without any post-fixation treatment or after immersion in 4% formaldehyde at 52 °C after acid-alkaline steps.

Results and discussion. Coriphosphine-O staining shows a red-orange colour on all autosomes in both species. The X chromosome also does not reveal differences between the species, the short arm showing a red-orange colour like the autosomes and the long arm the following pattern: the proximal and distal areas are red-orange, the interstitial region being bright yellow-green. The long arm of the Y chromosome reveals the same staining reaction in the 2 species and appears to be identical to the homologous portion of the X, while the short arm is grey-orange in *A. atroparvus* and bright yellow-green in *A. labranchiae*. This portion (figure), reveals a clearcut difference between the species.

Coriphosphine-O is a metachromatic dye already employed as stain for nucleic acids in fixed preparations[17,18]. A red-orange fluorescence is emitted when the fluorochrome is bound to RNA and a yellow-green fluorescence when it is bound to native, double-stranded DNA. Treatments producing denaturation and/or depolymerization of DNA also induce a red-orange fluorescence when the fluorochrome is bound to such a nucleic acid[19]. On the other hand, formaldehyde is known both to prevent reassociation of single polynucleotide chains after heat or high-pH denaturation, and to stabilize deoxyribonucleoproteins[20,21].

Control experiments show all chromosomal DNA in its native state (green colour) in untreated preparations, while the use of formaldehyde after the alkaline step shows denaturation of all DNA (red colour). We therefore assume that DNA strandedness is the fundamental factor causing the differential staining. Our results, then, show that in the short arm of the Y resides the only chromosomal difference demonstrable with a technique whose different staining reactions could reveal specific types of DNA or at least a particular DNA/protein packing. This cytological difference is particularly interesting when compared to the data of Jayakar et al.[22], who proposed an X-Y incompatibility mechanism as the genetical basis of sterility of the F_1 hybrid males which have an X chromosome of *A. atroparvus* and a Y chromosome of *A. labranchiae* or vice-versa. Fraccaro et al.[9], moreover, state that '... behavioural polymorphism stenogamy-eurygamy is obviously controlled by one or more genes located on the Y chromosome'.

We cannot say whether our results indicate a differential spiralization and heterochromatinization in the short arm of the Y chromosome between the 2 sibling species, given that the correlations between banding techniques and heterochromatin structure are intricate. Nor can we affirm that genetic differences between *A. atroparvus* and *A. labranchiae* are located in the short arm of the Y chromosome, although we believe that these results support the involvement of this chromosome in the differentiation of these 2 species.

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2 U. Bianchi, Riv. Parasit. *29*, 221 (1968).
3 J.B. Kitzmiller, Adv. Genet. *18*, 315 (1976).
4 G. Frizzi, Scientia genet. *3*, 67 (1947).
5 J.B. Kitzmiller, G. Frizzi and R.H. Baker, in: Genetics of Insect Vectors of Disease, p. 151. Ed. J.W. Wright and R. Pal. Elsevier, London 1967.
6 U. Bianchi, Nature Lond. *217*, 382 (1968).
7 M. Coluzzi, Misc. Publs ent. Soc. Am. *7*, 63 (1970).
8 A. Corradetti, Riv. Malar. *13*, 707 (1934).
9 M. Fraccaro, L. Tiepolo, U. Laudani, A. Marchi and S.D. Jayakar, Nature, Lond. *265*, 326 (1977).
10 R. Mezzanotte and L. Ferrucci, It. J. Zool., in press.
11 L. Tiepolo, G. Diaz and U. Laudani, Chromosoma *45*, 81 (1974).
12 M. Fraccaro, U. Laudani, A. Marchi and L. Tiepolo, Chromosoma *55*, 27 (1976).
13 A. Farci, U. Laudani and A.R. Lecis, Z. zool. Syst. Evolut.-Forsch *II*, 304 (1973).
14 F.E. Arrighi and T.C. Hsu, Cytogenetics *10*, 81 (1971).
15 S. Pimpinelli, G. Santini and M. Gatti, Chromosoma *57*, 377 (1976).
16 S.A. Keeble and R.F. Jay, Nature, Lond. *193*, 695 (1962).
17 D. Nash and W. Plaut, J. Cell Biol. *27*, 682 (1966).
18 J.E. Mayfield and J.R. Ellison, Chromosoma *52*, 37 (1975).
19 F.H. Kasten, in: Encycl. of Microscopy and Microtechnique, p. 5. Ed. P. Gray. Van Nostrand, New York 1973.
20 C.A. Thomas, Jr, and K.J. Berns, J. molec. Biol. *4*, 309 (1962).
21 R. Rigler, D. Killander, L. Bolund and N.R. Ringertz, Exp. Cell Res. *55*, 215 (1969).
22 S.D. Jayakar, U. Laudani, A. Marchi, L. Tiepolo and M. Fraccaro, J. Genet. *63*, 1 (1977).

