Plant Cell Reports (1983) 2:21–25

Plant Cell Reports

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Plant Regeneration Via Somatic Embryogenesis in Sugarcane [1]

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Received July 6, 1982/November 9, 1982

ABSTRACT

Plants, regenerated from callus cultures of sugarcane (Saccharum officinarum L.) clone 1J76-316, originated through somatic embryogenesis. Callus cultures were established from primordial leaves and apical meristems on Murashige and Skoog medium (MS) supplemented with 3 mg l^{-1} 2,4-dichlorophenoxy acetic acid and 100 ml l^{-1} coconut water (MSC_3). Nodular calli formed within 2 weeks of culture. Calli were maintained on MSC_3 medium by transfer every 3 to 4 weeks. Somatic embryogenesis occurred after 10 weeks culture of callus on MSC_3 medium. Somatic embryogenesis was also observed in cell suspension cultures initiated from calli maintained on MSC_3 and then cultured in half strength MS liquid medium supplemented with 0.5 mg l^{-1} 2,4-D. Somatic embryos produced coleoptiles and shoots 2 to 4 weeks after transfer to MS medium supplemented with 100 ml l^{-1} coconut water (MSC), and produced complete plantlets within 4 weeks of further culture on half-strengh MS medium (half-MS) with 30 g l^{-1} sucrose. Calli grown on MSC_3 medium, when transferred to half-MS medium containing 15 g l^{-1} sucrose, produced tiny plantlets, circa 4-10 mm, without forming coleoptiles, suggesting precocious germination of somatic embryos. The regenerates included morphological variants.

INTRODUCTION

Several graminaceous crop plants and forage grasses have been regenerated from tissue cultures. In tissue cultures, plants originate through formation of shoot primordia or somatic embryos. However, it also has been suggested that in cereals plants originate from the highly disorganized primary shoot primordium which proliferates adventitiously in the culture (King et al., 1978). Plant regeneration via somatic embryogenesis thus represents a distinct alternative to plant formation from adventitious bud formation. In grasses, plant regeneration through somatic embryogenesis was first reported in Bromus inermis Leyss (Gamborg et al., 1970). Since then, somatic embryogenesis has been shown to occur in a number of graminaceous species, e.g., ryegrass, Lolium spp. (Ahloowalia, 1978); annual ryegrass, Lolium multiflorum Lam. (Dale, 1980); sorghum, Sorghum bicolor (L.) Moench (Thomas et al., 1977; Dunstan et al., 1978; Wernicke and Brettall, 1980); pearl millet, Pennisetum americanum (L.) Schum. (Vasil and Vasil, 1980); napier grass, Pennisetum purpureum Schum. (Haydu and Vasil, 1980); and Panicum maximum Jacq. (Lu and Vasil, 1982).

Plant regeneration in sugarcane (Saccharum sp. hybrid) was first reported by Heinz and Mee (1969); however, the origin of regenerates was not investigated. Nadar et al. (1978) claimed the occurrence of "embryoids" in sugarcane callus cultures maintained on a high auxin (3 mg l^{-1} 2,4-D) medium and postulated that a low auxin concentration may be required for advanced stages of embryogenesis. However, these workers failed to show full somatic embryo development. In the present communication, we report regeneration of sugarcane plants through somatic embryogenesis.

MATERIALS AND METHODS

Callus cultures were initiated from apical meristems and young leaves of sugarcane cultivar 1J76-316 (Saccharum officinarum L.). Stem cuttings (5-8 cm long), and each with one bud, from field grown plants, were dipped in saturated Benlate solution to prevent fungal growth and were germinated in plastic boxes at 37-39°C. Two-week-old shoots, 2 to 5 cm long, were surface sterilized in diluted (1:3 v/v) aqueous solution of commercial Clorox (5.25% sodium hypochlorite as the active agent) for 25 min, dipped in 95% alcohol for 1 min, and rinsed twice in sterile distilled water. Young leaves and meristematic tissues were taken from the apical regions of the innermost 10-mm whorl, sliced into 2- to 3-mm pieces, and cultured on Murashige and Skoog (1962) medium (MS) supplemented with 3 mg l^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) and 100 ml l^{-1} (v/v) coconut water (MSC_3) (Heinz and Mee, 1969). Induced calli were maintained on MSC_3 medium by serial transfer every 3 to 4 weeks. Ten-week-old callus pieces were transferred to MS medium with 100 ml l^{-1} coconut water (MSC), and later to half-strength MS medium (half-MS) with and without coconut water (CW), 2,4-D, zeatin, and sucrose concentrations of 15, 30 and 60 g l^{-1}.

