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Rapid propagation of agave by in vitro tissue culture

L.T. Binh, L.T. Muoi, H.T.K. Oanh, T.D. Thang & D.T. Phong
Institute of Biology, NCSR Vietnam, Nghia-Do, Tu-Liem, Hanoi, Vietnam

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Abstract

A procedure for rapid propagation of Agave (*A. cantala* Roxb., *A. fourcroydes* Lem. and *A. sisalana* Perrine, (Agavaceae) have been developed. The explants were excised from stolon plantlets, sterilized and cultivated on Murashige and Skoog (MS) basal medium containing 2% sucrose, 10% coconut water and 0.8% agar. The addition of following combination of growth substances—0.075 mg l^{-1} naphthalenacetic acid (NAA) + 0.1 mg l^{-1} indolylbutyric acid (IBA) + 0.5 mg l^{-1} kinetin (KIN) caused an extensive proliferation of multiple shoot primordia. Subcultures of these on the same medium were successful for the multiplication with an index of 3–4 times per 4 weeks subculture period. Shoots were rooted on hormone free MS medium and then transferred into a sand bed for acclimation before field planting.

Introduction

Agave plants are most important in terms of the production of cordage fibers such as sisal, henequen and cantala, which are scraped mechanically from leaves of *Agave sisalana* Perrine, *A. fourcroydes* Lem. and *A. cantala* Robx., respectively [5].

Agave plants are conventionally propagated by the bubils which arise from the axilary meristems on the inflorescence after flowering. However, it takes approximately 20–30 years of vegetative growth until the adult plant reaches the generative stage and begins flowering. Most plants seldom set seeds, therefore sexual reproduction through true seeds is usually inconvenient [6]. Another way of propagation is based on the stolon cuttings. Each stolon terminates in a young plantlet. Under suitable conditions each adult Agave plant can only form a few stolons every year [unpubl. data]. For the new planting of large areas, a large number of seed plantlets (4000 plants per ha) is needed, therefore the development of a new propagation technique using in vitro culture methods could be of practical value.

In vitro culture has been reported in some species of Agavaceae [1–4, 7, 9, 10, 12]. This paper reports our results in developing a procedure for rapid propagation of three most important *Agave* species by using the in vitro tissue culture techique.

Materials and methods

Plant materials

Stolons were collected from adult field plants of three Agave species, *A. cantala* Robx, *A. fourcroydes* Lem. and *A. sisalana*, Perrine, cut into two-nodes segments (about 10 cm in length), set in sand and kept humid by watering every two days. After 2 months young plantlets arising from the nodal meristems were excised for in vitro culture.

Sterilization

Plant materials were first cleaned carefully under running water. All the leaves of the plantlet were removed, and only a segment of the basal part (a cylinder of 1.5 cm in height) was used. First it was rinsed in 70% ethyl alcohol for 30 sec, then sterilized in 0.05% mercuric chloride for 10 min

Table 1. Effects of different combinations of growth substances on callus initiation, single shoot formation and multiple shoot bud proliferation in Agave (*A. cantala* Robx.) shoot explant culture.

No.	Combination of growth substances[1]	Callus	Single shoot	Multiple shoot buds[2]
1.	2,4-D(0.1) + BAP(0.1)	+++	+	−
2.	2,4-D(0.1) + KIN(0.5)	+++	++	−
3.	NAA(0.1) + BAP(0.1)	++	+++	+
4.	NAA(0.1) + KIN(0.5)	+	+++	++
5.	IBA(0.1) + BAP(0.1)	+	+	−
6.	IBA(0.1) + KIN (0.5)	+	++	+
7.	NAA(0.075) + IBA(0.1) + KIN(0.5)	+	++	+++

[1] Figures in parentheses are concentrations in $mg\,l^{-1}$.
[2] Relative intensities: − no; + slight; ++ mediate; +++ extensive.

and finally rinsed 5 times in autoclaved distilled water. The exposed surface of the tissue was removed with a razor blade under aseptic condition, leaving a cylinder of 7–8 mm in height, which contained apical and axial meristems. The cylinder was then divided in 8 sections by 4 vertical cuts with the razor blade, each section was placed separately in an 100 ml-Erlenmeyer flask containing prepared culture medium.

Culture medium and culture conditions

The culture medium was Murashige & Skoog [8] basal composition containing 2% (w/v) sucrose, 10% (v/v) coconut water and 0.8% (w/v) agar, and supplemented with different combinations of growth substances (Table 1). Cultures were incubated at a temperature of 28°C and illuminated for 16 h per day at 2000 lux.

Subcultures of established multiple shoot buds were carried out in 4 weeks period after separating the multiple shoot bud clusters from the big shoots of the size up to 3 cm. The first were subcultured on fresh prepared medium containing the same growth regulator combination and the last were transferred into hormone free MS-medium for rooting.

