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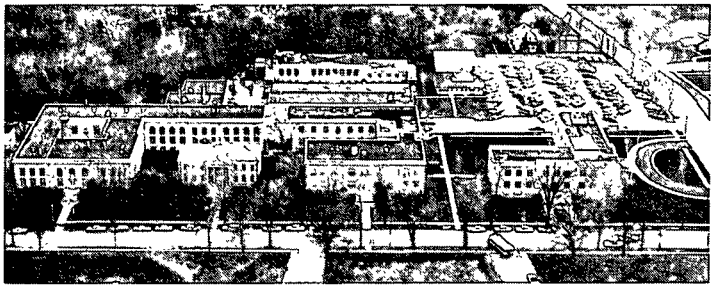

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NORWAY SPRUCE SOMATIC EMBRYOGENESIS: QUANTIFICATION OF SUSPENSION CULTURES DERIVED FROM MATURE EMBRYO CALLUS

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Norway Spruce Somatic Embryogenesis: Quantification of Suspension Cultures Derived from Mature Embryo Callus

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Norway spruce somatic embryogenesis: quantification of suspension cultures derived from mature embryo callus

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Key words; Picea abies, cell suspensions, somatic embryogenesis

Abstract. Embryogenic callus initiated from mature embryos of Picea abies (Norway spruce) have been established as rapidly growing suspension cultures. Maintained on half-strength modified Murashige-Skoog medium without NH_4NO_3 and supplemented with 5 mM glutamine, 2.2 µM N6-benzyladenine and 5.4 µM naphthalene-acetic acid, these cultures have continuously produced somatic embryos in early stages of development for over one year. The effect of media components on tissue growth and embryo yield was determined using quantitative growth measurements. During the linear phase of growth, the dry weight of the tissue doubled within 48 hours and embryo yield correlated with growth, increasing in density up to approximately 100 embryos/ml. Rapid growth and high embryo yield have been maintained by repeated subculture at 10-12 day intervals. Embryo maturation has been achieved, however, at a low frequency.

Introduction

The commercial success of conifer propagation via somatic embryogenesis will require a reproducible method for the efficient mass production of somatic embryos, high frequency maturation and the ultimate production of artificial seeds. Suspension cultures, which require the minimum of individual manipulations, have the potential to meet these goals as well as provide tissue sources readily adaptable for protoplast, biochemical and genetic improvement studies. The ease of maintenance and rapid growth of liquid culture systems, combined with the recent successes in the area of conifer somatic embryogenesis [1-5], have stimulated renewed interest in continuous cloning via suspension cultures.

Conifer embryogenic suspensions have been reported in Picea abies [1], Pseudotsuga menziesii [6], Picea glauca [7] and Pinus lambertiana [8]. However, quantitative data have not been presented. In our studies, we have initiated and maintained long-term embryogenic cultures as well as determined growth rates and the effect of time and medium on embryo induction and development. Using quantitative measurements, we can now present growth curves and embryo yields from rapidly growing embryogenic suspension cultures derived from Norway spruce mature embryo callus. The potential of the suspension grown somatic embryos to develop to plantlets is also demonstrated.

Methods and materials

Embryogenic callus was initiated from excised embryos of mature Norway spruce (Picea abies) as previously reported [9]. Briefly, Norway spruce seeds purchased from Quality Tree Seed, Inc., Brewster, N. Y. and stored at 4°C, were

surface sterilized in 30% H_2O_2 for 45 minutes, rinsed 3 times and imbibed overnight in sterile water. Embryos were excised and cultured on a half-strength modified MS basal medium, 0.5 BLG [10], supplemented with 10.7 μM NAA and 4.5 μM BA. Cultures were maintained at 23° (±) 1°C with 16 hr irradiance (10-40 μE $m^{-2}s^{-1}$) from cool-white fluorescent lights, and subcultured biweekly. Embryogenic callus has been maintained on solid medium for up to 1 year prior to transfer to liquid media.

Suspension cultures were initiated by transferring 1-2 callus masses (approximately 500-750 mg) to 125 ml Erlenmeyer flasks containing 25 mls of liquid medium of the same composition. Tissue readily dispersed when flasks were placed on a gyratory shaker (about 100 rpm). Suspended cells were maintained by subculture (1:10 dilution) at 10-14 day intervals in the same 16 hr photoperiod of diffuse light.

