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Callus induction from protoplasts of *V. unguiculata, V. sublobata* and *V. mungo*

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Summary. Protoplasts were isolated from hypocotyl of *V. mungo* (L.) Hepper or hypocotyl-derived callus of *V. sublobata* (*Phaseolus sublobata* Roxb.) and *V. unguiculata* (L.) Walp (syn. *V. sinensis* (L.) Saviex Hassk) using an enzyme solution comprising Cellulase 2.5%, Macerozyme, Hemicellulase and Driselase each at a 0.5% level in 0.5 M sorbitol. Isolated protoplasts were cultured in Murashige and Skoog's (1962) basal liquid medium supplemented with BA, NAA, 2,4-D (1 mg/l each) and sucrose (14%). After four weeks, protoplast colonies were transferred to the same medium with a reduced level of sucrose (7%). Colonies proliferated into actively growing calli. Further attempts to regenerate plants from such calli were not successful. However, protoclones of *V. unguiculata* differentiated roots on auxin/cytokinin supplemented media. Alternative methods for shoot differentiation from protoplast-derived cultures were tried by the use of *Agrobacterium tumefaciens* "shooter" strains pGV 2215 or pGV 2298 or wild type strain B6S3.

Key words: Protoplasts – Legumes – *Vigna mungo* – *V. unguiculata* – *V. sublobata* – *Agrobacterium tumefaciens* – "Shooter" mutant

Introduction

Somatic hybridization following protoplast fusion is a valuable tool to combine genotypes which cannot be crossed sexually. Since the first report of sustained division and regeneration of plants from protoplasts in *Nicotiana tabacum* (Takebe et al. 1971) success has been achieved in many plant families (Davey 1983).

Regeneration to plants in legumes is as yet restricted to a few members, such as forage crops, namely *Medicago sativa* (Kao and Michayluk 1980; Dos Santos et al. 1980; Johnson et al. 1981; Xu et al. 1982b; Lu et al. 1982), *M. coerulea* and *M. glutinosa* (Arcioni et al. 1982), *Trigonella foenum-graecum* (Shekhawat and Galston 1983; Xu et al. 1982b), *Trifolium repens* (Gresshoff 1980) and *T. rubens* (Grosser and Collins 1984). Nevertheless, grain legumes which constitute the chief food crop are retractable to available culture strategies due to their limited regeneration potential, and hence pose a difficulty in using protoplast technology for their improvement. Successful regeneration has been reported only in *Vigna aconitifolia* (Shekhawat and Galston 1983; Krishnamurthy et al. 1984; Gill and Eapen 1986) and *Psophocarpus tetragonolobus* (Wilson et al. 1985). In view of this, extensive work on the isolation and culture of protoplasts needs to be taken up to develop the protoplast technology in legumes.

We have started investigations on protoplast culture and plant regeneration in different species of the genus *Vigna* and herein report callus induction in *V. unguiculata, V. sublobata* and *V. mungo*.

Materials and methods

Plant material

Matured seeds of blackgram (*Vigna mungo* L. Hepper), CV-T-9 *V. sublobata* (*Phaseolus sublobata* Roxb.) and *V. unguiculata* (L.) Walp, (Syn. *V. sinensis* (L.) Saviex Hassk) cv. 'Pune' were used as the source material. The seeds were surface-sterilised with 70% ethanol for 30 s, followed with 0.1% mercuric chloride treatment for 5 min. Seeds were freed of sterilant by washing five times with sterile distilled water. The sterilised seeds were aseptically planted on Murashige and Skoog's (1962) basal medium for germination. Hypocotyls of blackgram were directly incubated in the enzyme mixture, while those of cowpea and *V. sublobata* were cut into fine pieces and cultured on Murashige and Skoog's (1962) basal medium supplemented with 2,4-D (2 mg/l) and coconut water (15%) for callus induction. One-week old, freshly initiated callus was used for the experiments.

