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IN VITRO PROPAGATION OF *SPILANTHES MAURITIANA* DC., AN ENDANGERED MEDICINAL HERB, THROUGH AXILLARY BUD CULTURES

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Summary

Spilanthes mauritiana DC., (Compositae), a East African medicinal herb containing pharmaceutically promising secondary metabolites, has successfully been raised *in vitro*. We have developed a clonal propagation protocol that uses juvenile plants as starting material. The addition of benzylaminopurine (BA) (1.0 μM) and naphthaleneacetic acid (NAA) (0.1 μM) to the culture medium resulted in maximum shooting response with minimal callusing. Shoots rooted best *in vitro* in MS medium supplemented with indole-3-acetic acid (IAA; 0.2 μM), and plants that had already developed roots showed better growth, with maximum survival rate, in the greenhouse after an initial hardening.

Key words: Spilanthes mauritiana; micropropagation; meristem culture; medicinal plant; plant growth regulator.

Introduction

Spilanthes mauritiana DC., a monogeneric-endangered herb belonging to the Compositae family, is a native of Eastern Africa and is used in the local pharmacoepia to cure infections of the throat and mouth (Watt and Brayer-Brandwijk, 1962), and as remedy for stomach ache and diarrhea (Kokwaro, 1976). Kamba tribes in Kenya chew the flower of *S. mauritiana* for the relief of toothache and the treatment of pyorrhea (Watt and Brayer-Brandwijk, 1962), and an infusion of the herb is used as a febrifuge (Dalziel, 1937). In the Cameroons the plant is used as a snake-bite remedy and in the treatment of articular rheumatism (Dalziel, 1937). In India the plant has been used as a remedy for kidney stones, and bladder and kidney infections (Dragendorff, 1898). In contrast, the flowering head is reported to produce stupefaction of fish and to be used as fish poison (Dragendorff, 1898). So far the only isolated active principle in *S. mauritiana* is an antiseptic alkaloid, spilanthol, present at a concentration of as much as 1.25% in the flowers (Watt and Brayer-Brandwijk, 1962). Spilanthol is effective against blood parasites at extremely low concentrations, and indeed is a poison to most invertebrates, while remaining harmless to the majority of vertebrates (Watt and Brayer-Brandwijk, 1962). Researchers have shown preliminary antimicrobial activity in the crude extract from roots and flower heads of *S. mauritiana* (Fabry et al., 1996, 1998).

One of the essential requirements for the successful application of plant propagation technology to agriculture is the capacity to regenerate elite plantlets. During the past decade, the demand for elite plantlets has undergone a steep rise. To meet this ever-growing commercial need, the realization of *in vitro* production of a large number of clonal plants with improved characteristics has become necessary. Additionally, clonal propagation of elite plantlets still remains the only way to conserve endangered plant species. The accelerating loss of plant species as a result of destruction of their tropical habitat has yielded a revival of interest in the propagation of endangered plants (Moncousin, 1991). Given that clonal propagation also preserves the genetic stability of the progeny, collections are important *ex situ* germplasm reservoirs.

In order to study and characterize the bioactive compounds in *S. mauritiana*, a constant source of this plant is needed, yet the natural habitat of the plant is under severe pressure and is constantly shrinking. The present communication reports for the first time the clonal propagation of *S. mauritiana* through axillary bud cultures.

Materials and Methods

Plant material. Healthy plants of *S. mauritiana* were collected from Lugane District in Western Kenya and were authenticated by Simon Mathenge of the University of Nairobi Herbarium, Kenya, where duplicate voucher specimens are held for reference (reference no. 96/2001).

Chemicals. Plant growth regulators were obtained from the Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical and HPLC grade.

In vitro *culture conditions.* Axillary buds of *S. mauritiana* were washed under running tap water to remove superficial contamination. Single bud explants (1 cm each; upper portion) were washed with Tween-20 (5% v/v) for 5 min followed by thorough washing under running tap water for 15 min. The explants were surface-sterilized with 5.0% (v/v) commercial bleach (Albertson Inc., Boise, ID) for 15–25 min and later rinsed four or five times with sterile distilled water. For all the experiments, MS (Murashige and Skoog, 1962) basal culture media was used. The pH was adjusted to 5.8 ± 0.2 using 1 *N* HCl/1 *N* NaOH before adding 0.8% (w/v) of agar (Sigma). The media was subsequently autoclaved under 105 kPa at a temperature of 121°C for 15 min. Explants were placed in a culture tube (50 ml) with 20 ml of MS media and kept under controlled conditions at 25 ± 2°C, with a photoperiod of 16 h under cool white light (Phillips Co.,