To quantify cell growth and embryo yield, tubes containing liquid medium were inoculated with stock suspension cultures at 10-15 μL/ml and rotated at 10 rpm on a roller drum under same environmental conditions. Inoculation density was determined by packed cell volume (centrifugation at 100 x g for 5 min.) Sufficient tubes were prepared to provide 3-4 replications at from 8-10 time periods. Fresh and oven-dry weight determinations were obtained by filtering tissue from tubes onto preweighed Whatman microfibre filters. Fresh weights were obtained immediately and dry weights were recorded after 24 hours at 65°C.

The number of somatic embryos at each time period was determined by pipetting samples from each of 3-5 tubes onto either developmental agar medium plates which were counted immediately, or imbedded into a 1% merthiolate/agar medium for later determinations. Embryos were counted by placing the Petri

dish on a background grid over the lighted stage plate of a Nikon dissecting microscope viewed at 20 X. Growth and yield were measured at 2 levels of growth regulators: 10.7 µM NAA/4.5 µM BA versus half-strength concentrations, 5.4 µM NAA/2.2 µM BA. To promote maturation, somatic embryos were transferred en masse or by individual selection to HM medium (9) containing 1 µM indole-3-butyric acid (IBA) and 1-5 µM abscisic acid (ABA) as either liquid treatments in roller-drum tubes, agar plates or multiwells containing saturated cheese cloth pads.

Results and discussion

Embryogenic tissues derived from subcultured mature embryo callus have been maintained as suspension cultures for over 12 months. The embryogenic suspensions consist of a complex mixture of dense, spherical cell aggregates, elongated vacuolated cells and somatic embryos in various stages of development or dedifferentiation. Figure 1 illustrates the typical somatic embryo stages observed in proliferative suspension medium. The smooth embryonal heads composed of small, dense meristematic cells are associated with suspensor masses of elongated vacuolated cells similar to the early embryos observed in callus cultures.

Tissue growth, determined by the fresh weights of a 2 month old suspension culture, increased from 2 mg/ml at day 0 to approximately 90 mg/ml at day 14 (Fig. 2). Results suggest optimum subculture time to be at 10-12 day intervals. A second study confirmed the same growth pattern determined by dry weights and correlated the growth rate with embryo density (Fig. 3). Tissue dry weight doubled within 48 hrs during the linear phase of growth, and the number of

early stage embryos peaked at approximately 100/ml, just prior to maximum tissue growth.

Growth or embryo yield was not increased by any medium/protocol variation studied. There were no significant differences in either tissue growth (Fig. 4) or embryo yield (Table 1) over time when cultured in 0.5 BLG medium with either full or half-strength levels of NAA/BA. Cultures have therefore been maintained on the lower levels of NAA/BA, 5.4 µM/2.2 µM, respectively. Final inoculation density of 15 µl/ml produced optimum and consistent results in all studies. Growth and embryo development, however, have been observed when inoculated at from 5-40 µl/ml. The increase of sucrose from 1 to 3%, or inositol to levels reported by Gupta and Durzan (1986), produced no significant increase in growth or embryo numbers.

Maturation of the suspension-grown bipolar embryos has been achieved following removal from liquid media to abscisic acid treatments. Smooth early-stage embryos developed cotyledonary nodes and a clearly visible shoot apex 21 days after transfer from suspension culture to cheese cloth pads saturated with liquid HM (9) medium containing 1 µM IBA and 5 µM ABA (Fig. 5A-C). Initial studies suggest development can be enhanced by sequential treatments. Elongated embryos and plantlets have been produced by subculture from proliferation medium to liquid ABA maturation treatments followed by transfer to agar medium lacking growth regulators (Fig. 6A, B). Plantlet development has occurred only outside the liquid environment.

Summary

Our results show that Norway spruce suspensions can routinely be established and maintained as rapidly growing long-term cultures. The maintenance medium provides for continuous production of early-stage somatic embryos which correlate in

number with total tissue growth. Studies to increase maturation and conversion are in progress.