Isolation and culture of protoplasts

The hypocotyls or hypocotyl-derived calli were incubated overnight at 25 ± 1 °C with 20 ml of enzyme mixture comprising 2.5% Cellulase (Onozuka R-10) and 0.5% each of Macerozyme, (Onozuka R-10) Hemicellulase (Sigma Chemical Co.) and Driselase (Kyowa Hakko Kogyo), in 0.5 M sorbitol at pH 5.8. The undigested cells were removed by passing the mixture through a steel sieve (100 μ) and later the protoplasts were washed thrice by repeated centrifugation with sorbitol (0.6 M) at 300 g. Protoplasts were purified by floatation over 25% sucrose followed by centrifugation at 100 g for 10 min. Purified protoplasts were pipetted out using pasteur pipettes and cultured in Murashige and Skoog's basal medium (1962) containing benzyladenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthalene acetic acid (NAA) each at 1.0 mg/l, supplemented with sucrose (14%) at pH 5.8. Protoplasts were cultured on liquid medium in Petri dishes, sealed with Parafilm and incubated at 25 ± 1 °C under continuous light (1,000 lux). After a month of culture, the protoclones were pipetted out and cultured on top of agar medium (0.6%, Difco Agar) with a reduced level of sucrose (7%). The colonies were picked up at the end of two months and subcultured on MS medium with 2,4-D (2 mg/l), CW (15%) and sucrose (3%) for rapid proliferation. For organogenesis the colonies were transferred to same basal medium but randomly supplemented with 2,4-D, indoleacetic acid (IAA), NAA, BA or zeatin.

Bacterial strains

Agrobacterium tumefaciens wild type strain B6S3 and "shooter" mutants pGV 2215 and pGV 2298 were used (Steffen et al. 1986). Bacteria were grown in liquid medium containing NaCl (5 gm/l) yeast extract (5 gm/l) and Bacto-tryptophan (8 gm/l) on a gyratory shaker. Bacterial suspensions at the late log phase were co-cultivated with protoplast-derived colonies.

Co-cultivation

Four-day-old freshly subcultured protoplasts derived colonies growing in MS medium supplemented with 2,4-D (2 mg/l), CW (15%) and sucrose 3% were incubated with individual strains for 2 days at 25 ± 1 °C under diffuse light on a gyratory shaker. Afterwards, the mixture was centrifuged and the pellet was washed five times with medium by repeated centrifugations. Cells were transferred to fresh medium of the same composition but supplemented with carbenicillin (500 mg/l) and cultured under identical conditions as described before.

