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Embryoid and Plantlet Formation from Leaf Segments of *Dactylis glomerata* L.

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Summary. The purpose of this investigation was to demonstrate callus induction and plantlet formation from cultured leaf segments of 12–15 week-old *Dactylis glomerata* L. (orchardgrass) plants. Flat half-leaf sections, approximately 2–3 mm square, from the three innermost (youngest) leaves were isolated and individually plated serially beginning at the leaf base on a solid SH medium containing 30 μM of 3,6-dichloro-o-anisic acid (dicamba). Callus formed on leaf sections from all 50 plants used in the study. After transfer to SH medium with 1 μM dicamba, plantlets formed from leaf sections of 9 of the 50 plants. In most cases plantlets formed from embryogenic callus but in a few cases embryoids formed directly on the leaf surface without an intervening callus state. These developed into plantlets when transferred to low auxin medium. The response for both callus and plantlet formation decreased with increasing distance both spatially and temporally from the shoot apex. Histological examination of embryogenic callus revealed the presence of non-zygotic embryos in various stages of development. The results provide further support for compentency (if not totipotency) of Gramineae leaf cells.

Key words: *Dactylis glomerata* Orchardgrass – Tissue culture – Embryoid – Dicamba

Introduction

Immature or mature embryos are commonly used as explants to initiate in vitro cultures in Gramineae (Conger 1981). Until recently, there were few reports of plantlet formation from cultured leaf tissue in important cereals and grasses. Bhojwani et al. (1977) spent considerable effort culturing leaf tissue of wheat, maize and Sorghum, but a callus, regardless of leaf age and medium composition, was never obtained. Saalbach and Koblitz (1978) obtained callus from basal leaf segments of 5–6 day-old barley seedlings excised 3 to 5 mm above the meristem and one explant exhibited plantlet formation. These authors utilized a seedling extract in the medium.

Recently, plantlet regeneration via somatic embryogenesis from serially cultured seedling leaf segments was reported in *Sorghum bicolor* (Wernicke and Brettell 1980) and rice (*Oryza sativa*) (Wernicke et al. 1981). Plantlet regeneration, also via somatic embryogenesis, was reported from cultured whorls of the youngest leaves in *Pennisetum purpureum* Schum. (Haydu and Vasil 1981) and *Panicum maximum* Jacq. (Lu and Vasil 1981). Histological information presented for the latter species supported the somatic embryogenesis concept.

Leaves and leaf tissue are of interest as explants because of their potential use for protoplast isolation and culture. However, the difficulties and problems of culturing Gramineae leaf mesophyll protoplasts are well known and have been extensively discussed (Potrykus et al. 1976; Vasil and Vasil 1980). Therefore, plantlet regeneration from cultured leaf tissue is an important first step in the demonstration of cellular totipotency in these species. The objectives of this study with *Dactylis glomerata* L. (orchardgrass) were to (1) demonstrate plantlet formation from cultured individual leaf sections and (2) determine the mode of plantlet formation by histological techniques.

Materials and Methods

Single tillers were selected from each of 50 individual plants of 'Potomac' orchardgrass grown from seed in the greenhouse. Sampling was repeated at weekly intervals over a period of 4 weeks so that a total of four tillers were obtained from each plant. The plants were 12-weeks-old at the beginning of the sampling period. The outer leaves were removed and the three innermost (youngest) leaves were individually separated. Since the leaf sheaths of *Dactylis* are flattened with united edges rather than convolute, individual leaves can be separated easily and the identity of each readily maintained. This also

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eliminates any possibility of including the shoot apex with any leaf section. Leaves were split longitudinally down the midrib and one-half portion of each leaf was discarded. The remaining half was surface sterilized by placing it in a 2% (v:w) detergent (Alconox) solution for 2 minutes and then into 2.5% sodium hypochlorite for 2 minutes. Leaves were then rinsed three times with sterile distilled water.

Since the plants were 12 to 15-weeks-old and some of the initial seedling leaves had senesced, the innermost leaf was designated as leaf No. 1 and the second and third leaves outward from the shoot apex were designated as leaves No. 2 and No. 3, respectively. This is opposite to the normal designation of leaf number but was the most convenient classification method in the present study. Leaves were cut into 2–3 mm sections and the first six sections from the basal end upward were plated serially onto a solid SH (Schenk and Hildebrandt 1972) medium containing 30 μM 3,6-dichloro-o-anisic acid (dicamba) and 0.8% agar. The medium was previously sterilized by autoclaving for 15 min at 121 °C. Incubation was in the dark at 25 °C.