Acknowledgment

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References

1. Hakman I, Fowke WC, von Arnold S, Eriksson T. (1985) The development of somatic embryos in tissue cultures initiated from immature embryos of Picea abies (Norway spruce). Plant Sci. 38:53-9

2. Nagmani R, Bonga JM (1985) Embryogenesis in subcultured callus of Larix decidua. Can J For Res 15:1088-91

3. Gupta PK, Durzan DL (1986) Plantlet regeneration via somatic embryogenesis from subcultured callus of mature embryos of Picea abies (Norway spruce). In Vitro 22:685-8

4. Nagmani R, Becwar MR, Wann SR (1987) Single cell origin and development of somatic embryos in Picea abies (L.) Karst. (Norway spruce) and P glauca (Moench) Voss (white spruce). Plant Cell Report 6: 157-9

5. Lu Chin-Yi, Thorpe TA (1987) Somatic embryogenesis and plantlet regeneration in cultured immature embryos of Picea glauca. L Plant Physiol 128: 297-302

6. Durzan DJ, Gupta PK (1987) Somatic embryogenesis and polyembryogenesis in Douglas-fir cell suspension cultures. Plant Science 52:229-235

7. Hakman I, Fowke LC (1987) An embryogenic cell suspension culture of Picea glauca. Plant Cell Reports 6:20-22

8. Gupta PK, Durzan DJ (1986) Isolation and cell regeneration of protoplasts from sugar pine (Pinus lambertiana). Plant Cell Reports 5:346-348

9. Verhagen SA, Wann SR (1988) Norway spruce somatic embryogenesis: high frequency induction from light initiated mature embryo callus. Submitted to Plant Cell, Tissue and Organ Reports, April, 1988

10. Amerson HV, Frampton LJ, McKeand SE, Mott RL, Weir RJ (1985) Loblolly pine tissue culture: IN: Tissue Culture in Forestry and Agriculture, Basic Series 32(19):271-87, Plenum Press, NY

FIGURE CAPTIONS

Fig. 1. Proliferating Norway spruce somatic embryos in suspension maintenance medium. Note dense embryonal heads composed of small cytoplasmic cells and suspensors with elongated vacuolated cells. Bar = 0.5 mm.

Fig. 2. Fresh weight growth curve of newly established (2 mo. old) Norway spruce embryogenic culture. (0.5 BLG medium with 10.7 µM NAA and 5.4 BA).

Fig. 3. Growth curve and somatic embryo yield of Norway spruce suspension culture grown on 0.5 BLG medium with 10.7 µM NAA and 5.4 µmM BA.

Fig. 4. Dry weight growth curves of Norway spruce suspension culture grown on 0.5 BLG with 2 levels of growth regulators, NAA/BA.

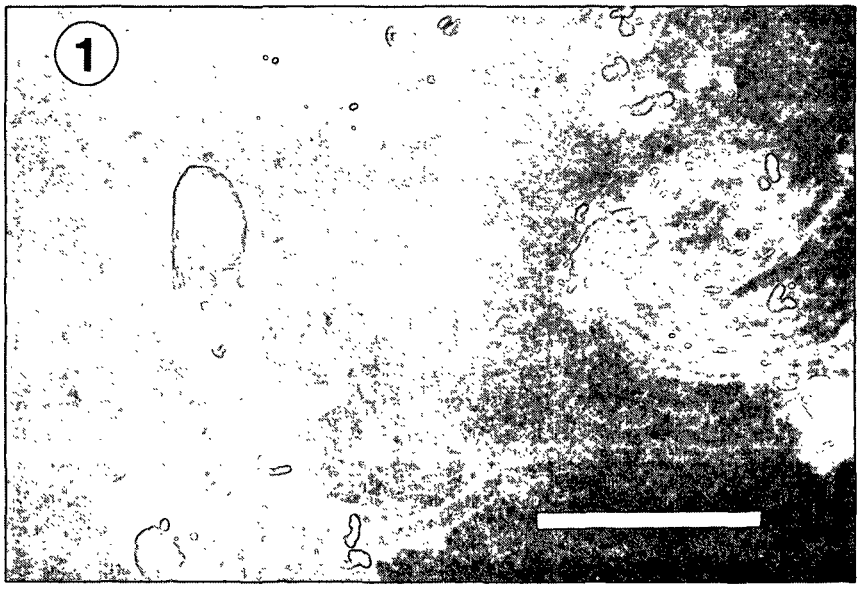
Fig. 5. (A) Selected bi-polar early somatic embryo transferred from liquid 0.5 BLG proliferation medium to cheese cloth pad saturated with HM maturation medium containing 1 µM IBA and 5 µM ABA. (B) Embryo development after 15 days. (C) Development after 21 days. Bar = 0.5 mm.

