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Isolation and Culture of Protoplasts from Callus of Four Cultivars of *Hibiscus rosa-sinensis*

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The genus *Hibiscus* (family Malvaceae) is comprised of about 300 species, among them the most well known and extensively cultivated is *Hibiscus rosa-sinensis*, an ornamental plant producing large and bright red blooms[1]. But this species has weak response to cold tolerance[2], and it requires the protection of a glasshouse to endure the coldness of winter in temperate zones. In contrast, *Hibiscus syriacus* L., a perennial deciduous shrub, can withstand a considerable amount of frost[3]. Genetic improvement of *H. rosa-sinensis* for increased chilling tolerance by cell fusion with *H. syriacus* requires an effective protoplast isolation, culture, and regeneration system. Moreover, the selection of certain cultivars of *H. rosa-sinensis* which are easier to be manipulated *in vitro* is the first step of the establishment of *in vitro* protoplast system.

Four cultivars of glasshouse grown *Hibiscus rosa-sinensis*, Brilliant, Brilliant Red, Okinawa 1 and Okinawa 2, were used in this study.

Healthy young leaves were excised from plants and surface-sterilized by 0.5% sodium hypochlorite solution, washed three times with sterile distilled water and then cut into approximately 1×1 cm pieces. Callus was initiated on MS medium[4] supplemented with 0.5 mg/*l* 2, 4-dichlorophenoxyacetic acid, 0.1 mg/*l* kinetin, 3% sucrose and 0.8% agar at pH 5.8 (MS-DK) and incubated in the dark at 25°C. Callus was subcultured periodically at one week intervals on the same medium.

For protoplast isolation, 2-3 g of one-week-old callus cultures were incubated in CPW solution[5] with 10% sorbitol (CPW-10S), containing 0.5% cellulase Onozuka RS and 0.2% pectolyase Y-23 and agitated at 30 rpm for 15 h in the dark at 25°C. Protoplasts were purified by filtration through a nylon mesh (100 μm) and washed three times with CPW-10S by gentle centrifugation (80×*g*, 5 min.). The viability of protoplasts was examined by staining with fluorescein diacetate (FDA)[6].

Protoplasts at a density of 5×10^4 per m*l* were cultured in 1 m*l* of MS-DK liquid medium containing 0.4 M mannitol, with or without supplement of 2% dimethyl sulfoxide (DMSO)[7] in the dark at 25°C. During incubation, 0.25 m*l* each of fresh dilution medium (the same as culture medium but mannitol was omitted, and 5 mg/*l* vitamine C and polyvinylpyrrolidone was included)[8] was added to the culture at 5th and 10th day to reduce the mannitol concentration.

Protoplasts were reproducibly obtained from callus cultures of four cultivars after treatment with the enzyme solution. The number of protoplasts isolated from Okinawa 1, Okinawa 2 and Brilliant Red calli was similar, ranging in 1.1-1.6×10^6 /g, whereas the yield from the callus of Brilliant was

