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Isolation and characterization of intergeneric somatic hybrids in the Apocynaceae family

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Summary. Protoplasts were isolated from callus cultures of *Rauwolfia serpentina* Benth., *Rhazya stricta* Decaisne, and *Catharanthus roseus* (L.) G. Don, or from leaves of *Vinca minor* L. Protoplast isolation, culture, and fusion techniques as well as hybrid screening systems were developed for these species, and hybrids were obtained. Hybrid combinations were *Rauwolfia* + *Vinca*, *Rauwolfia* + *Catharanthus*, *Rauwolfia* + *Rhazya*, and *Catharanthus* + *Vinca*. For hybrid isolation, the physiological complementation method was utilized. Analyses of the material obtained included a cytogenetic study of the chromosomes, a study of multiple molecular forms of transferases and esterases, and the blot hybridization of restricted nuclear DNA using ribosomal DNA as a probe. Hybrids were identified in all species' combinations tried. A ten-fold increase in the accumulation of raucaffricine (relative to the parental *Rauwolfia* strain) was observed in one cell line of the *Rauwolfia* + *Vinca* hybrid. Our studies indicated the genetic stability of the great majority of the hybrid cell lines over a period of more than 20 months of in vitro growth. No shoot morphogenesis has so far been observed in this material.

Key words: Protoplast fusion – Somatic hybrid – Indole alkaloid – Isozyme spectrum – Ribosomal DNA

Introduction

Several species of the Apocynaceae family (*Rauwolfia serpentina* Benth., *Catharanthus roseus* (L.) G. Don, *Vinca minor* L., etc.) are used as the raw material for antihypertonic and antineoplastic medicine preparation (Balsevich 1988; DeLuca and Kurz 1988). Secondary metabolites detected either in intact plants or in callus and suspension cultures of these species represent a large and diverse group of the monoterpene indole alkaloids (Balsevich 1988). Other Apocynaceae species, such as *Rhazya stricta* Decaisne, has been used in folk medicine in Pakistan and India (Chopra et al. 1956). Despite various cytological, biochemical, and physiological studies on in vitro cells of *R. serpentina* (Zenk and Deus 1982; Kutchan et al. 1988), *C. roseus* (Cresswell et al. 1988), and *V. minor* (Takeuchi and Komamine 1981; Sakushima and Nishibe 1988), there has been little or no work conducted on protoplasts or somatic hybridization in Apocynaceae (Gleba and Sytnik 1984). This paper presents the results of studies on protoplast culture that were conducted for four Apocynaceae species: *R. serpentina*, *C. roseus*, *V. minor*, *Rh. stricta*. Also, somatic hybridization was studied using protoplast fusion in: *R. serpentina* + *V. minor*, *R. serpentina* + *C. roseus*, *R. serpentina* + *Rh. stricta*, and *C. roseus* + *V. minor*.

Materials and methods

Callus strains of *R. serpentina*, *Rh. stricta* and *C. roseus* were cultivated in the dark at 26 °C on 4 × (Sidorov et al. 1985) solid medium containing 2.0 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mgl^{-1} indole-3-acetic acid (IAA), 0.5 mgl^{-1} 1-naphthaleneacetic acid (NAA), 0.2 mgl^{-1} kinetin, and 8.0 gl^{-1} agar (Serva). *V. minor* plants were grown in the greenhouse at 25 °C, 2000–3000 lux, and a 16-h photoperiod.

Pieces of calli (0.5–1 cm) were placed into the following enzyme mixtures: (a) *R. serpentina* and *C. roseus* – 0.15% cellulase "Onozuka R-10" (Serva Feinbiochemica, Heidelberg, FRG), 0.20% "Driselase" (Sigma Chemical Co, St Louis, Mo., USA), 0.10% "Macerozyme R-10" (Serva), 50 ml 0.45 *M* sucrose, and 50 ml W5 medium (Medgyesy et al. 1980); (b) *Rh. stricta* – 0.75% cellulase "Onozuka R-10" (Serva), 0.35% cellulase "Cellulysin TM" (Calbiochem, USA), 0.25% "Macerozyme

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R-10" (Serva), 0.25% "Macerase" (Serva), and 1% sucrose. All components were dissolved in W5 medium. Enzyme treatment was carried out in the dark at 26 °C for 18–24 h.