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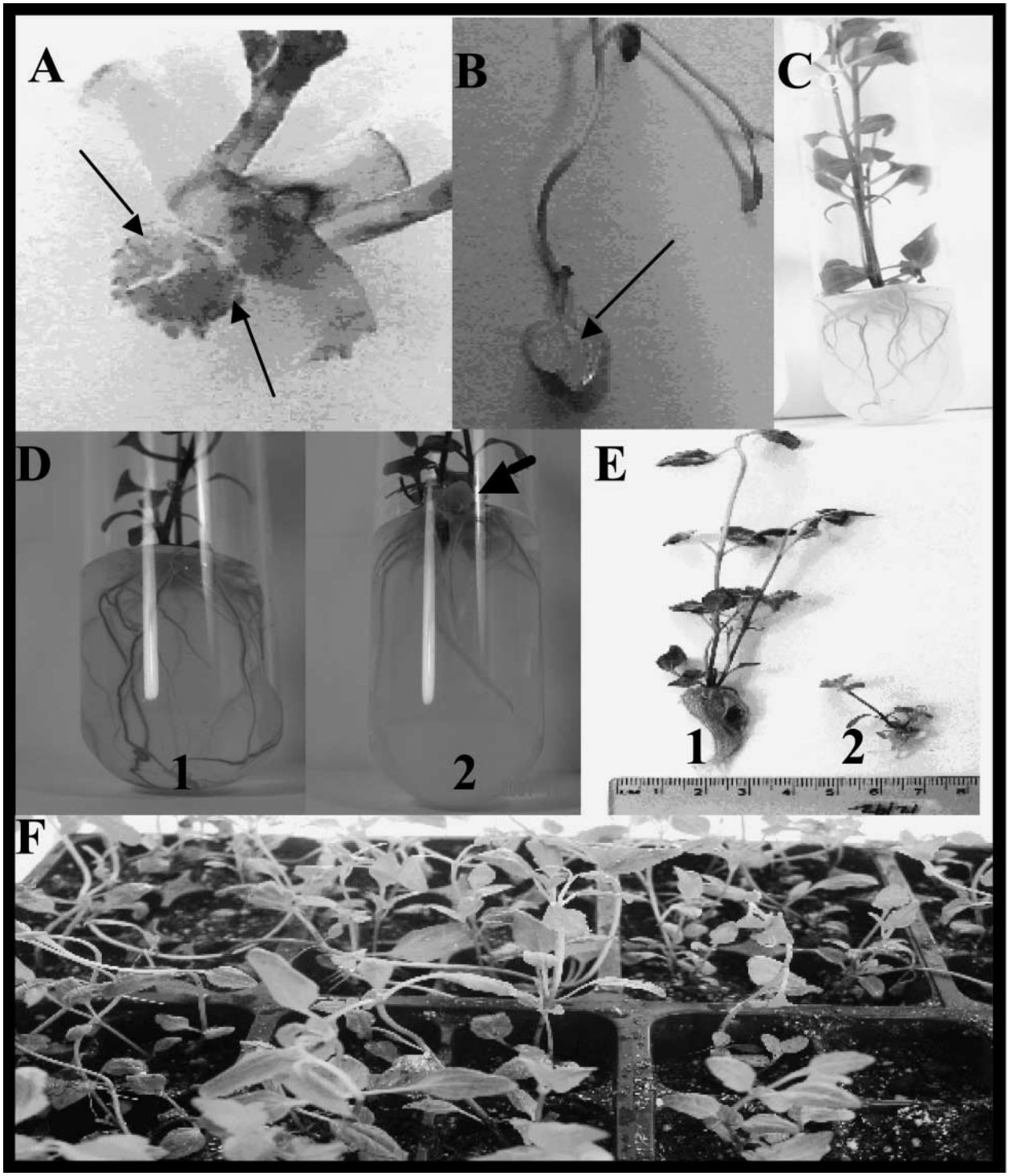

FIG. 1. Micropropagation of *Spilanthes mauritiana* DC. through axillary bud cultures. *A*, Swollen shoot tips represent the first response of cytokinins. *B*, Increased callusing on shoot tips at higher concentrations of BA (1.80 μ*M*). *C*, Multiple shooting in *S. mauritiana* with BA (1.0 μ*M*) and NAA (0.11 μ*M*). *D*, *In vitro* rooting with *S. mauritiana*: (1) profuse rooting with IAA at 0.20 μ*M*; (2) hindered rooting showing intervening callusing with IAA at 0.01 μ*M* concentration. *E*, Visible difference in shooting and rooting differentiation with optimal and extreme concentrations of cytokinins: (1) BA (1.0 μ*M*) and NAA (0.11 μ*M*); (2) BA (1.80 μ*M*) and NAA (0.1 μ*M*). *F*, *In vitro*-raised plants 1 mo. after transplantation.

