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Isolation and Culture of Cereal Protoplasts

Part 2: Embryogenesis and Plantlet Formation from Protoplasts of *Pennisetum americanum*

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Summary. Protoplasts isolated from embryogenic suspension cultures derived from immature embryos of pearl millet (*Pennisetum americanum*) gave rise to cell masses. These cell masses upon transfer to a hormone-free medium formed embryoids, which further developed into plantlets with roots and shoots.

Key words: Pearl millet – *Pennisetum americanum* – Cereals – Protoplasts – Embryogenesis – Plant regeneration

Introduction

Totipotency of plant protoplasts was first demonstrated in 1971 (Takebe et al. 1971). Since then plants have been regenerated from protoplasts of about 35 different species, most belonging to the family Solanaceae (Vasil et al. 1979). Isolation and culture of cereal and legume protoplasts, the two most important groups of plants for man and his domesticated animals, have proved most difficult, and there are no reports of plant regeneration from protoplasts of such species (Potrykus et al. 1976, Galston 1978). The techniques of somatic hybridization by protoplast fusion, and the possibility of genetic modification by induced uptake of cell organelles, plasmids, DNA, etc., can not be applied to cereals and legumes until the technology to regenerate plants from their protoplasts is available. We have recently reported high and reproducible plating efficiency of pearl millet (*Pennisetum americanum* (L.) K. Schum., var. Gahi 3) protoplasts resulting in callus formation (Vasil and Vasil 1979), and now describe the formation of embryoids (somatic embryos produced in tissue cultures) and plantlets from protoplasts isolated from suspension cultures of pearl millet.

Materials and Methods

Immature embryos, isolated 10-20 days after pollination, were grown in vitro on Linsmaier and Skoog's (1965) medium, supplemented with 2.5 mg/l 2,4-D and 5% coconut milk, to form a callus tissue, which was placed in liquid medium to initiate suspension cultures. The latter were subcultured every 5-6 days in 35 ml of the medium in 250 ml Erlenmeyer flasks on a Gyrotory shaker at 150 rpm, in the dark at 27°C. The suspension cultures are embryogenic and on plating give rise to embryoids, shoots, roots and plantlets (unpublished observations).

Protoplasts were isolated by mixing 10 ml of a 4-5 days old suspension culture with 60 ml of a filter-sterilized enzyme mixture (2% Cellulysin, 1% Macerozyme, 0.5% Driselase, 0.5% Rhozyme, 0.25M sorbitol, 0.25M mannitol, 250 mg/l glucose, 3mM MES buffer, prepared in the hormone free medium of Linsmaier and Skoog at pH 5.6), incubated for 1 hr at room temperature, followed by 19 hr at 14°C in the dark. The protoplast/enzyme mixture was filtered through a layer of Miracloth, and through 100 and 50 μm stainless steel filters to remove undigested cells and other cellular debris. Protoplasts were collected and washed three times with fresh nutrient medium by low speed centrifugation (100 g × 3 min). At the end of each centrifugation cycle the protoplasts floating at the top of the nutrient medium and those which had pelleted were collected and mixed. After the final wash, the floating and pelleting protoplasts were either mixed and cultured together, or separately. Protoplasts were cultured in liquid droplets (0.25 to 0.3 ml) or in very thin layers of nutrient medium in Falcon Petri dishes (35 × 10 mm), at a density of 2×10^4 or 10^5/ml. Petri dishes were sealed with Parafilm, incubated in diffused light in a growth chamber at 27°C, with a 16/8 hr day/night cycle. Kao and Michayluk's (1975) nutrient medium was used for the washing and culture of protoplasts, without the free amino acids, nucleic acid bases, riboflavin, and vitamin B_{12}, but containing 0.4M glucose, 1250 mg/l sucrose, 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyl amino purine (BAP). After 15- to 20-celled colonies had been formed from the protoplasts, fresh medium with 1% glucose and 2% sucrose was added, and after 4-5 weeks the resulting cell masses were transferred to the same medium solidified with 0.8% agar.