Protoplasts were purified by filtration-centrifugation and then washed 3 times with W5 medium. Young leaves of *V. minor* were sterilized in 70% ethanol (30–50 sec) and 3% sodium hypochlorite (5–10 min), washed in sterile distilled water, and transferred onto solid MS hormone-free medium or MS medium containing 0.2–1.0 mgl^{-1} kinetin (Murashige and Skoog 1962). They were incubated for 1–5 days under dark or light conditions (2000–3000 lux, 16 h photoperiod) and then put into an enzyme mixture containing 0.3% cellulase "Onozuka R-10", 0.4% "Driselase", 0.2% "Macerozyme R-10", 0.45 *M* mannitol, 0.10% glucose, 0.10% sucrose, and 0.055% calcium chloride. Enzyme treatments and protoplasts washing were performed as previously described. Nutrient media (1) MS, (2) 4×, (3) Schenk and Hildebrandt (modified), and (4) Caboche (1980) (modified) were tested for protoplast culture. Protoplasts of *C. roseus* and *Rh. stricta* synthesized cell walls and formed cell colonies on media (2) and (4). *R. serpentina* protoplasts were capable of dividing (irregularly and at a low frequency) only on (2) medium. Medium (3) was superior for *Vinca* protoplasts.

Protoplast fusion was induced using the high PEG – high Ca^{++}-high pH method. To select hybrids in the *Rauwolfia* + *Vinca* combination, we used the PEG (MW 4000–6000)-high pH protocol, which eliminated sensitive *Vinca* mesophyll protoplasts, and then cultivated the remainder in medium (3) where *Rauwolfia* protoplasts did not develop. In other combinations, the treatment with monoiodacetate or iodacetamide (Serva), 0.10–0.15% solution in W5 (Sidorov et al. 1980) of *C. roseus* and *Rh. stricta* protoplasts prior to fusion, was successful.

The following analyses were performed on the material obtained: a study of the transferases and esterases (Brewer 1970), blot hybridization of restricted nuclear DNA using ribosomal DNA as a probe (Shure et al. 1983; Kolosha and Fodor 1986), and a cytogenetic study of chromosomes (combination 1, ten independent lines). Callus and root tips for chromosome analysis were treated with 0.05% colchicine (Fluka, Switzerland), fixed with acetoalcohol (1:3), and stained with 1.5% acetoorcein (Merck, FRG).

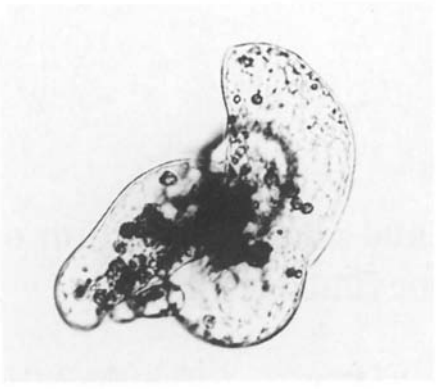

Fig. 1. Cell colony developed from *Rauwolfia* protoplast. Obj. ×20, oc. ×6.3

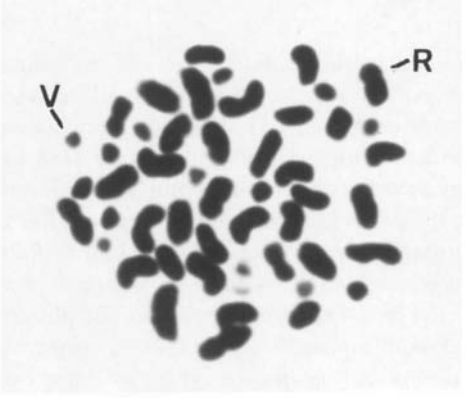

Fig. 2. Metaphase chromosomes of hybrid cell (*Rauwolfia serpentina* + *Vinca minor*). *R Rauwolfia* chromosomes, *V Vinca* chromosomes

Results and discussion

Protoplast isolation and culture

Isolated callus protoplasts of *Rauwolfia* when cultured in liquid 4× medium supplemented with 0.5 *M* mannitol divided irregularly and at a low percentage (0.01–0.05%). First divisions were usually observed on the 20–22nd day after culture initiation. By the end of the 6th week, cell colonies (Fig. 1) could be transferred to solidified 4× medium. Isolated callus protoplasts of *Catharanthus* and *Rhazya* divided in 4× medium on the 15–17th and 17–20th days, respectively; after 5–6 weeks, they were transferred to solidified 4× medium. *Vinca* leaf mesophyll protoplasts were successfully cultured in modified Schenk and Hildebrandt's medium (2 mg/l 2,4-D, 0.5 mg/l I-NAA, 0.5 mg/l 3-IAA, 0.2 mg/l kinetin, 0.2 mg/l 6-BAP), and first divisions were detected after 17–22 days (after 2 weeks, new portions of fresh medium were added). By the end of the 2nd month, cell colonies were formed that could be transferred to solidified media.

