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Regeneration of the tropical legume *Aeschynomene sensitiva* Sw. from root explants

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Abstract

Regeneration of *Aeschynomene sensitiva* Sw. after callogenesis was obtained from small (2–5 mm long) root explants of 30-day-old seedlings aseptically cultivated on Murashige and Skoog medium supplemented with various concentrations of growth regulators. After 4 weeks, the best results were observed with 0.54 μM α-naphthaleneacetic acid and 2.22 μM benzyladenine. On this medium, the rate of regeneration depended on seedling age and agar concentration. The highest number of shoots per explant was obtained with small cuttings from 30-day-old seedlings grown on a medium containing 8 g l^{-1} of agar. Regeneration success was also dependent on explant size. When longer explants (7–20 mm) were cut from the main root, direct regeneration was obtained in two weeks. These cuttings also generated shoots through callogenesis in four weeks but always in lower quantities than with direct regeneration, whatever the seedling age. Here also, the best regeneration was obtained with cuttings from 30-day-old seedlings maintained on a medium with 8 g l^{-1} of agar. Regenerants were rooted on growth-regulator-free Murashige and Skoog medium and then acclimatized in a greenhouse. A better survival to transplantation was observed when plantlets were inoculated with the photosynthetic *Bradyrhizobium* strain ORS 278. Stem and root nodules developed on the inoculated plantlets and were able to fix nitrogen.

Abbreviations: BA – benzyladenine; 2,4-D – 2,4-dichlorophenoxyacetic acid; NAA – α- naphthaleneacetic acid; MS – Murashige & Skoog (1962) medium

Introduction

The genus *Aeschynomene* contains about 160 species of tropical legumes (Kretschmer & Bullock, 1980) spread throughout America and Africa, with a few species occurring in Southeast Asia and in the Pacific Islands. Some species are able to form both stem and root nitrogen-fixing nodules with *Rhizobia* (Dreyfus *et al.*, 1984; Loureiro *et al.*, 1994). The stem infection always occurs at predetermined sites which correspond to root primordia (Dreyfus & Dommergues, 1981). Most of the *Bradyrhizobium* strains capable of inducing stem nodules are of great significance because of their unusual ability to produce bacteriochlorophylla (Lorquin *et al.*, 1993) and to realize photosynthesis during heterotrophic growth (Eaglesham *et al.*, 1990). Moreover, these stem-nodulating legumes are used as green manure in paddy-fields because of their high nitrogen accumulating potential, their tolerance to flooding and their fast growing ability (Alazard & Becker, 1987). No techniques of propagation have so far been described for this genus and particularly for *Aeschynomene sensitiva*. The paper reports an efficient system of tissue culture for rapid plantlet regeneration from root explants.

Materials and methods

Seeds of *Aeschynomene sensitiva* Sw. obtained from Casamance (South of Senegal) were surface sterilized (30 min in concentrated sulfuric acid followed by 5 rinses with distilled water) and then aseptically germinated by dropping them in test tubes on a filter paper