When callus developed and/or embryoid structures appeared, usually within 3 to 4 weeks after plating, either the entire leaf section with callus or individual embryoids were carefully removed and individually transferred to a solid SH medium containing 1 μM dicamba. These cultures were incubated in a 16 hr/8 hr light/dark cycle at 25°/10 °C. Light was from cool-white influorescent bulbs. When plantlets developed, they were transferred to culture tubes containing one-half strength SH agar medium without auxin. After plants had developed a root system they were transferred to soil in pots and maintained in the greenhouse.

Tissues for histological studies were fixed for 24 hr (under vacuum) in 2.5% glutaraldehyde buffered at pH 7.0. Fixed tissues were dehydrated in a t-butanol series (Johansen 1940), embedded in Paraffin (Paraplast TM 56–57 °C) and sectioned at 10 μm. Ribbon segments were floated on distilled water and placed on slides without adhesive. Tissue sections were dewaxed in xylene and rehydrated in a descending ethanol series. They were then triple stained using safranin 0 and aniline blue-orange G according to the procedure of McDaniel et al. 1982.

Results

Leaf sections a few days after plating are shown in Fig. 1 a. Within 3 to 4 weeks embryoids formed directly on the surface as well as on the edges of a few leaf sections (Fig. 1 b). These embryoids could be removed and plated singly onto low auxin medium (1 μM dicamba) where approximately 40% of them developed into plantlets (Fig. 1 d). Still other leaf sections developed embryogenic callus with multiple embryoids (Fig. 1 c). Many shoots developed from these explants (Fig. 1 e). These plantlets as well as those obtained from isolated embryoids were established in culture tubes (Fig. 1 f) and eventually transferred to soil in pots.

Although some explants were lost due to fungal or bacterial contamination, all 50 plants exhibited callus formation form one or more leaf sections. The highest percentage was obtained from the basal section of the innermost leaf (Table 1). Percent callus formation decreased as sections were taken further from the leaf base and from the second and third leaves outward. Plantlets were obtained from leaf sections of only nine of the 50 plants. Although the data were more variable, percentage of leaf sections from which plantlets were obtained showed the same trend as for callus formation with respect to leaf number and position of leaf section (Table 2). The highest percentage of plantlets was obtained from the basal section of the innermost leaf. No plantlets were obtained from sections of the third leaf.

Histological information on embryogenesis is shown in Fig. 2. Embryoids in different stages of development obtained 3 weeks after plating are shown in Figs. 2a through 2c. Note the suspensor-like structures (as indicated by arrows) on some of the presumptive proembryos in Fig. 2a. A developing embryo-like structure is shown in Fig. 2b. Distinct leaf primordia and root and shoot apices are evident on one of the young embryoids in Fig. 2c and leaf primordia and a shoot apex are visible on another embryoid. A more highly developed embryoid 3 weeks after transfer to low auxin medium and 6 weeks after initial plating is shown in Fig. 2d. Many structures present in mature seed embryos such as coleoptile, leaf primordia, radicle, coleorhiza and scutellum are present in this embryo.

Table 1. Percent callus formation from different leaf sections of the three innermost (youngest) leaves of *Dactylis glomerata*. Data are based on four tillers from each of 50 plants. Leaf No. 1 represents innermost leaf. Leaf section No. 1 represents the basal section; sections extending outward from the basal section are denoted by correspondingly higher numbers

Leaf No.	Leaf Section No.					
	1	2	3	4	5	6
1	87.2	74.8	42.9	17.3	9.8	5.3
2	54.8	40.7	16.8	10.8	3.9	1.5
3	9.7	9.5	5.7	2.4	1.5	0.8

Table 2. Percent of cultured leaf sections from three innermost (youngest) leaves of *Dactylis glomerata* plants exhibiting shoot formation. Data are based on four tillers from each of 50 plants. Leaf No. 1 represents innermost leaf. Leaf section No. 1 represents the basal section; sections extending outward from the basal section are denoted by correspondingly higher numbers