Suspension cultures were initiated from 12-week-old callus pieces in 125-ml flasks containing 50 ml half-MS liquid medium supplemented with 0.5 mg l^{-1} 2,4-D. Cell suspensions were maintained by replacing 25-30 ml of suspension with fresh medium every 8-10 days. Cultures were shaken at 150 rpm on a rotary shaker.

For each experiment, a minimum of five replicates (bottles, flasks, or petri-dishes) were inoculated with callus or cell suspension cultures. All media were adjusted to pH 5.7 and autoclaved for 25 minutes. All cultures were

[1] Published with the approval of the Director as Paper No. 000 in the Journal Series of the Experiment Station, Hawaiian Sugar Planters' Association

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maintained at 25°C under continuous diffused lighting provided by two 40-W warm fluorescent lights suspended at 35 cm above the bench surface.

The regenerated plantlets were potted in a mixture of peat and soil and kept for the first week under high humidity by covering trays with thin plastic and under continuous fluorescent lighting as described above.

Samples of cells, callus, and somatic embryos were stained and preserved in a solution of 1% alcoholic safranin, glycerol, and water (1:2:1).

RESULTS

Callus induction and proliferation:

Shoot meristem and primordial leaves initiated nodular calli within 2 weeks of culture on MSC_3 medium. Calli were maintained by subculture every 3 to 4 weeks on MSC_3 medium. Calli stayed regenerative for more than 6 months. Calli (5-10 mm) when subcultured on MSC_3 medium nearly doubled in size within 30 days. Calli continued to grow for more than 17 weeks without retransfer to fresh medium. Such callus cultures developed green pin-head-sized structures scattered among the mass of white nodules. These calli produced shoots and eventually plantlets on transfer to half-MS medium containing 15 g l^{-1} sucrose.

Somatic embryogenesis & plantlet regeneration:

Transfer of 2-week-old callus to MSC_3 medium and prolonged culture for 10 weeks initiated somatic embryogenesis. In these cultures, structures resembling globular to torpedo stage embryoids (Fig. 11-13) were observed. Subculture of this callus on MSC medium produced, within 2 weeks, massive growth of cup-shaped scutellum-like structures with folds and invaginations (Fig. 1). In each of the cup-shaped structures, a single, pale-colored coleoptile developed which became green and grew in some cases to 10 to 12 mm before the emergence of the first leaf (Fig. 2-4). Somatic embryos formed complete plantlets within 4 weeks of culture on MSC medium (Fig. 5-6). Root elongation was inhibited in most of these plantlets on MSC medium. A further subculture of plantlets on half-strength MS medium containing 15 or 30 g l^{-1} sucrose within 4 weeks produced plants 10 to 15 mm tall with 2 to 3 leaves and a single root (Fig. 7-8). Plantlets at this stage were transferred to soil in 6-cm diameter peat pots and kept at high humidity for 4 weeks before transplanting into larger pots.

Medium composition had a marked effect on somatic embryogenesis (Table 1). Callus, maintained on MSC_3 medium for 10 weeks and then transferred to half-strength MS medium with 15 g l^{-1} sucrose (treatment 4), produced plantlets within 4 weeks of culture. On this medium, somatic embryos germinated precociously; i.e., in most cases coleoptile did not form. Instead, plantlets 3 to 4 mm tall with a shoot and a root were obtained. Higher sucrose concentration (30-60 g l^{-1}) and addition of growth regulators (treatments 5 through 10) (Table 1) strongly influenced the growth and development of callus and somatic embryos. Whereas differentiated callus produced plantlets on half-MS medium with 15 or 30 g l^{-1} sucrose (treatment 4 and 5), on half-MS medium with 60 g l^{-1} sucrose (treatment 6), massive root formation was observed on callus surface. The roots grew upwards, callus turned purple, and shoot primordia were completely suppressed. Addition of 0.1 or 0.5 mg l^{-1} 2,4-D and 60 g l^{-1} sucrose (treatments 7 and 8) inhibited callus growth; the calli became purple and necrotic and plantlets failed to differentiate. On the other hand, calli maintained on MSC_3 medium and then transferred to half-MS medium containing 15 g l^{-1} sucrose, and supplemented with 0.5 mg l^{-1} 2,4-D, 50 ml l^{-1} coconut water and with or without 2.0 mg l^{-1} zeatin (treatments 9 and 10), showed excellent shoot growth, good callus enlargement, and good scutellum development and embryo growth. In callus cultures continuously maintained on MSC_3 medium, the differentiated embryos became deformed and developed secondary calli from both the shoot and root zone. Plantlets differentiated on MSC medium showed better growth and rooting on transfer to half-MS medium containing 30 g l^{-1} sucrose (treatment 3) than either 15 or 60 g l^{-1} sucrose (treatments 4 and 6).