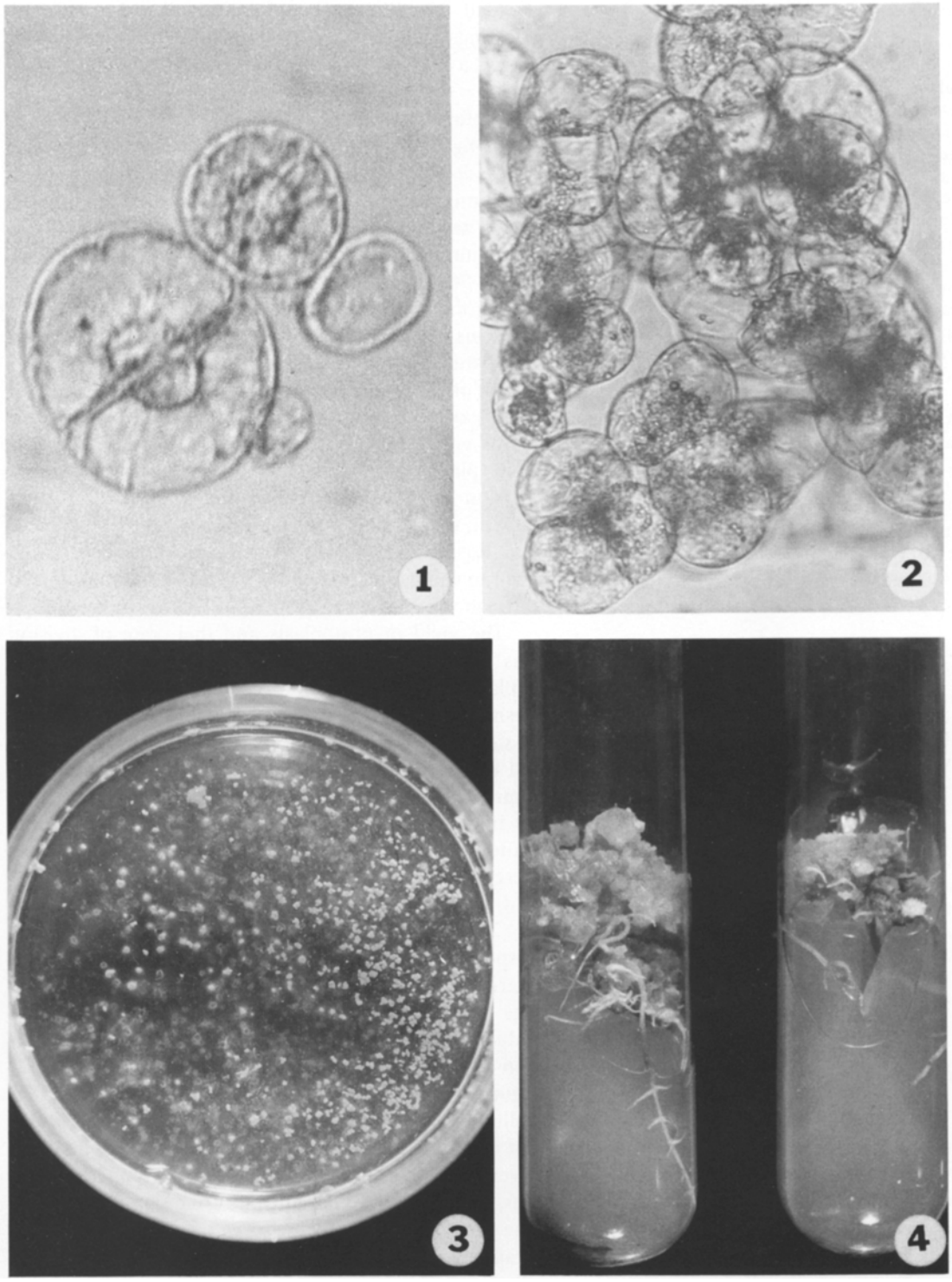
Results and discussion

A good number of protoplasts could be isolated from all three *Vigna* species under identical incubation conditions ($5–6 \times 10^5$ per g of explant). The protoplasts, either derived directly from the hypocotyl i.e. (*V. mungo*) or the hypocotyl-derived callus (*V. unguiculata* and *V. sublobata*), could easily be harvested by centrifugation. This is in contrast with the earlier reports on *Vigna sinesis* where attempts to recover them by centrifugation were met with failure (Bharal and Rashid 1980). To avoid losses of protoplasts by the above method, Bharal and Rashid (1980) recovered them instead by decanting the enzyme followed by subsequent washing with osmoticum and finally shredding the tissue with gentle shaking. Davey et al. (1974) also avoided centrifugation and harvested protoplasts by allowing them to settle by gravity. We, however, observed a loss of 20–30% protoplasts during centrifugation.

Protoplasts either isolated from hypocotyl pieces (*V. mungo*) or hypocotyl-derived callus (*V. sublobata* and *V. unguiculata*) divided within 3–4 days and developed into colonies (Figs. 1, 2, 3). Regeneration of protoplasts was not found to be critically dependent upon the illumination of plants or protoplasts themselves. About 70% of the protoplasts underwent first division during the first week irrespective of either being kept at 1,000 lux or under diffuse light (ca. 400 lux). Our observations regarding the growth behaviour of protoplasts of *V. unguiculata* in this respect are different from earlier report where protoplasts obtained from plants grown at 1,500 lux never divided while the ones from plants grown at 3,500 lux underwent frequent division to form 4–10 celled aggregates. For further growth, the pH had to be raised from 5.5 to 6.0 (Bharal and Rashid 1980). However, we did not observe the need to change the pH and a pH of 5.8 was maintained throughout.

The yield of protoplasts and their rate of division was found to be critically dependent on the age of explant. Only 4–5 day old hypocotyls of *V. mungo* and freshly initiated, one-week old callus of cowpea and *V. sublobata* yielded pure protoplast preparations. With old explants, protoplast preparation tended to be contaminated heavily with cell debris.

In cowpea, although 70% of the freshly isolated protoplasts showed first division, subsequent divisions were restricted to about 50% only, whereas in *V. mungo* and *V. sublobata* the division rate was between 10 to 20%. Multicellular calli started turning brown despite repeated subculture and the addition of charcoal. A factor which was favourable for enhanced rate of division in all three legumes was the gradual reduction of the level of osmoticum. This observation is in accordance with earlier reports on grain legumes (Davey et al. 1974; Bharal and Rashid 1980; Gill and Eapen 1986). Protoclones of cowpea did not differentiate shoot buds and plantlets despite using several auxin/cytokinin combinations. Root formation was, however, predominant in all the cultures (Fig. 4). The protoclones which were co-cultivated with "shooter" strains developed roots and did not show regeneration of shoots. These strains have been reported to produce shoots in protoplast-derived cultures of *Physalis minima* and *Nicotiana paniculata* where otherwise shoot regeneration was never achieved by conventional regeneration methods (Steffen et al. 1986). It is possible that legumes are less responsive to shooter strains. An alternative method for shoot differentiation using low dosages of radiation is in progress.



Figs. 1–4. Callus induction from protoplasts of *Vigna unguiculata.* **1** Initiation of cell division; **2** Multicellular colonies; **3** Callus; **4** Differentiation of roots

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