Houston, TX) with an intensity of 45 μmol m^{-2} s^{-1} and 60–70% relative humidity.

Shoot proliferation. Various plant growth regulators (PGR) viz., benzylaminopurine (BA) (0.5–1.8 μ*M*) and α-naphthaleneacetic acid (NAA) (0.02–0.2 μ*M*) were tried individually or in combination to obtain the most suitable combination for the proliferation of shoots in established explants. Experiments were performed with a minimum of six replications and repeated twice. Observations were recorded after an interval of 4 wk.

In vitro *rooting*. For root induction, shoot tips with three fully expanded leaves from *in vivo*-grown plants were cut. Shoot tips were surface-sterilized as described above. The basal rooting media (RM) contained MS salts without PGR or with indole-3-acetic acid (IAA; 0.01–0.4 μ*M*) supplemented with similar sucrose and agar concentrations given for shoot proliferation media. The plants were subcultured after 1 mo. on the same culture media. Experiments were performed with a minimum of six replications and repeated twice.

Greenhouse establishment. Rooting efficiency was calculated as the percentage of shoots producing roots after 4 wk of culture for each treatment. Rooted plantlets were removed from the media, freed of agar by washing in running water, and planted in a sand–compost mixture (1:2) at about 90% relative humidity and under greenhouse conditions. The plantlets were hardened for 20 d and then transplanted to the field. After an additional 15 d the survival percentage was determined.

Statistical analysis. All experiments were repeated at least twice with 24 replicates for the same clone under each treatment. Data were analyzed by one-way ANOVA calculated using SPSS 10.0 (SPSS Inc., Chicago, IL) software. Significant differences between means were assessed by Duncan's test at $P < 0.05$.

Results

Shoot induction and propagation. No shoot development was observed when nodal explants were cultured on medium without added PGR. Nodes began to swell after 5 d on media supplemented with PGR (Fig. 1*A*), but bud breaking was only observed after an additional 10 d on PGR-containing medium. The number of shoots per explant and the percentage of explants per vessel with shoots were significantly different when using BA versus NAA (Table 1). Excessive callus growth negatively interfered with the propagation process (Table 1; Fig. 1*B*). The application of supraoptimal concentrations of BA seemed to result in higher callusing rates at the bases of the plants or even led to deformed plants (Fig. 1*B*, *E*). In the presence of BA at 1.0 μ*M* and NAA (0.1 μ*M*), 96.7% of the explants per vessel produced shoots, with an average of 5.6 shoots per explant (Table 1; Fig. 1*C*, *E*). Treatments containing BA and NAA alone did not perform as well for shoot regeneration compared to treatments that combined them (Table 1). Further shoot differentiation and growth were observed after the time of data acquisition (Fig. 1*E*).

Induction of rooting and transfer to the greenhouse. Rooting is generally a very slow process for *S. mauritiana*, and after 1 mo. of subculturing roots were still rarely longer than 6 cm, but were profuse (Fig. 1*C*). Rooting was not observed on medium without PGR (Table 2). The rooting process was inhibited by supraoptimal concentrations of IAA, with callusing at the shoot tip intervening with growth in the treatments with higher concentrations of IAA (Fig. 1*D*; Table 2). Maximum rooting was achieved with IAA (0.2 μ*M*; Fig. 1*D*), in which the percentage per vessel of explants with roots was greater compared to the other treatments (Table 2). Plants rooted with IAA (0.2 μ*M*) showed the highest field survival rate (79.8%) under greenhouse conditions (Table 2; Fig. 1*F*).