Table 1. Effect of medium composition on callus growth and differentiation in sugarcane.

Tr't No.	Medium	Supplementation: sucrose g l^{-1}	2,4-D mg l^{-1}	cw ml l^{-1}	zeatin mg l^{-1}	Growth & Differentiation Response*: callus	somatic embryos	shoots	roots	Remarks
1	MS	20	3.0	10	-	+++	0	0	0	nodular pale callus
2	MS	20	-	10	-	+++	+++	++	0	promotes somatic embryogenesis
3	MS	15	0.1	-	-	+++	++	++	+	promotes callus growth
4	Half-MS	15	-	-	-	0	0	+++	+	promotes shoot growth
5	Half-MS	30	-	-	-	0	0	+++	+++	promotes shoot & root growth
6	Half-MS	60	-	-	-	0	0	+	+++	purple callus, with abundant root formation
7	Half-MS	60	0.5	-	-	+	+	0	+	purple callus
8	Half-MS	60	0.5	10	-	+	+	0	+	purple callus
9	Half-MS	15	0.5	5	-	++	+++	++	+	promotes embryogenesis
10	Half-MS	15	0.5	5	2.0	+++	+++	+++	+	promotes growth & embryogenesis
11	Half-MS (liquid)	15	0.5	-	-	++	+	0	0	promotes cell division

* Growth response excellent = +++, good = ++, poor = +, and none = 0.