Acclimation and plantation

After 4 weeks the rooted shoots from 5–8 cm, in size, each with at least 1 healthy root were transferred into a sand bed for acclimation of further 4 weeks. The acclimated plantlets were planted at a density of 20–25 plants per m^2. After 12 months they had grown to the size of 45–50 cm for field planting.

Results and discussion

Establishment and multiplication of multiple shoots

The use of plantlets developed from stolon segments in a sand bed as the initial material had the advantage that only a low degree of contamination in the initial cultures occurred (less than 10%). After the first harvest of plantlets, the second meristems of each 2-nodes stolon segment could be grown into new plantlets, more rapidly so these could be used within 4 weeks.

Callus formation on the cut surfaces of explants was observed after two weeks incubation on culture media. The addition of $0.1\,mg\,l^{-1}$ 2,4-dichlorphenoxyacetic acid (2,4-D) and $0.1\,mg\,l^{-1}$ 6-benzylamino purine (BAP) stimulated an extensive proliferation of callus tissue, from which shoot could be regenerated. To avoid morphogenetical diversities of differentiated plants as described in other plants, for example in pineapple [10] direct proliferation of shoot buds was preferred in our culture system.

Independent of the extensive callus initiation more than 60% of the non-infected explants tended to form a single shoot from the apical or axial meristem. These single shoots appeared to inhibit the development of other meristems occurring in the same explants.

From a total of more than 120 combinations of different auxins (2,4-D; IAA; NAA; and IBA) and cytokinins (KIN; BAP; and Zeatin) at various concentrations (from 0.01 to $10\,mg\,l^{-1}$) that have been tested, the medium No 7 (Table 1) based on MS salts and organic compounds containing 2% sucrose, 10% coconut water and 0.8% agar and supplemented with $0.075\,mg\,l^{-1}$ NAA, $0.1\,mg\,l^{-1}$ IBA and $0.5\,mg\,l^{-1}$ KIN proved to be the most suitable for establishment of in vitro multiple