Results and Discussion

The suspension cultures are comprised of two distinct cell types, one large and highly vacuolated, the other small and densely cytoplasmic. Protoplasts are formed first from the large vacuolated cells, and float to the surface of the enzyme solution. The cytoplasmic contents of these protoplasts are clumped and pushed together against the

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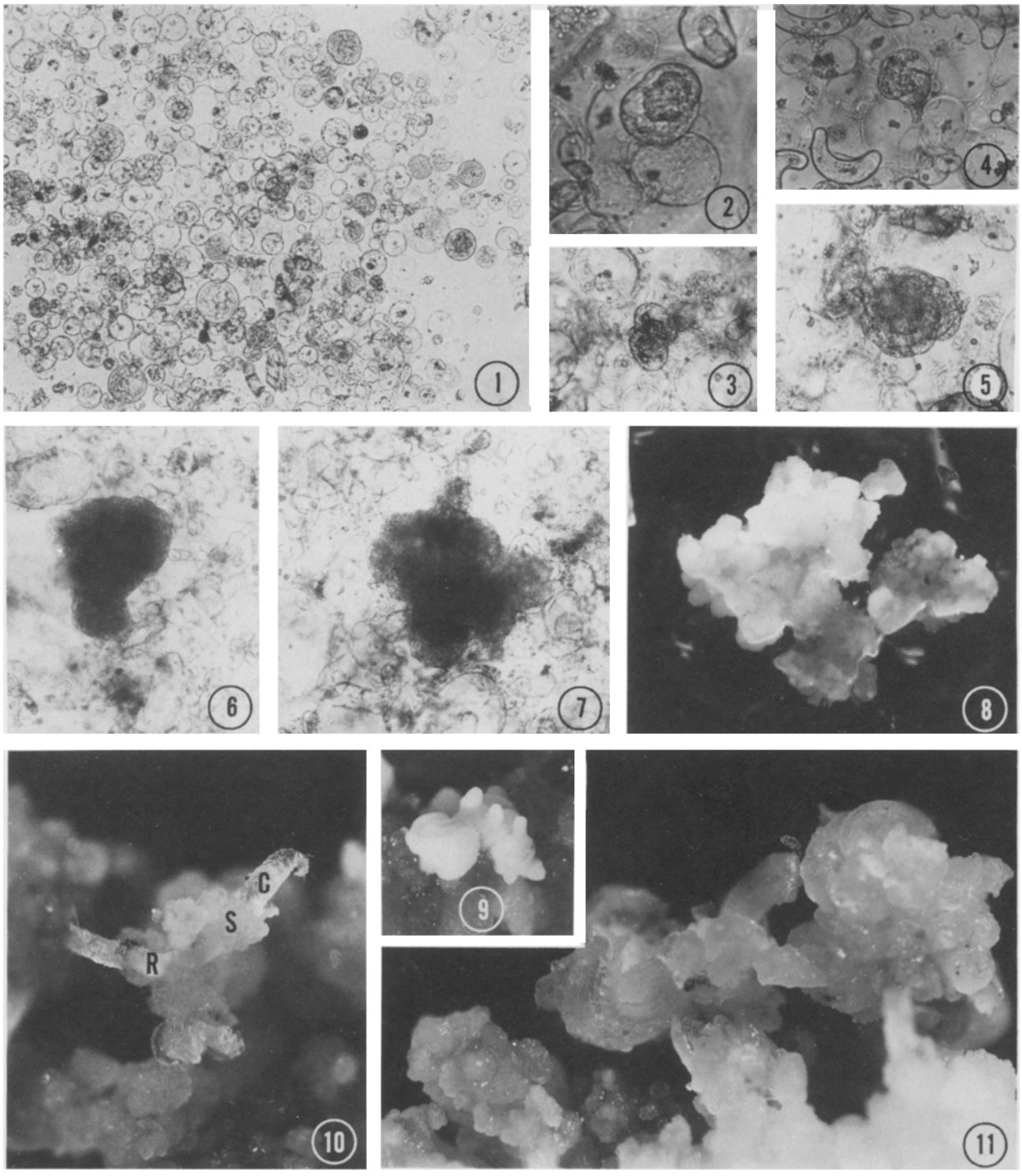