Regeneration of plants from mesophyll protoplasts of *Atropa belladonna*[1]

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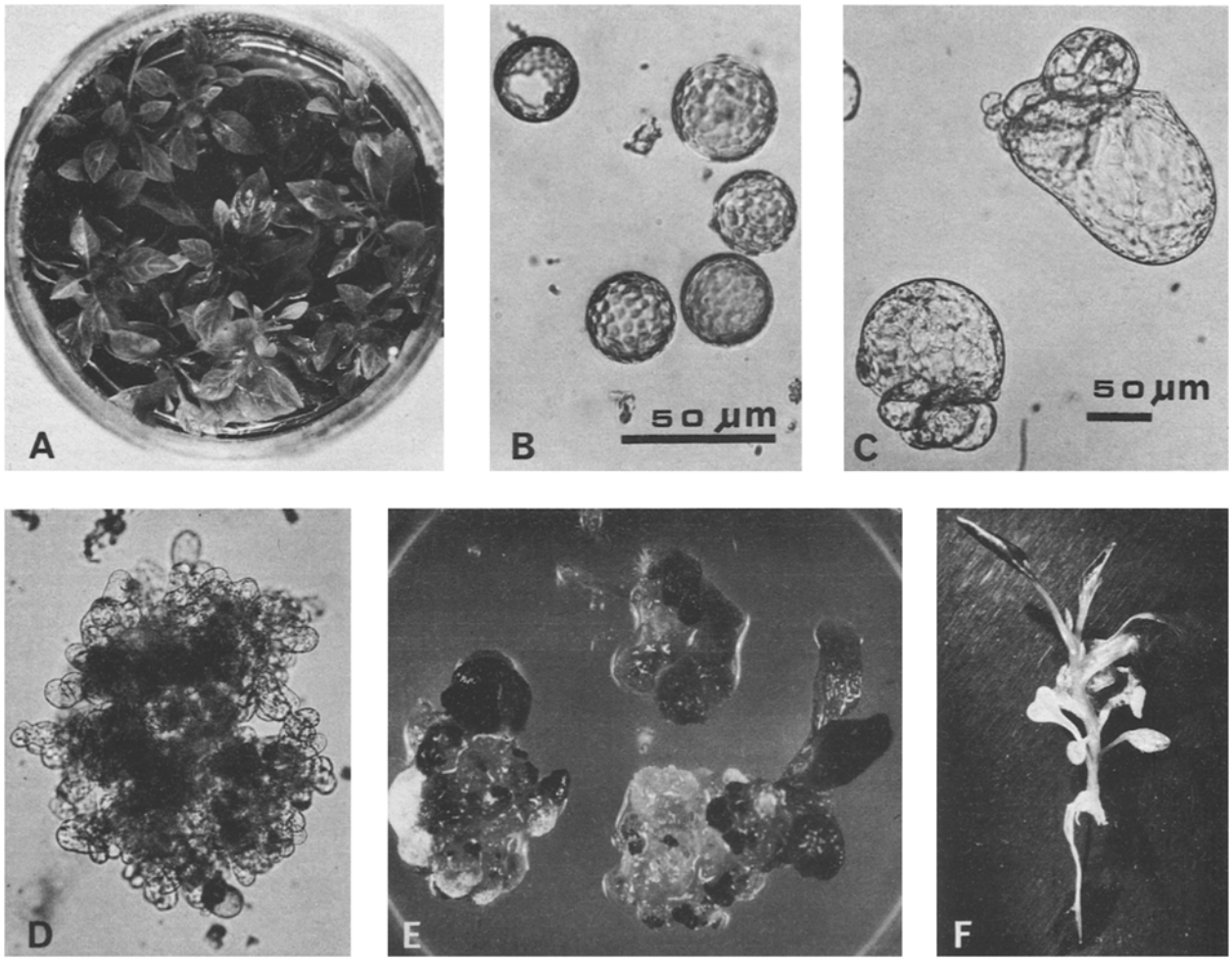
Summary. Isolated leaf mesophyll protoplasts of *Atropa belladonna* when cultured in defined liquid culture media regenerate cell walls, divide and form calli. Subsequent induction of shoot and root organogenesis leads to plantlets which grow to maturity after transfer to soil.