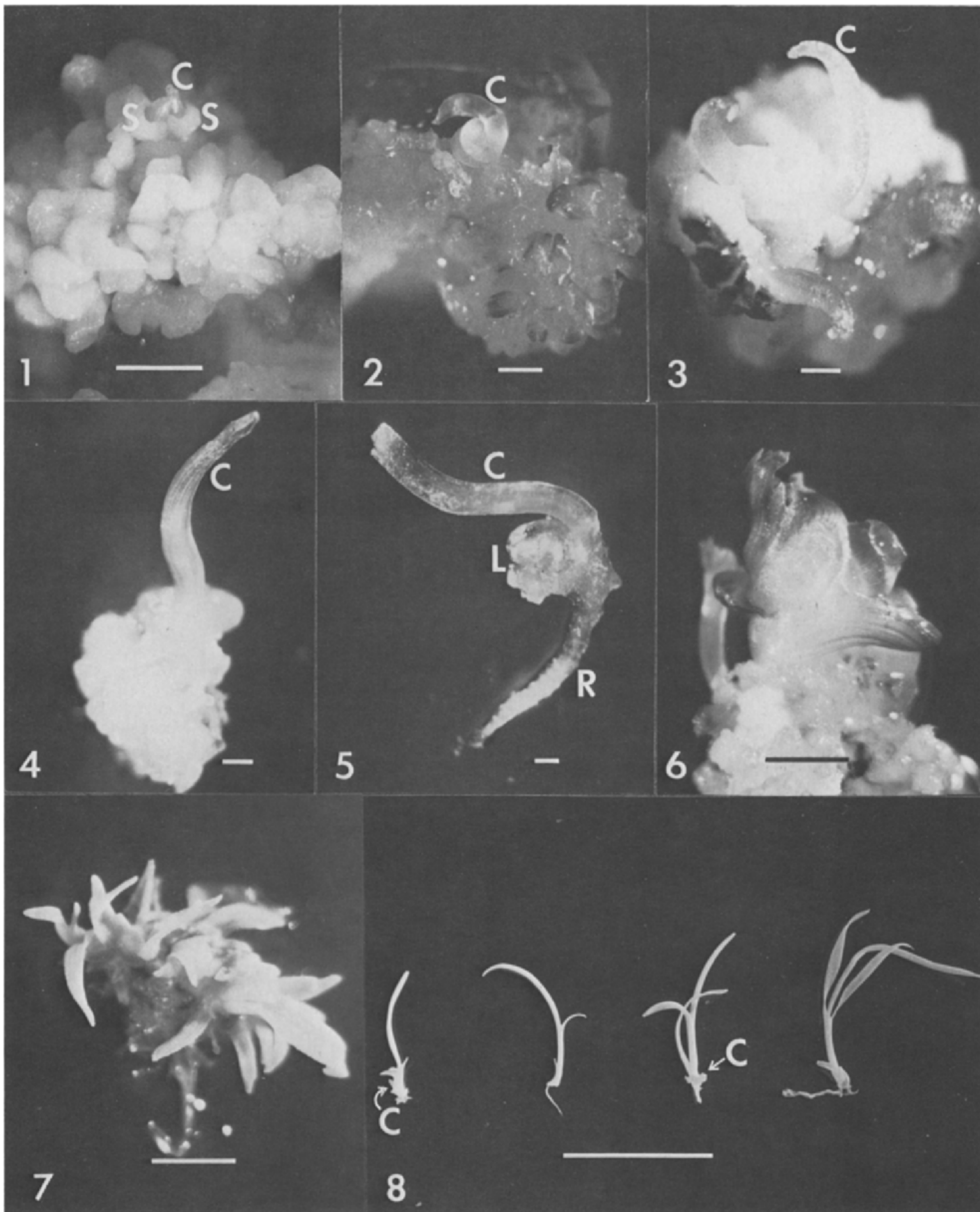

Fig. 1-8. Somatic embryogenesis in sugarcane. Fig. 1. Cup-shaped scutellum (S) with coleoptile (C). Fig. 2-4. Coleoptile (C) development on MSC medium. Fig. 5. Formation of complete plantlet -- coleoptile (C), folded leaves (L), and root (R). Fig. 6-8. Embryo to plantlet formation on half-MS medium containing 15 g l^{-1} sucrose. Fig. 6. Elongated coleoptiles and formation of shoots on callus surface. Fig. 7-8. Plantlets obtained following precocious germination of somatic embryos. Coleoptile remains are visible at the base of shoots in Fig. 8. Bars represent 1 mm in Fig. 1 through 6 and 10 mm in Fig. 7 & 8.