TABLE 1

EFFECT OF PGR COMBINATIONS ON MICROPROPAGATION OF *SPILANTHES MAURITIANA*

	MS full strength + PGR (μ*M*)		Percentage of explants per tube with shoots[a]	Number of shoots[a]	Percentage of explants per tube with callus[a]
	BA	NAA			
Control	0	0	0	0	0
1	0.45	0.02	46.8 ± 3.5	2.3 ± 0.1	23.8 ± 1.7
2	0.6	0.02	39.8 ± 2.9	2.1 ± 0.1	31.6 ± 2.3
3	0.8	0.02	54.8 ± 4.1	2.5 ± 0.1	19.8 ± 1.4
4	1.0	0.02	56.9 ± 4.2	3.1 ± 0.2	21.8 ± 1.6
5	1.8	0.02	19.6 ± 1.4	1.1 ± 0.08	93.5 ± 7.0
6	1.0	0.11	96.7 ± 7.2	5.6 ± 0.4	1.6 ± 0.09
7	1.0	0.2	65.1 ± 4.8	3.4 ± 0.2	18.6 ± 1.3
8	1.2	0.2	61.6 ± 4.6	2.9 ± 0.2	16.8 ± 1.2
9	1.45	0.2	52.8 ± 3.9	2.6 ± 0.1	19.8 ± 1.5
10	1.0	0.2	51.9 ± 3.8	2.4 ± 0.3	29.8 ± 2.5
11	1.0	0.02	55.9 ± 4.1	1.8 ± 0.04	39.6 ± 4.1
12	1.0	0.04	51.8 ± 3.8	1.6 ± 0.03	31.8 ± 3.2
13	0.45	–	19.6 ± 1.1	1.1 ± 0.06	98.6 ± 8.9
14	0.6	–	16.9 ± 1.2	1.1 ± 0.04	89.9 ± 7.5
15	0.8	–	12.1 ± 0.9	1.1 ± 0.03	91.2 ± 7.9
16	1.0	–	29.6 ± 0.8	1.1 ± 0.04	92.3 ± 7.8
17	1.8	–	12.1 ± 0.6	1.1 ± 0.07	90.1 ± 7.9
18	–	0.02	7.8 ± 0.8	1.1 ± 0.07	93.8 ± 7.4
19	–	0.11	5.3 ± 0.2	1.1 ± 0.09	91.2 ± 5.9
20	–	0.20	4.8 ± 0.3	1.1 ± 0.02	91.9 ± 5.6
21	–	0.22	3.2 ± 0.1	1.1 ± 0.03	93.4 ± 4.9

[a] Mean ± SE of 24 replicates.

TABLE 2

FACTORS INFLUENCING *IN VITRO* ROOTING OF *SPILANTHES MAURITIANA*

MS full strength + IAA (μM)	Percentage of explants per tube with roots[a]	Rooting efficiency[ab]	Length of primary root[a] (cm)	% Field survival
0	0	0	0	0
0.01	25.6 ± 1.9	12.8 ± 0.9	1.2 ± 0.01	25.8
0.15	28.9 ± 2.5	13.9 ± 0.5	1.6 ± 0.03	29.6
0.20	91.8 ± 6.8	28.9 ± 1.3	3.8 ± 0.6	79.8
0.40	43.4 ± 3.9	15.8 ± 0.9	1.9 ± 0.04	52.0
0.44	19.8 ± 2.5	6.8 ± 0.06	0.9 ± 0.01	21.6

[a] Mean ± SE of 24 replicates.
[b] Rooting efficiency was calculated as number of roots per explant in all the treatments.

Discussion

The objective of the study presented here was the establishment of *in vitro* techniques for the cultivation of *S. mauritiana*, especially the development of a micropropagation system and a process for the transfer of *in vitro*-raised plants to greenhouse field conditions. Shoot proliferation was promoted by a PGR combination of BA (1.0 μM) and NAA (0.1 μM) without simultaneously inducing callus growth; the latter would restrict the subsequent rooting and hardening processes. Earlier reports have demonstrated that shoot proliferation is promoted by a PGR combination of NAA and BA without simultaneously inducing callus growth (Bringmann and Rischer, 2001). A simple but efficient rooting method consisted of the optimal concentration of IAA (0.2 μM), which induced root growth in most of the explants; a subsequent transfer to MS basal liquid media with shaking resulted in profuse rooting. These *in vitro*-rooted plants were transferred to the greenhouse with a higher survival rate than that of unrooted cuttings. The resulting plants show great vigor and have well-developed roots and thereby are suitable for re-establishment purposes in their native countries.

Spilanthol is the only value-added secondary metabolite characterized from *S. mauritiana* showing insecticidal activity (Jondiko, 1986). Lack of ethnobotanical knowledge and the unavailability of *S. mauritiana* has led to lacunae in the knowledge of uncharacterized compounds in *S. mauritiana*. As a prerequisite for further experiments on the biogenesis of secondary metabolites, a higher BA concentration will be used to enhance callusing to elucidate the occurrence of novel metabolites in cell suspension cultures of *S. mauritiana*. In this communication we provided an example of conserving an endangered medicinal plant by axillary bud propagation, which may lead to increased availability of the plant for its medicinal usage.

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