Isolated plant protoplasts with high viability and regeneration capacity (totipotency) are a prerequisite for genetic modification studies. Further progress in somatic hybridization between incompatible non-related species is likely in the near future within the family of Solanaceae, since protoplasts of various members of this group can already be regenerated to plants. Plantlet formation from *Atropa belladonna* cell culture-derived protoplasts has also been described[2]. However, because of desired visible markers for protoplast fusion studies and possible genetic instability of long-term cell cultures[3], we have concentrated our efforts on mesophyll protoplasts.

In vitro shoot cultures[4] were established from sterile seedlings and were subcultured at 4 weeks intervals on MS medium[5] supplemented with 0.5 mg/l BAP[6] and maintained at 25 °C, 12 h 3000 lux. 2–3 weeks after subculture of the shoots, excised leaves were cut into 0.5 mm strips and incubated for 2.5 h at 27 °C in enzyme mixture (0.5% macerozyme R 10 + 0.5% cellulase R 10 + 0.2% hemicellulase) dissolved in osmoticum (0.4 M mannitol + 5 mM



Plant regeneration from *Atropa belladonna* mesophyll protoplasts. *A* In vitro shoot culture; *B* freshly isolated protoplasts; *C* cell growth and first cell divisions; *D* protoplast derived callus in liquid medium; *E* differentiation of shoot primordia; *F* protoplast-derived plantlet before transfer to soil.

$CaCl_2$, pH 5.7). Resulting protoplasts were filtered through 100 and 50 µm steel sieves and thereafter washed twice with osmoticum and once with culture medium by repeated centrifugation at $50 \times g$ for 5 min.

Culture experiments were performed with different basal media (AE, DPD, KM, MS, NN, NT, V47)[5]; suitable phytohormone concentrations were screened in a 10-step dilution series. The protoplasts were cultured in thin layers of liquid culture medium in petri dishes, kept in humidified plastic boxes, first day at 12 °C in the dark, thereafter at 24 °C, 12 h 800 lux. While survival of protoplasts and cell wall regeneration occurred in different media, sustained divisions were more frequently observed in NT medium[5] when supplemented with 4 mg/l KIN, 1 mg/l CPA and 0.4 M mannitol. Under these conditions, over a wide range of protoplast density more than 90% of the plated protoplasts formed a cell wall and enlarged in size. Cell division however, when tested in a protoplast density dilution series, was restricted to a range of $1.3\text{–}3 \times 10^5$ protoplasts/ml. At this population density 20–30% of regenerated cells divide between the 5th and 12th day of culture and form cell colonies. Fast growing regenerants (about 5 mm in diameter) were transferred about 3–4 weeks after protoplast isolation from the primary culture medium immediately to differentiation media. Small calli were kept for further growth in NT medium without osmoticum and half-strength phytohormone concentration. Appropriate media to induce shoot organogenesis were either MS media containing only cytokinin as phytohormone (0.1–0.2 mg/l) or a combination of auxin and kinetin (4 mg/l IAA and 2.5 mg/l KIN). By subsequent culture on Knop medium supplemented with thiamine · HCl (1 mg/l) or hormone-free half strength MS medium, adventitious roots were formed. Rooted plantlets were then transplanted to soil and grown to maturity.

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2 G. Gosch, Y.P.S. Bajaj and J. Reinert, Protoplasma *86*, 405 (1975).

3 F. D'Amato, Proc. 4th int. Congr. Pl. Tissue Cell Culture, Calgary 1978 (in press).

4 H. Binding, Physiologia Pl. *35*, 225 (1975).

5 Culture media abbreviations: AE S. Arnold and T. Eriksson, Physiologia Pl. *39*, 257 (1977); DPD J. Durand, I. Potrykus and G. Donn, Z. Pflanzenphysiol. *69*, 26 (1973); KM K.N. Kao and M.R. Michayluk, Planta *126*, 105 (1975); MS T. Murashige and F. Skoog, Physiologia Pl. *15*, 473 (1972); NN J.P. Nitsch and C. Nitsch, Science *163*, 85 (1969); NT T. Nagata and I. Takebe, Planta *99*, 12 (1971); V47 H. Binding, Z. Pflanzenphysiol. *74*, 327 (1974).

6 Phytohormone abbreviations: BAP 6-benzylaminopurine; CPA p-chlorophenoxy acetic acid; IAA indole acetic acid; KIN 6-furfurylaminopurine.