Leaf No.	Leaf Section No.					
	1	2	3	4	5	6
1	8.0	4.5	0.9	0.0	0.0	1.8
2	2.7	0.9	2.7	0.0	0.0	0.0
3	0.0	0.0	0.0	0.0	0.0	0.0

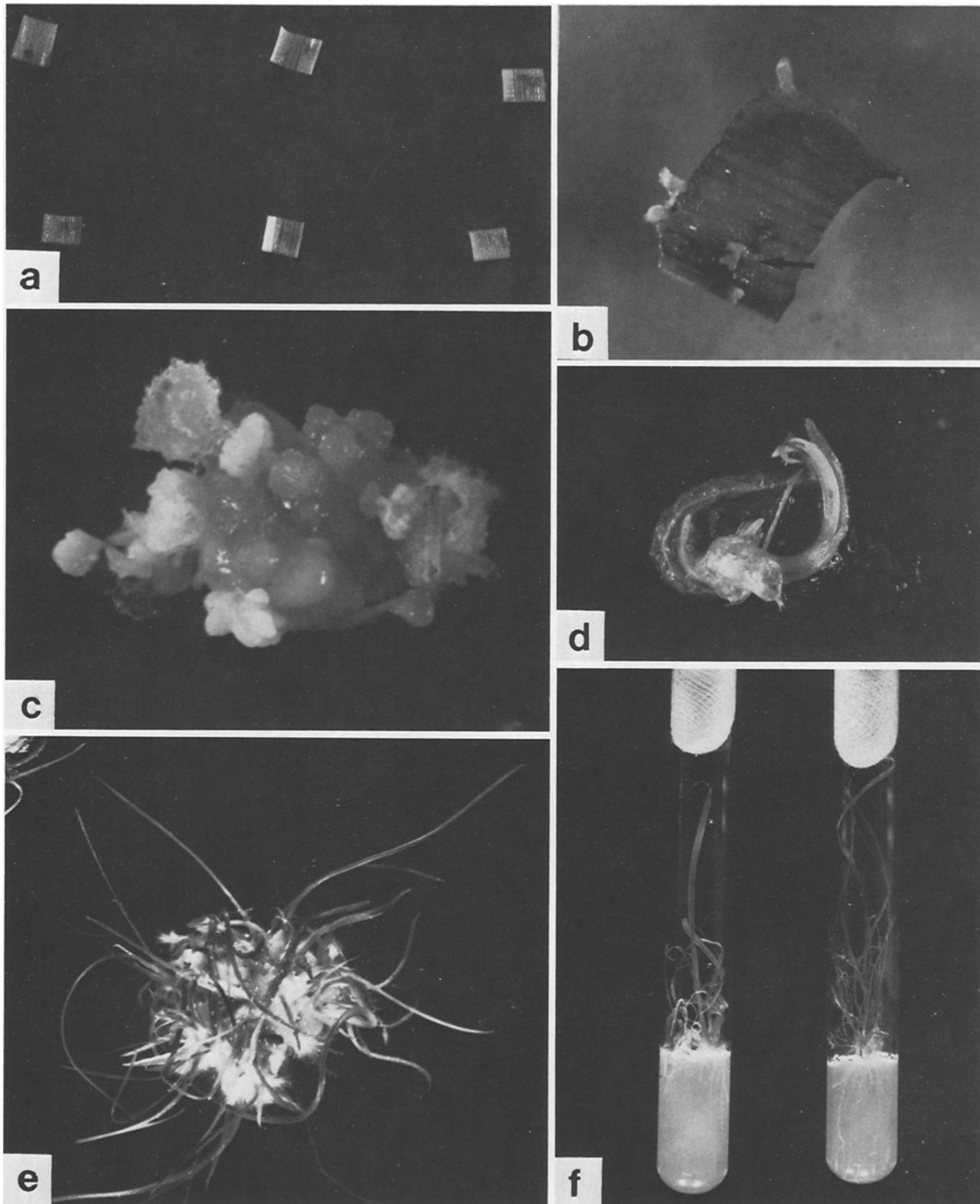

Fig. 1a–f. Embryoid and plantlet formation from cultured leaf segments of *Dactylis glomerata*. **a** Leaf segments (approx. 3 mm square) shortly after plating; **b** Embryoid development on adaxial surface of 3-week-old cultured leaf section (×7); **c** Embryogenic callus forming on 3-week-old cultured leaf section (×10); **d** Plantlet developing from individually cultured embryoid 3 weeks after transfer to low auxin medium (×10); **e** Shoot formation from embryogenic callus 3 weeks after transfer to low auxin medium (×7); **f** Seedling development in tubes