Somatic hybridization and analysis of the obtained material

In our preliminary experiments, *Rauwolfia* protoplasts would not divide in the modified Schenk and Hildebrandt's medium. On the basis of this information and by eliminating the majority of *Vinca* mesophyll protoplasts with "strong" PEG treatment, we expected the survival of hybrid fusion products only in this species combination. First divisions were observed on the 9–14th days after fusion. In total, about 5,000 colonies were obtained.

Esterase and transferase studies indicated clear differences in the PAAG isozyme patterns between parental species. More than 200 cell lines obtained from the fusion of *Rauwolfia* and *Vinca* protoplasts were studied: 51.2% were identified as hybrids (Fig. 3A), 26.8% exhibited pure specific *Rauwolfia* bands (although in control cultures, protoplasts of *Rauwolfia* failed to divide), and 4.8% demonstrated *Vinca*-type transferase spectra. Cytogenetic studies revealed mixoploidy in hybrid cell lines as well as in the *Rauwolfia* parental cell line.

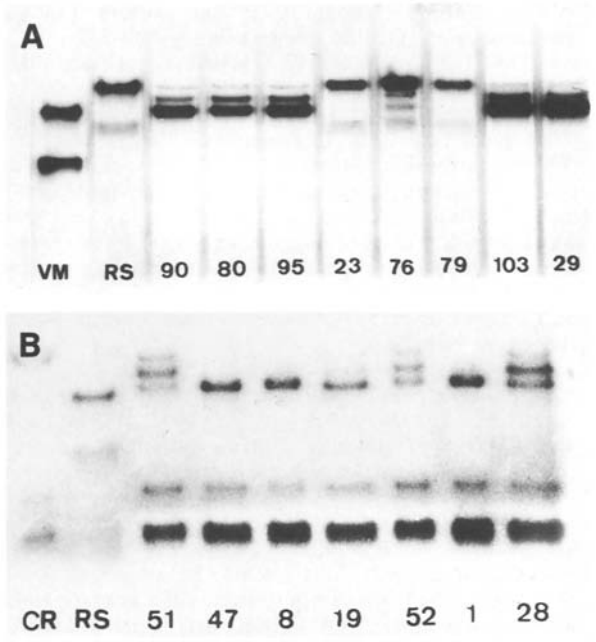

Fig. 3. **A** Aspartate aminotransferase patterns: *RS Rauwolfia serpentina, VM Vinca minor, 90, 80, 95, 23, 76, 79, 103* are clones derived from protoplast fusion. **B** Aspartate aminotransferase isozyme patterns of *Catharanthus roseus* (*CR*), *Rauwolfia serpentina* (*RS*) and their somatic hybrids (clones *51, 47, 8, 19, 52, 1, 28*)

The presence of both parental chromosomes with quantitative predominance of *Rauwolfia* chromosomes has been demonstrated with chromosome numbers varying between 30 and more than 200 (Fig. 2). Hybrid clones were verified by blot hybridization analysis using fragments of lemon rDNA (Kolosha and Fodor 1986) as a probe. In this study, one cell line (line 76) was found that apparently exhibits rDNA recombination (Fig. 4A).

In *Rauwolfia* + *Catharanthus* and *Rauwolfia* + *Rhazya* we developed a physiological complementation method for hybrid isolation based on the low viability of *Rauwolfia* protoplasts and the inactivation of protoplasts of the second fusion partner with IIA or MIA (Sidorov et al. 1980). Of the 78 cell lines analyzed in *Rauwolfia* + *Catharanthus* (Fig. 3B), 53 hybrids were detected, whereas only 4 hybrid cell lines out of the 70 lines tested in the *Rauwolfia* + *Rhazya* combination were identified on the basis of data obtained from transferase isozyme studies.

Similarly, 8 hybrid clones were identified after the screening of 15 cell colonies developed from the fusion of *Catharanthus* + *Vinca*.

