medium. This induced great frequency (100 %) of root induction, the maximum number (31.4 ± 3.2) of roots per shoot and the root length (3.0 ± 0.2 cm). At high level of IAA (10 µM), the frequency of explant responding for rhizogenesis and number of roots per explant declined. Also at these concentrations of IAA callus was produced from the cut end of nodal explants in the first or two weeks of culture. The number of roots per explant on MS medium supplemented with 1.25 µM NAA and 1.25 µM IAA was 18.5 ± 1.8 and 29.5 ± 1.9 respectively.

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