Fig. 6. Norway spruce somatic embryo development. (A) Elongating embryo and (B) developing plantlet after transfer to agar medium. Bar = 1 mm.

Table 1. Effect of NAA/BA levels on somatic embryo yield (number/ml) over time. Basal medium = 0.5 BLG.

Time, day	NAA/BA (μM)	
	10.7/5.4	5.4/2.2
0	14.6 ± 11.9	14.6 ± 11.9
1	23.3 ± 4.5	19.0 ± 4.4
6	41.7 ± 17.0	68.3 ± 14.2
12	97.3 ± 13.0	79.7 ± 33.7
18	51.7 ± 36.3	61.7 ± 34.7

$\bar{x}$ ± SD, N = 3



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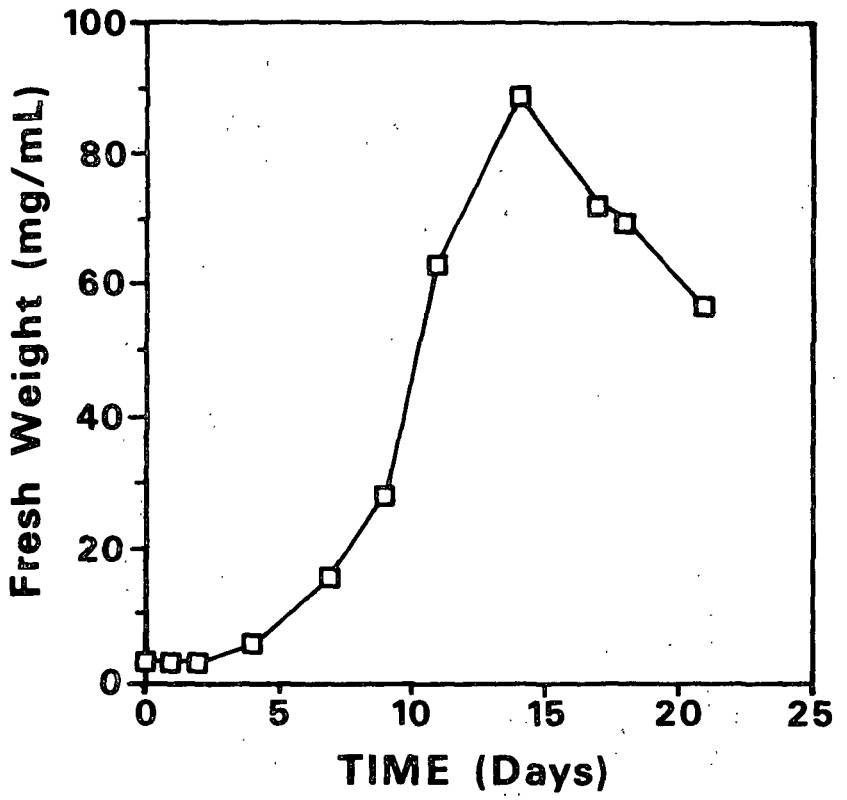

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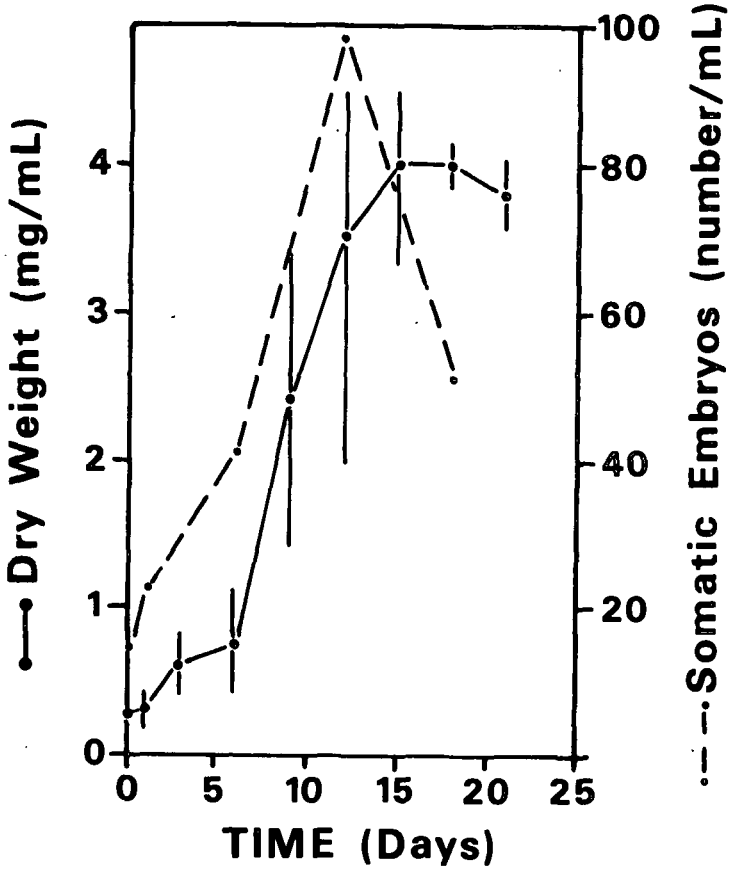