Relatively long periods (9–15 days) were required for all fusion combinations before the first cell divisions were observed. During the first 3–4 weeks, cell microcolonies grew slowly. It was necessary to dilute them 1–3 times

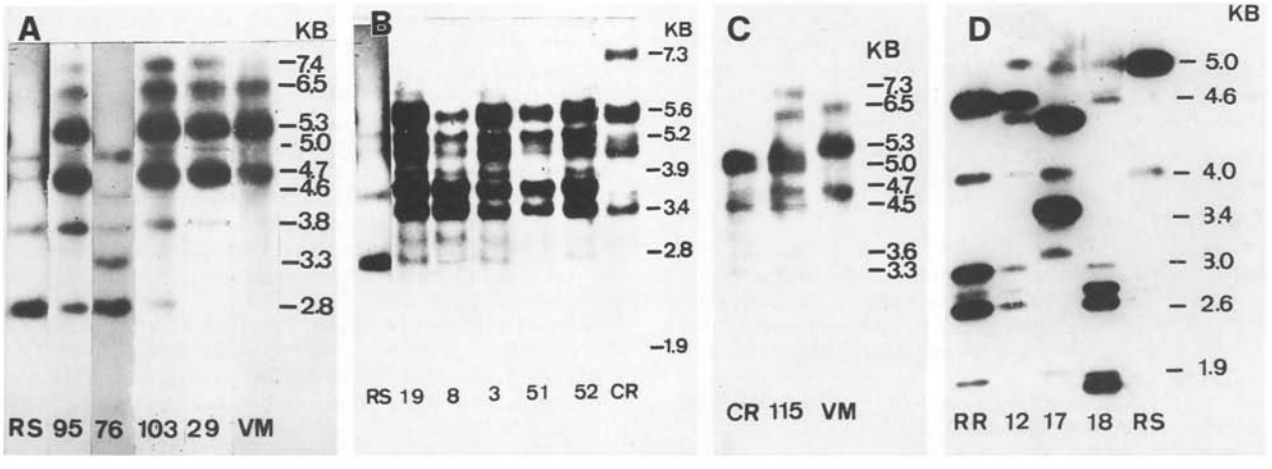

Fig. 4. **A** Blot hybridization of lemon rDNA probe with BamHI- and EcoRV-restricted nuclear DNA from cells of hybrid clones produced by protoplast fusion in *Rauwolfia* + *Vinca* (clones *95, 76, 103, 29*), and from parents (*VM Vinca minor, RS Rauwolfia serpentina*). **B** Blot hybridization of lemon rDNA as a probe with BamHI- and EcoRV-restricted nuclear DNA from cells of hybrid clones produced by protoplast fusion in *Rauwolfia* + *Catharanthus* (*19, 8, 3, 51, 52*), and from parents (*Rauwolfia serpentina* (*RS*) and *Catharanthus roseus* (*CR*)). **C** Blot hybridization of lemon rDNA as a probe with BamHI- and EcoRV-restricted nuclear DNA from cells of the hybrid clone produced by protoplast fusion in *Catharanthus* + *Vinca* (*115*), and from parents (*Catharanthus roseus* (*CR*) and *Vinca minor* (*VM*)). **D** Blot hybridzation of lemon rDNA as a probe with EcoRI- and EcoRV-restricted nuclear DNA from cells of hybrid clones produced by protoplast fusion in *Rauwolfia* + *Rhazya* (*12, 17, 18*) and from parents (*Rauwolfia serpentina* (*RS*) and *Rhazya stricta* (*RR*))

with portions of fresh medium with a gradually reduced mannitol concentration before transferring them to solidified 4 × or modified Schenk and Hildebrandt media. The callus cell strains formed differed in their morphology, speed of growth, spontanious root formation, and sensitivity to growth regulators and to light, especially in first combination. In some cases these differences were parallelled by differences in their biochemical and blot hybridization patterns. All attempts to regenerate hybrid plants have so far failed. In blot hybridization studies on all combinations, the presence of parental characteristic bands in the tracks of the hybrid cell lines was observed (Fig. 4 B, C, D).

A wide diversity in the characteristics of cultivated plant cells and somatic hybrids, especially during undifferentiated growth in vitro, has bee reported in a variety of plant material (Kao et al. 1974; Nagy et al. 1983; Fujita et al. 1985). Some were studied in detail by isozyme, chromosome number, and secondary metabolite analyses (Petiard et al. 1985; Kobayashi 1987).

Thus, the stability of hybrid callus cell lines is of interest in somatic hybridization studies. During 20–22 months of culture, transferase isozyme and blot hybridization analyses were repeated several times for a total of 52 cell lines representing all possible fusion combinations. The characteristic patterns of the absolute majority of all cell lines during this period remained constant.

Preliminary studies of indole alkaloid content indicated a ten-fold increase in accumulated raucaffricine in clone 27 of *R. serpentina* + *V. minor* when compared to the parental *Rauwolfia* callus line (data not presented).

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