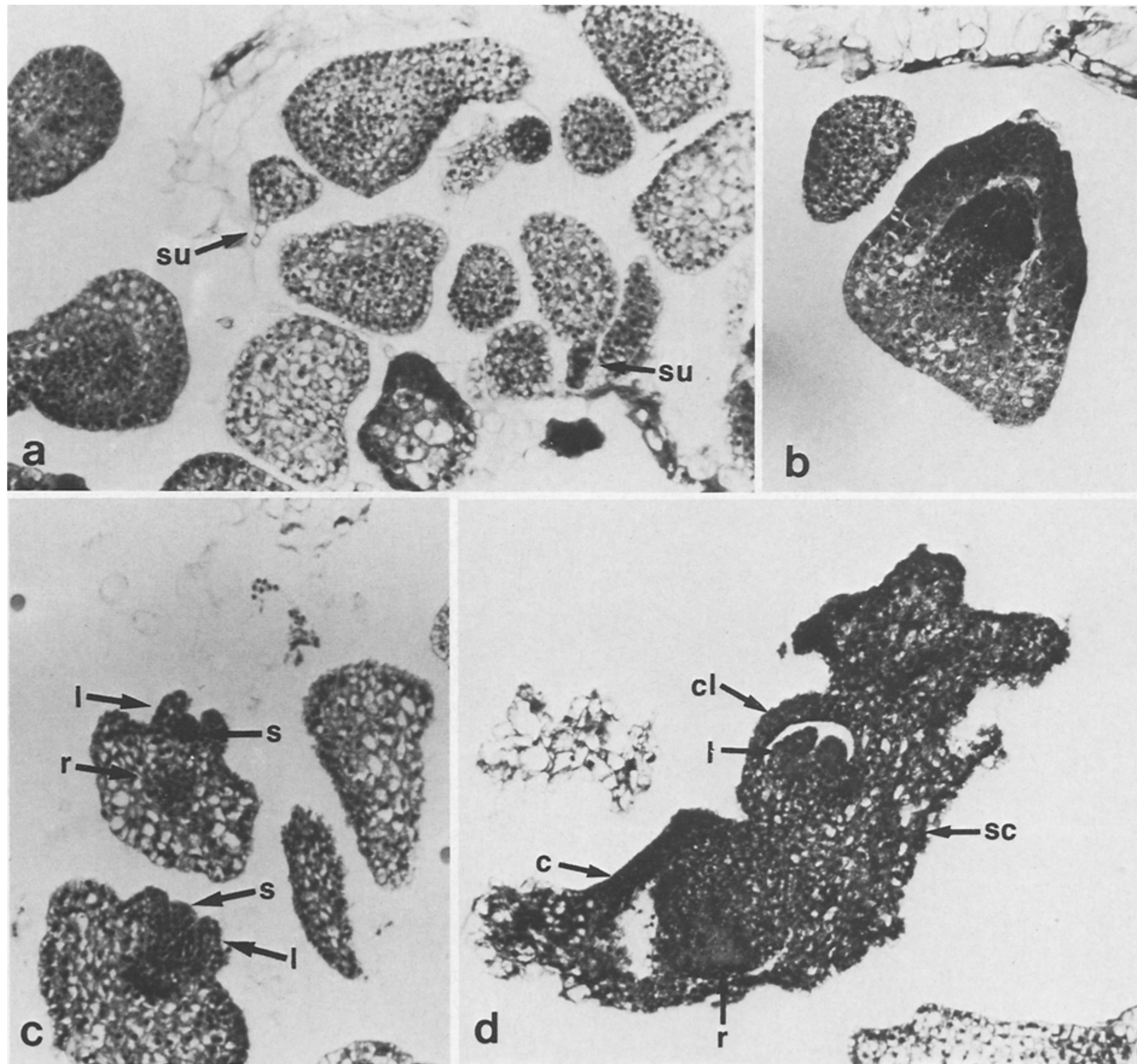

Fig. 2a–d. Histology of embryoid development from cultured leaf sections of *Dactylis glomerata* L. **a** Presumptive proembryos with suspensor-like structures (su) in embryogenic callus 3 weeks after plating (× 100); **b** developing embryo-like structure 3 weeks after plating (× 110); **c** Young embryoids with leaf primordia (l) and root (r) and shoot (s) apices three weeks after plating (× 110); **d** Developed embryoid with coleoptile (cl), leaf primordia (l), scutellum (sc), radicle (r) and coleorhiza (c) 3 weeks after transfer to low auxin medium (× 120)