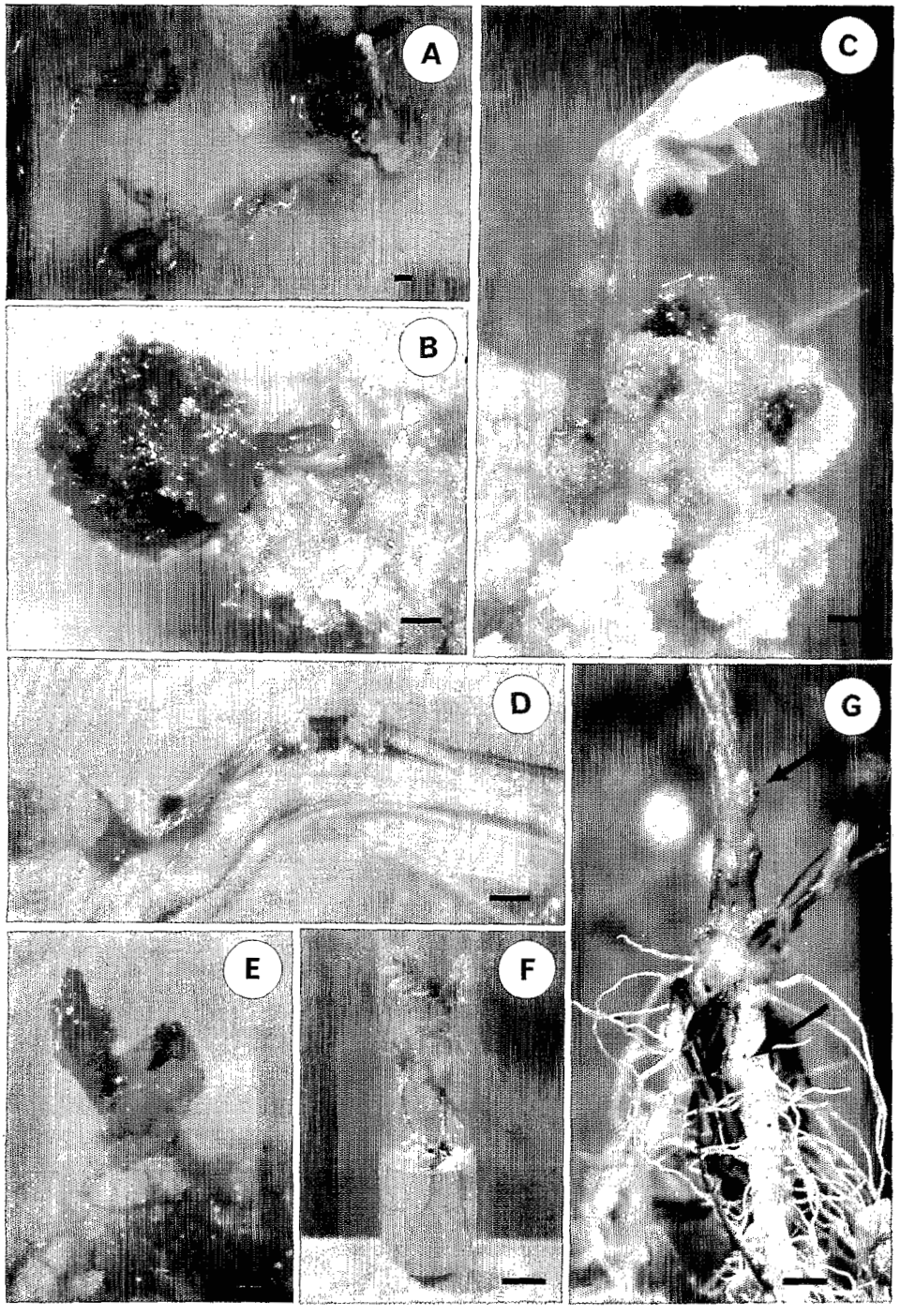

Fig. 1. Regeneration from root explants of *Aeschynomene sensitiva* Sw. On small cuttings (2–5 mm) from 20-day-old seedlings cultivated on NB medium (*A*) green callus initiation on yellowish callus after 15 days of culture. (*B*) shoot initiation in a green callus and (*C*) shoot elongation. On long cuttings (7–20 mm) of main root from 30-day-old seedlings cultivated on NB medium. (*D*) bud formation by direct regeneration on the root axis and (*E*) shoot differentiation. (*F*) Root formation on regenerated shoot after transfer onto MS medium free of growth regulators. (*G*) Regenerated plantlets inoculated with the rhizobium strain ORS 278 after 8 weeks of culture in greenhouse. Note the location of the nodules (arrows) both on stem and root of the plantlet. Bars represent 1 mm in A-D, 0.5 mm in E, 1 cm in F and 0.5 cm in G.

Table 1. Effect of combinations of 2,4-D and kinetin on callus development and shoot regeneration of small root cuttings from 30-day-old seedlings of *Aeschynomene sensitiva*. Sw.

2,4,D (μM)	Kinetin (μM)	Explants with callus (%)	Calluses with shoots (%)	Number of shoot explant^{-1}
0.45	0.46	13a[1]	0a	0a
	2.32	9a	0a	0a
2.26	0.46	100d	0a	0a
	2.32	82c	28b	0.64c
4.53	0.46	100d	91c	1.91d
	2.32	37b	25b	0.34b

[1]Each value represents the mean of 3 replicates each with 10–12 root cuttings (2–5 mm). Callus formation was scored after 2 weeks and shoot regeneration after 4 weeks. Means within the same column followed by different letters are significantly different. ($p \leq 0.05$) according to one way ANOVA analysis.

Table 2. Effect of combinations of NAA and BA on callus development and shoot regeneration of small root cuttings from 30-day-old seedlings of *Aeschynomene sensitiva* Sw.