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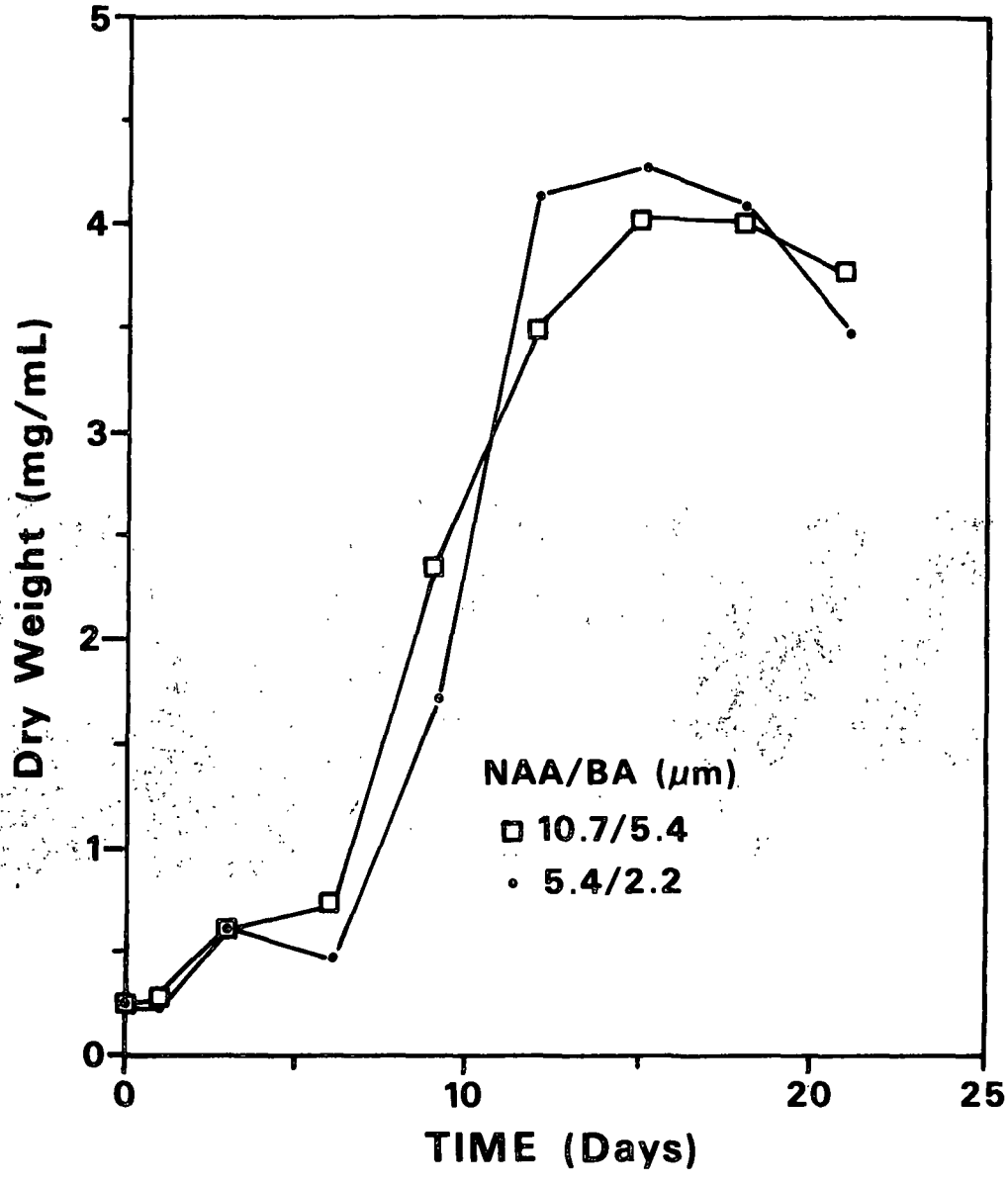
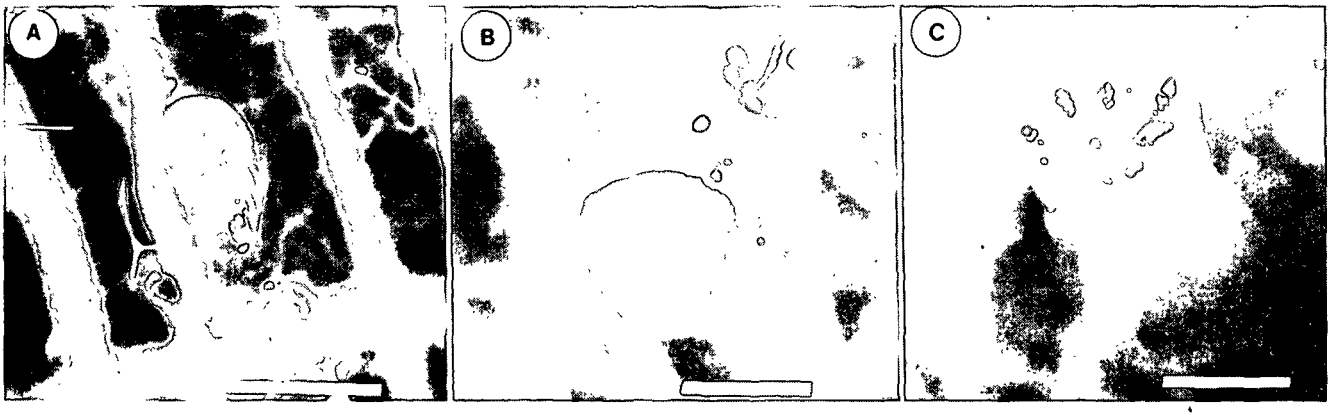