Discussion

Plantlet formation from embryo-derived callus in orchardgrass was reported earlier (Conger and Carabia 1978). Furthermore, a histological examination of calli 84 days and two subcultures after the initial plating revealed somatic embryogenesis (McDaniel et al. 1982). In the present study, plantlet formation was demonstrated for in vitro cultures of individual flat leaf sections obtained from greenhouse-grown orchardgrass plants. The external appearance of structures occurring both on callus and directly on explants 3 to 4 weeks after plating and a histological study of these structures indicated that at least some of the plantlets formed by somatic embryogenesis.

The results provide further corroboration for competency and possibly also for totipotency of Gramineae leaf tissues and cells. The evidence for somatic embryogenesis supports previous reports in *Sorghum* (Wernicke and Brettell 1980), rice (Wernicke et al. 1981), *Pennisetum* (Haydu and Vasil 1981) and *Panicum* (Lu and Vasil 1981). In the studies with *Pennisetum* (Haydu and Vasil 1981) and *Panicum* (Lu and Vasil 1981) segments of 1–4 of the youngest whorled leaves were sectioned transversely and plated. The leaves of orchardgrass are not whorled around each other which allows for easy separation and culture of individual leaves. Leaves, including

the coleoptile, were separated and individually cultured in the studies with *Sorghum* (Wernicke and Brettell 1980) and rice (Wernicke et al. 1981); however, the age of the source plants was only 10 days. The present study shows, as was reported with *Panicum* (Lu and Vasil 1981) and *Pennisetum* (Haydu and Vasil 1981), that leaves from much older plants will form embryogenic callus and regenerate plants. Observations that the ability of different leaf segments to produce both callus and plantlets decreases with increasing distance, both spatial and temporal, from the apical meristem confirms those reported in *Sorghum* (Wernicke and Brettell 1980) and rice (Wernicke et al. 1981).

Although calli were obtained from leaf sections of all 50 plants, the quality of callus varied from watery and gelatinous to firm and friable. The latter had a higher tendency to become embryogenic. Obtainment of plantlets from leaf sections of only nine of the 50 plants indicates genotypic differences for regeneration capacity such as reported in other plant species, including several in the Gramineae (Lu and Vasil 1981; Haydu and Vasil 1981; Wernicke and Brettell 1980; Green and Phillips 1975). Since orchardgrass is a highly heterozygous, self-incompatible, out-crossing species, each caryopsis within a cultivar and even from the same plant may vary greatly in genotype. Hence, each individual plant used in the study must be considered to be of a different genotype. Preliminary results show that leaf sections taken from regenerated plants exhibit a much higher potential for producing embryogenic callus and plantlet formation than those taken from plants at random.

The cellular origin of embryogenic callus and embryoids was not determined. However, previous studies with embryo-derived callus indicated that somatic embryos arose de novo from undifferentiated parenchyma (McDaniel et al. 1982). Interpretation and discussion of those observations in relation to the development and anatomy of the grass embryo by Brown (1965) provided further support for the de novo hypothesis. However, the single cell origin of somatic embryos from either embryo or leaf-derived callus remains to be resolved.

According to Haccius (1978), non-zygotic embryos in angiosperms may develop in vitro directly from segmenting single cells or indirectly from a preliminary proembryonal cell complex. Furthermore, her definition of embryos arising as new individuals from a single cell is equally true in both cases of non-zygotic embryogenesis. In the present study, suspensor-like structures were apparent in presumptive proembryos. Haccius (1978) interprets the suspensor as a rudimentary proembryonal cell complex from which new embryos may develop.

The production of embryoids directly on the adaxial surface of a few leaf segments without an intervening callus state and the development of these into plantlets is a significant observation and has not been previously reported in culture of grass leaf sections. Further studies will yield information concerning the cellular origin of these embryoids, as well as that of embryogenic callus, and on the ability of leaf tissues and cells to produce embryogenic suspension cultures and protoplasts.

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