NAA (μM)	BA (μM)	Explants with callus (%)	Calluses with shoots (%)	Number of shoot explant^{-1}
0.54	0.44	0a[1]	0a	0a
	2.22	96d	83c	3.6c
2.68	0.44	21b	79c	1.25b
	2.22	8a	0a	0a
5.37	0.44	89d	28b	1.05b
	2.22	48c	9ab	0.2a

[1]Each vlaue represents the mean of 3 replicates each with 10–12 root cuttings (2–5 mm). Callus formation was scored after 2 weeks and shoot regeneration after 4 weeks. Means within the same column followed by different letters are significantly different ($p \leq 0.05$) according to one way ANOVA analysis.

maintained at the surface of distilled water. Cultures were then incubated with a 14-h photoperiod (77 μmol m^{-2} s^{-1} from Osram L36W/11 day-light fluorescent lamps) at 27–23 °C (± 1 °C) day-night temperatures. After 20 days of culture, the main root reached 3 to 6 cm in length. At this stage, both main and adventitious roots were cut into 2–5 mm sections referred to hereafter as small cuttings. After 30 to 45 days of culture, the main root reached 6 to 9 cm in length. The upper part of the main root (4 cm under the collar) was cut into 7–20 mm sections referred to hereafter as long cuttings. The lower part of this main root and the adventitious roots were treated as those from 20-day-old seedlings (small cuttings). The effect of some plant growth regulator combinations (2,4-D in combination with kinetin and NAA in combination with BA) on callogenesis and regeneration was investigated on small cuttings from 30-day-old seedlings. These explants were then cultivated in Petri dishes containing MS medium supplemented with agar at 8 g l^{-1} (Agar-Agar Prolabo). The growth regulators were tested at different concentrations as indicated in Tables 1 and 2. The effect of explant age was evaluated by comparing shoot regeneration of small cuttings from 20-, 30- and 45-day-old seedlings grown on MS medium containing NAA and BA at 0.54 and 2.22 μM, respectively (NB medium). In addition, the influence of explant size on regeneration was studied using the same NB medium and long cuttings obtained from the upper part of the main root from 30- and 45-day-old seedlings. The influence of water availability was also investigated by cultivating 30-day-old seedling explants (small and long cuttings) on the following agar concentrations : 4, 6, 8 and 10 g l^{-1}. Experiments were carried out in three replicates containing 10–12 explants each. After 2 and 4 weeks of culture, explants were observed for callogenesis and regeneration.

The regenerated plantlets were transferred to growth regulator free medium for rooting. When the rooted shoots reached the 7–8 leaf stage, one hundred (50 were obtained from small cuttings of 30 day-old seedlings and 50 were obtained directly from long cuttings) were distributed into three pots containing a mix of sterilized sand and vermiculite (1:1). Two pots were inoculated after 3 days of acclimatization with 1 ml of a 5-day-old photosynthetic *Bradyrhizobium* ORS 278 liquid culture containing 10^8 bacteria ml^{-1} (Vincent, 1970). The last pot was a control containing 30 non-inoculated plantlets. All plants were covered with polypropylene screw caps during the first week of culture. After transferring the plants into soil, the ability of stem and root nodules to fix nitrogen was estimated by the acetylene reduction assay (Hardy *et al.*, 1973).

Results

Influence of growth regulators on shoot regeneration

Various combinations of growth regulators were tested on small cuttings from 30-day-old seedlings of *A. sensitiva*. Some of them allowed both callus induction and shoot development (Table 1, 2). However, regeneration only occurred after callogenesis. Bud formation and shoot proliferation occurred on green calli which differentiated from yellowish calli after 2 weeks of cul-

Table 3. Effect of seedling age on shoot regeneration from small root cuttings (2–5 mm) of *Aeschynomene sensitiva* Sw. after 4 weeks of culture on NB medium.

Seeding age (days)	Explants with shoots (%)	No of shoot explant^{-1}
20	69 ± 18 [1]	1.46 ± 0.26
30	83 ± 11	3.60 ± 0.70
45	41 ± 9	1.48 ± 0.43

[1]Means ± standard deviation of 3 replicates each with 10–12 root cuttings.