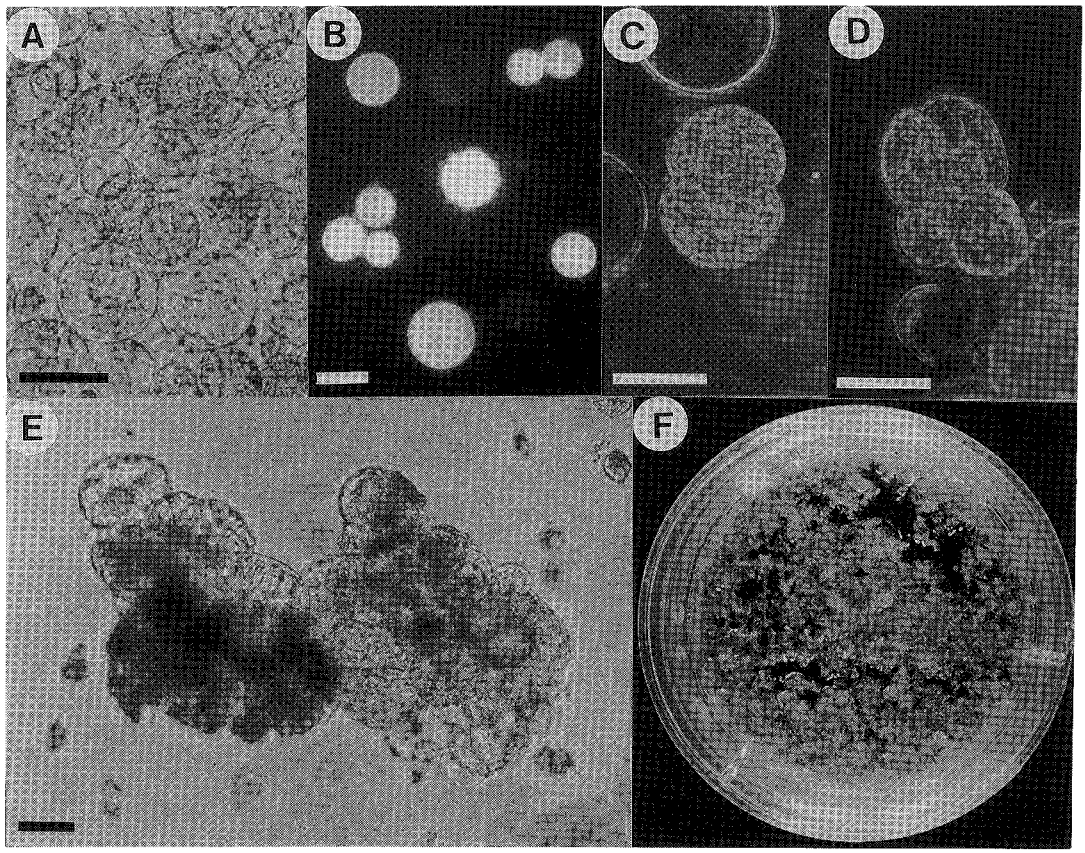

Fig. 1 Isolation and culture of protoplasts of *Hibiscus rosa-sinensis* cv. Okinawa 1 (bar=50 μm).
A : Freshly isolated protoplasts.
B : Fluorescence of protoplasts stained with FDA.
C : First division of protoplast at 3rd day.
D : Second division at 7th day.
E : Small colonies formed after 20 days.
F : Callus formed after 40 days.

lower, being 7.5×10^5/g (**Table 1**). Freshly isolated protoplasts were spherical (**Fig. 1-A**, only Okinawa 1 is shown as an example) and about 95% protoplasts showed the viability by vigorously exhibiting fluorescence under a fluorescence microscope (**Fig. 1-B**).

By culture in medium containing DMSO, some protoplasts became oval shaped at 2nd day, indicating new cell wall formation. Protoplasts of Okinawa 1, Okinawa 2 and Brillinat Red underwent first cell division at the 3rd day (**Fig. 1-C**). The frequency of first cell division of Okinawa 1 at 6 days was 19.4%, being the highest among three cultivars (**Table 1**). On the medium lacking DMSO, however, the first cell division of protoplasts was observed only in Okinawa 1 cultures (**Table 1**). Protoplasts of Brilliant showed recalcitrant response to both DMSO minus medium and DMSO plus medium (**Table 1**). After reducing the concentration of mannitol in the medium, only Okinawa 1 protoplasts cultured on medium containing DMSO could undergo 2nd cell division at day 7 (**Fig. 1-D**) and continued cell division thereafter. On the other hand, although protoplasts of Okinawa 2 and Brilliant Red showed first cell division on medium containing DMSO, they ceased growing afterwards and subsequently became necrotic. Colonies of Okinawa 1 were observed after 20 days (**Fig. 1-E**) and callus was formed at 40 days (**Fig. 1-F**).

Table 1. Protoplast yield, first cell division frequency, and colony formation of four cultivars of *H. rosa-sinensis*.

Cultivar	Yield ($\times 10^5$)	First division frequency (%)		Colony formation	
	protoplasts/g fresh weight	MS medium −DMSO	MS medium +DMSO	−DMSO	+DMSO
Okinawa 1	12	5.7	19.4	−	+
Okinawa 2	16	nd	6.0	−	−
Brilliant Red	11	nd	3.0	−	−
Brilliant	7.5	nd	nd	−	−

nd: no cell division at 6 days.

The function of DMSO on protoplasts had been elucidated by Hahne and Hoffmann[7] that DMSO could initiate division of protoplasts by promoting cortical microtubule assembly. Of four cultivars examined so far, only Okinawa 1 protoplasts were able to continue cell division in MS inquid medium containing 2% DMSO and formed callus. DMSO had no/little effect on protoplast division of Brilliant, Brilliant Red and Okinawa 2. Although the reason for this phenomenon was unclear, protoplasts from different genotypes certainly had different responses to nutritional conditions. This study clearly showed that Okinawa 1 could be a good candidate for protoplast culture and cell fusion studies. Since there are multiple factors involved in protoplast culture and regeneration processes, therefore it is necessary to improve Okinawa 1 protoplast culture system by testing various culture conditions.

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We dedicate this work to the memory of late Mr. Tadashi Yonehara who did a pioneering work of *Hibiscus* cell culture in our laboratory.

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《和文要約》

ハイビスカス(*Hibiscus rosa-sinensis*)の4品種のカルス由来プロトプラストの単離と培養

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ハイビスカスの4品種(沖縄1, 沖縄2, Brilliant, Brilliant Red)のカルスを酵素液(0.5% cellulase Onozuka RS, 0.2% pectolyase Y-23 及び 10% sorbitol を含む CPW 溶液)で処理し，プロトプラストを単離した．これを 2% DMSO, 0.5 mg/*l* 2, 4-D, 0.1 mg/*l* kinetin, 0.4 M mannitol を添加した MS 液体培地で培養したところ，3日目に沖縄1, 沖縄2, Brilliant Red のプロトプラストで第1回目の細胞分裂が観察された．その後は，沖縄1の細胞のみが分裂を続けカルスを形成した．