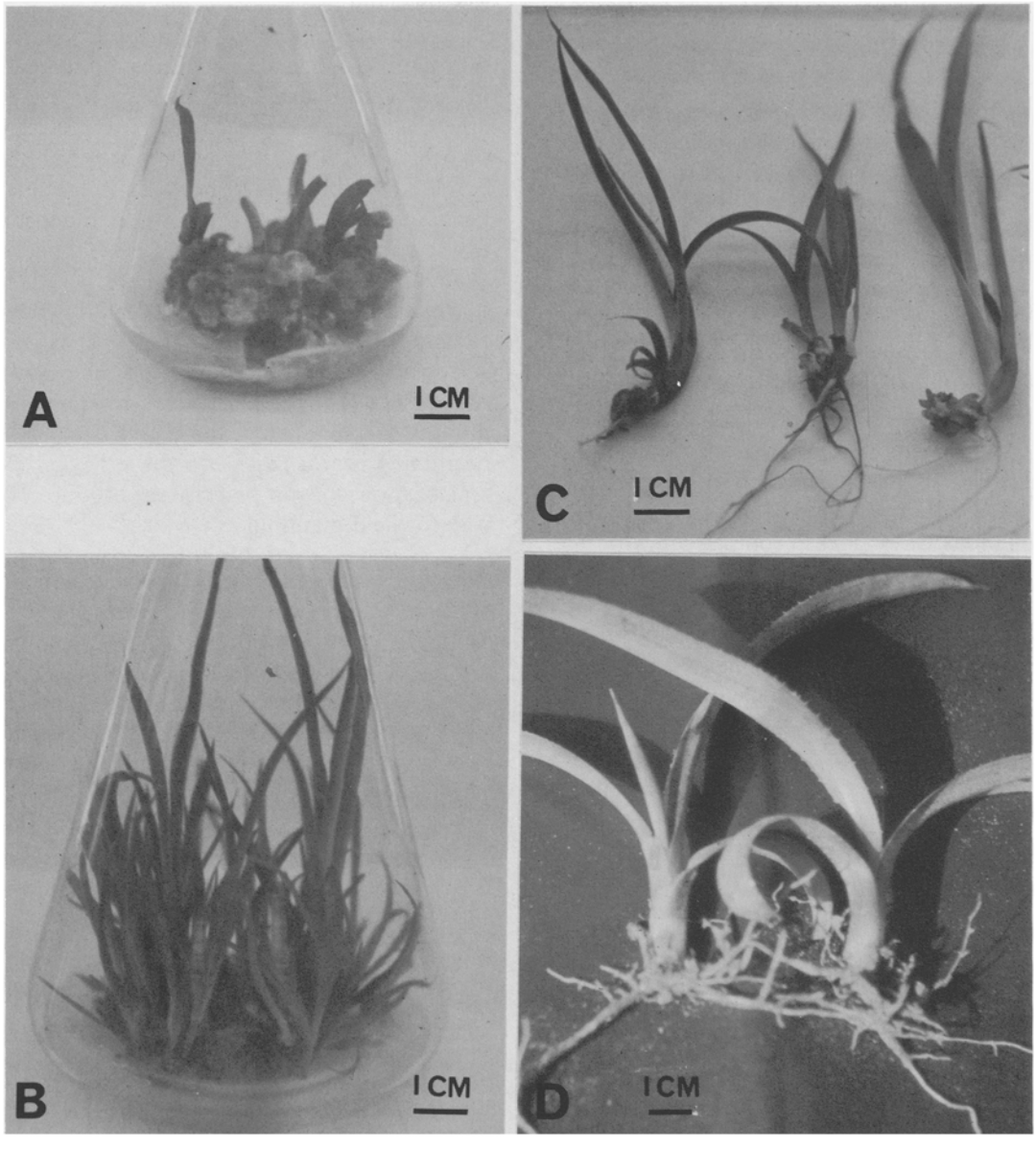

Fig. 1. Agave cantala Robx. — in vitro proliferation of multiple shoot buds, rooting and acclimation. (*A*) at the beginning and (*B*) after 4 weeks subculture, (*C*) plantlets from the rooting medium, and (*D*) 12 weeks later (4 weeks of acclimation on sand bed and 8 further weeks in the soil).

shoots. On this medium the explant formed at first a single shoot, then numerous new shoot primordia soon appeared on the tissues of the base part of the shoot, so clusters of multiple shoots and shoot buds were established within 4 weeks of culture. This mode of growth and development of the multiple shoots seemed to be similar to that described in *A. arizonica* [8].

For subculture only young multiple shoot clusters were used. Each 250 ml-Erlenmeyer flask

Table 2. Multiplication and rooting efficiencies of Agave (*A. cantala* Robx.) in in vitro cultures on subculture and rooting medium, respectively.

Time (day)	Multiplication No. shoot buds/flask[1]	Index[2]	Rooting No. shoot rooted	%
0	8.3	1	0	0
10	12.5	1.51	2.5	20.8
20	25.5	3.02	7.5	62.5
30	32.5	3.92	11.7	97.8
40	40.3	4.86	12	100

[1] Only buds up 2 mm were counted.
[2] Actual bud number/initial bud number (8.3).

was inoculated with 3 young clusters (Fig. 1). The multiplication index ranged between 3 and 4 for 4–5 weeks subculture period (Table 2). This multiplication index was higher in comparison with that observed for *A. arizonica* [8].

By comparing the growth patterns and multiplication indexes of all 3 tested Agave species (*A. cantala* Robx., *A. fourcroydes* Lem. and *A. sisalana* Perrine) no evident difference could be observed (data not shown).

All shoots of the size up to 3 cm were separated from the bud clusters and transferred into rooting medium. The MS medium without any growth substances was suitable for rooting. Nearly 100% of the shoots formed 2–5 healthy roots within 4–5 weeks (Table 2). If IBA at a concentration of $0.1\ mg\ l^{-1}$ was added to the rooting medium, the same rooting effect could be obtained, but a slight callus growth was also observed, whereby the survival of plantlets during the acclimation was negatively affected (data not shown).

Agave plants are generally drought resistant. The in vitro propagated plantlets were also able to resist a slight water deficit, but not a water excess. The use of only sand as bed material for acclimation stage was much better than a mixture with soil in 1:1 (v:v) ratio due to the low humidity and better air exchange. It was necessary to leave the sand bed on which in vitro plantlets were transferred uncovered. High humidity, not only in the sand bed, but also in the surrounding air caused putrification of the plantlets, especially in the wounded tissues (Table 3). Four weeks of acclimation was the necessary time for about 95% of the plants to form new roots.

Table 3. Survival and growth of in vitro propagated Agave (*A. cantala* Robx.) plantlets after 4 weeks of acclimation.

Bed material and treatment		Plants (%)		
		Putrefied	Formed new roots	Survived
Sand	covered	35.5	62.4	64.8
	uncovered	2.6	96.4	97.6
Sand + soil (1:1/v:v)	covered	46.3	55.8	62.5
	uncovered	18.2	76.8	82.7

Acclimated young plants were then planted in the soil at a density of 20–25 plants per m^2. After 12 months they had grown to a size of 40–50 cm in height and nearly 1 kg per plant in weight. Several tens of thousands of plants were produced by this way for field planting.

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