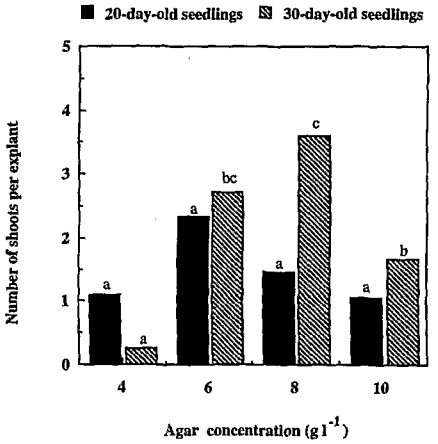

Fig. 2. Influence of the agar concentration on shoot production from small cuttings (2–5 mm) of root explants from 20 and 30-day-old seedlings of *Aeschynomene sensitiva* Sw. after 4 weeks of culture on NB medium. Means within the same series mentioned by different letters are significantly different ($p \leq 0.05$) according to one way ANOVA analysis.

ture (Fig. 1A, B and C). The highest callogenesis and shoot regeneration frequencies (100 and 91%, respectively) were observed on medium containing 2,4-D at 4.52 µM and kinetin at 0.46 µM (Table 1). The highest level of regeneration, in terms of shoot number per explant, was obtained on medium containing NAA at 0.54 µM and BA at 2.22 µM (Table 2). The average shoot number per callus was 3,6 which was 2-fold higher than for any other regenerating medium. This latter medium (called NB medium) was considered to be the most suitable for regeneration.

Effect of seedling age and agar concentration

The comparison of regeneration rates between small cuttings obtained from 20, 30 and 45-day-old seedlings cultivated on NB medium supplemented with 8 g l^{-1} of agar showed that the explants from 30-day-old seedlings had the best ability to regenerate shoots.

Table 4. Effect of seedling age on shoot regeneration from long cuttings (7–20 mm) of main root of *Aeschynomene sensitiva* Sw. after 4 weeks of culture on NB medium.

Seedling age (days)	Direct regeneration (No. of shoot explant^{-1})	Regeneration through callogenesis (No. of shoot explant^{-1})
30	2.70 ± 0.51 [1]	0.84 ± 0.29
45	2.10 ± 0.40	0.41 ± 0.27

[1]Means ± standard deviation of 3 replicates each with 8–10 root cuttings.

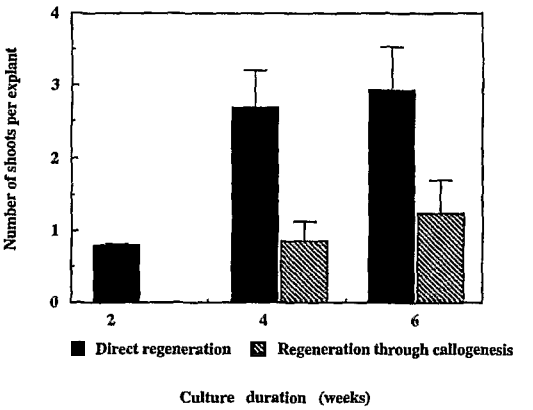

Fig. 3. Evolution of the number of shoots per explant obtained directly or after callogenesis on long cuttings (7–20 mm) of the main root from 30-day-old seedlings of *Aeschynomene sensitiva* Sw. cultivated on NB medium. Bar indicates the standard deviation of the mean.

These cuttings presented the highest rate of callogenesis (2-fold higher than for explants from 45-day-old seedlings) and the highest number of shoots per explant (Table 3). The regeneration rate was also dependent on the agar concentration of the culture medium. The best results were obtained with 6 and 8 g l^{-1} of agar for cuttings from 20 and 30-day-old seedlings (Fig. 2). However, these results were significantly different ($p \leq 0.05$) only for 30-day-old seedlings.

Effect of explant size

The culture on the NB medium of long cuttings from the main root gave direct shoot formation on the root axis after the second week of culture (Fig. 1D, E). These explants were also able to form calluses located at the cut ends. On these calluses, bud formation was noticed after 3 weeks of culture and this allowed shoot isolation after 4 weeks. The seedling age seemed to have a low impact on direct regeneration (Table 4). However, as observed with small cuttings, the rate of