Figs. 1-11. 1 freshly isolated vacuolated and embryogenic protoplasts from suspension cultures of pearl millet (*Pennisetum americanum*), 2-5 formation of a cell mass from embryogenic protoplast, 6 embryoid, 7, 8 cell masses and embryoids, 9 nearly mature embryoids, 10 young plantlets showing coleoptile (C), root (R) and fresh cell proliferation from the scutellum (S), 11 stages of plantlet formation from embryoids

plasmalemma during isolation and washing, but on culture they assume normal appearance within hours, and display a prominent nucleus, suspended by cytoplasmic strands that show streaming. Such protoplasts regenerate a cell wall within a few days, but were never seen to divide under the conditions described here. A few thick walled cells were also able to survive the enzyme treatment, but these too never underwent any division in culture, and eventually degenerated along with the large, vacuolated protoplasts.

The second type of protoplasts are richly cytoplasmic, non-vacuolated and always pellet during centrifugation (Fig. 1). Many starch grains are seen in them, and their nucleus is generally obscured from view owing to the high density of cell organelles in the dense cytoplasm. These protoplasts, which are derived from embryogenic cells in the suspension cultures, regenerate cell walls and form 15- to 20-celled masses within 2 weeks after isolation and culture. Some of the early stages of cell mass formation are illustrated in Figures 2-5. The protoplast-derived cell masses are tightly packed with small and richly cytoplasmic cells with thin walls. Further cell divisions follow in rapid succession, without significant cell enlargement, resulting in the formation of organized structures which are reminiscent of early stages of embryogenesis (Figs. 6-8). Within 4-5 weeks large cell masses, visible to the naked eye, are formed and consist of tightly packed embryogenic cells. Each cell mass is comprised of small, rounded and nodular mounds. At this time some loose and vacuolated cells are formed in each cellular mass on its periphery, and continue their association with the embryogenic masses.

The highest plating efficiency obtained by us in the culture of embryogenic protoplasts was 3-4% when 1 mg/l each of 2,4-D and BAP were present in the medium. Such cultures were often accompanied by the production of polyphenols which were detrimental to the continued growth of protoplast-derived cell masses. A plating efficiency of 1-2% was obtained with 2.5 mg/l 2,4-D, but without any polyphenol production.

Protoplast-derived cell masses were transferred to the same medium for continued growth after they had achieved a size of 1-2 mm. The transfer of cell masses from the liquid medium to an agar medium without any growth substances resulted in the growth of cells in tight and discrete groups, and finally led to the formation of embryoids and plantlets with shoots and roots within 4-5 weeks. The embryoids look like monocotyledonous embryos, with a distinct scutellum enveloping the embryonal axis (Fig. 9). The coleoptile shows typical hair-like structures and forms a tubular sheath around the young shoot which eventually pushes through it during further growth. The embryoids continue to grow and eventually form plantlets (Fig. 10, 11). The scutellar cells often proliferate resulting in the formation of secondary embryoids and plantlets. During the early stages of embryoid formation, the scutellum appears as a cup-shaped structure, with shoot meristem at the open end and the root meristem at the close end, which shows a few root hair-like outgrowths even at an early stage. A large number of plantlets is formed in each petri dish, starting from a few cell colonies derived from protoplasts. The fact that plantlet formation in the present system is through embryogenesis, and not after extensive callus growth preceding the organization of separate shoot and root meristems, is of added significance and advantage in the recovery of normal plants.

This first demonstration of totipotency and embryogenesis of cereal protoplasts shows that cereals are no different than many other species in which protoplasts have been cultured successfully, and should encourage vigorous and renewed efforts with other cereal species so that somatic hybridization and genetic modification technology can be adopted for the improvement of cereal crops.

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