Fig. 4. Dry weight growth curves of Norway spruce suspension culture grown on 0.5 BLG with 2 levels of growth regulators, NAA/BA.



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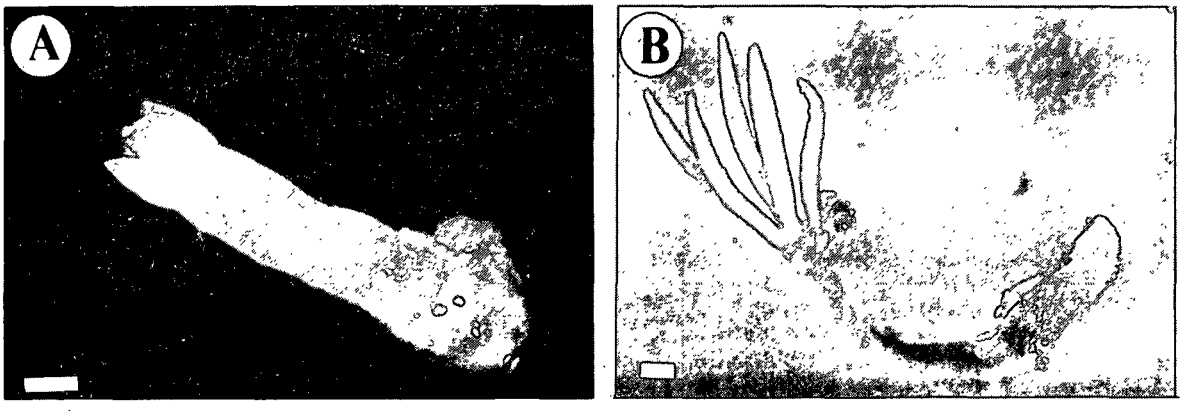

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