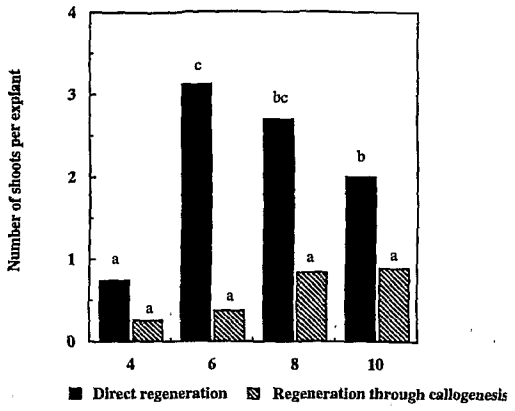

Fig. 4. Influence of the agar concentration on shoot production by direct regeneration or after callogenesis on long cuttings (7–20 mm) of the main root from 30-day-old seedlings of *Aeschynomene sensitiva* Sw. after 4 weeks of culture on NB medium. Means within the same series mentioned by different letters are significantly different ($p \leq 0.05$) according to one way ANOVA analysis.

regeneration after callogenesis decreased significantly when explants were cut from 45-day-old seedlings; the number of shoots formed per explant was 2-fold lower than with 30-day-old seedlings. As observed during the culture of small cuttings, the modification of the agar concentration induced variations on the rate of regeneration (Fig. 4). Direct regeneration was more effective with an agar concentration of 6 g l^{-1} (average of 3.1 shoots per explant). However, even if the number of shoots obtained after callogenesis increased with the agar concentration, the number of shoots obtained directly from roots was always higher.

Roots, transplanting and inoculation

All the isolated shoots rooted when transferred onto MS medium free of growth regulators (Fig. 1F). After transfer into pots and inoculation with the photosynthetic strain *Bradyrhizobium* ORS 278, most of the plantlets grew well (92 and 86% of the plantlets obtained directly and after callogenesis, respectively). In contrast, numerous control plantlets declined and turned yellow, so that only 44% were able to develop. The formation of stem and root nodules was noticed only on plantlets which had been inoculated (Fig. 1G). Nitrogen-fixing activity was observed in the root nodules of each plant tested.

Discussion

This study showed the capacity of *Aeschynomene sensitiva* Sw. to regenerate plantlets from root explants. Using NB medium (MS medium supplemented with NAA at 0.54 µM and BA at 2.22 µM), these root explants were able to develop shoots either directly or through callogenesis. Explant size and agar concentration seemed to be determining factors on the mode of regeneration. As for shoot regeneration from petunias leaf discs (Beck & Camper, 1991), a minimum size of explant seemed to be required to obtain direct regeneration. In this work, root cuttings shorter than 5 mm gave regenerated shoots only after callus production. Longer cuttings regenerated shoots directly on the root axis but for these explants, high agar concentrations promoted regeneration through callogenesis at the expense of direct regeneration. As the gelling agent concentration acted essentially on the water availability of the medium, with a matric potential 20% higher at 4 than at 10 g l^{-1} Owens & Wozniak, 1991), these results showed that a certain water availability and a certain explant length were needed to obtain direct regeneration. In return, the highest levels of regeneration after callogenesis were obtained for small cuttings from 30-day-old seedlings on media presenting a low water availability. The explant age also seemed to be a factor interfering with the level of regeneration. The best results for direct or indirect regeneration were obtained with root explants from 30-day-old seedlings. The root system of 20-day-old seedlings was not actually well developed. The small diameter of the main root and the fineness of the lateral roots were probably unfavourable factors to establish a complete regeneration process. In our *in vitro* culture conditions, seedlings of *A. sensitiva*, and more particularly their foliar axis, grew well and rapidly occupied most of the vessel volume. This fast growth favoured the accumulation in test tubes of volatile compounds as ethylene which could alter the regeneration capacities, as was observed with the oldest seedlings. It has been shown that ethylene influences the capacity of shoot regeneration, since the presence of ethylene inhibitors such as cobalt or silver nitrate stimulate shoot regeneration from cotyledon explants of *Helianthus annuus* (Chraibi *et al.*, 1991) or *Brassica campestris* (Palmer, 1992). However, culture of long root explants from 30-day-old seedlings on NB medium supplemented with 8 g l^{-1} of agar quickly gave a good level of shoot regeneration. After transplanting, acclimatization of the regenerated plantlets was improved by inoculation

with the *Bradyrhizobium* strain ORS 278. This could be attributed to a higher disponibility for the plant in nitrogen source, the symbiotic fixation constituting another nitrogen source for nitrogen metabolism. Root explants of *Aeschynomene sensitiva* Sw. seem to be suitable material for shoot regeneration and could be an interesting tool for plant transformation.

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