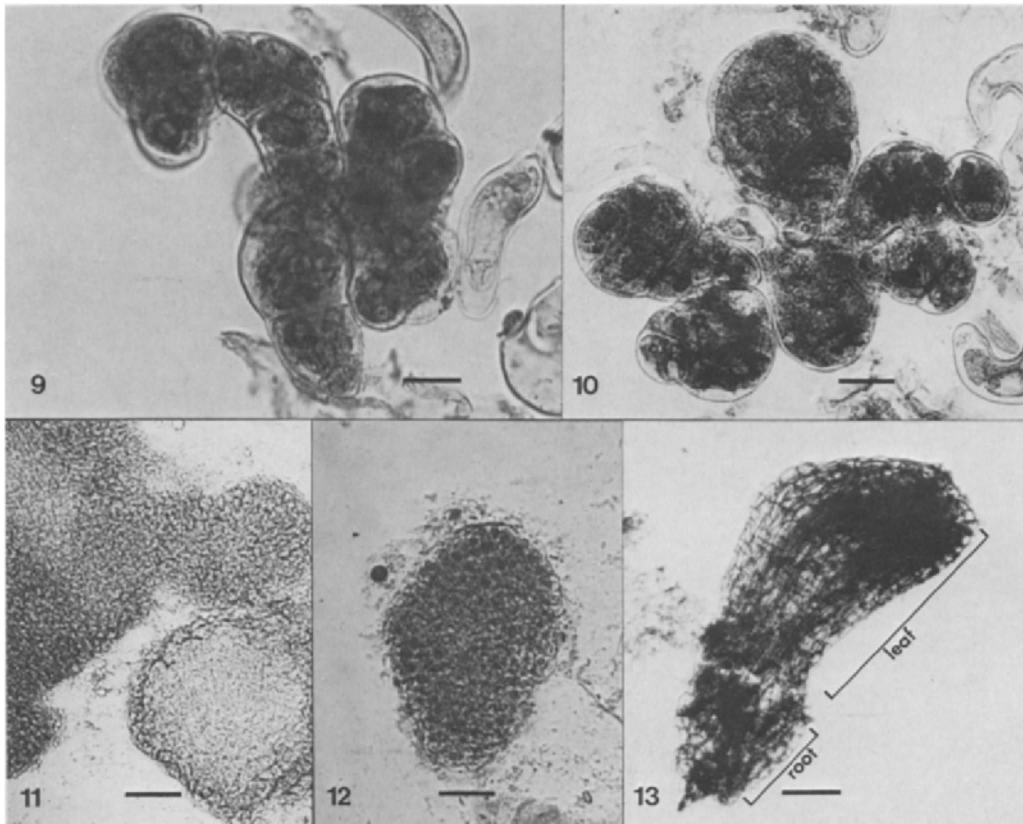

Fig. 9-13. Somatic embryogenesis in sugarcane. Fig. 9 & 10. Early stages in differentiation of embryos in liquid suspension culture. Fig. 9. shows five groups of meristematic cells at 4 to 8 cell stage. Fig. 10. Shows a single meristematic cell and several bipolar globular embryoids at various stages of development. Fig. 11-12. Somatic embryos at globular stage from cultures on solid MSC_3 medium. Fig. 13. A bipolar mini-plantlet with expanded leaf and root. Bars represent 17 µm and 27 µm in Fig. 9 & 10; 100 µm in Fig. 11 & 12, and 130 µm in Fig. 13.

Cell suspension culture:

Callus, maintained on MSC_3 medium for 10 weeks, was transferred to liquid medium (half MS + 0.5 mg l^{-1} 2,4-D), and small aliquots of the supernatant were examined at 10- to 15-day intervals. Most cells in suspension were single, amoeba-shaped, or tubular and elongated. Such cells appeared thin-walled, hyaline, and did not divide any further. However, a small percentage (less than 5%) of these cells were meristematic with dense cytoplasm. The meristematic cells were considerably smaller in size than the hyaline cells and divided to form either colonies (Fig. 9) or differentiated into bipolar embryoids (Fig. 10). Some of these cells produced a suspensor-like cell colony, the terminal portion of which formed an embryoid. Embryoids up to globular stage (Fig. 10) were recovered from cell suspension cultures and represented the younger stages of somatic embryos obtained on MSC_3 solid medium (Fig. 11-13).

Cell suspensions, 3-5 ml, were plated on solid media with various growth regulators. Cell colonies developed best when grown either on MSC medium or on half-MS medium supplemented with 0.5 mg l^{-1} 2,4-D, 50 ml l^{-1} coconut water and 2.0 mg l^{-1} zeatin. However, these cell colonies did not show any further differentiation.

Somaclonal variation:

Morphological variants differing in leaf shape, color, and plantlet vigor were present among the 300 or more plants regenerated from callus subculture. Color variants included albino, pale green, dark green, and purple-sheath and purple-leaf types. Some plantlets had extremely narrow leaves, and others had wide, short, thick leaves.

DISCUSSION

Tissue cultures of graminaceous plants give rise to plantlets by differentiation into shoot primordia, proliferation of adventitious buds, and somatic embryogenesis (King et al., 1978). Although each type of differentiation results in plantlets, there is a subtle distinction in the mode of plantlet formation. Differentiation of callus into a shoot primordium produces a leaf first; adventitious buds, if present, form a clump of leaves, but a somatic embryo produces a coleoptile first. The shoot and adventitious bud primordia are initially unipolar and develop roots later. If shoot and root primordia differentiate simultaneously, continuity between the shoot and root zones is lacking. Somatic embryos are, however, bipolar at a very early stage of embryogenesis. Somatic embryoids at globular stage can be easily confused with the spherical shoot-meristems of monocots. However, in the present study on sugarcane, the somatic embryoids at the globular stage showed the formation of typical epidermal layer formed by shield-like cells (Fig. 10 and 12) which completely surrounded the dense meristematic cells. Such embryoids at or soon after the globular stage developed hyaline cells at one end (Fig. 10) which are destined to produce a short suspensor or hypoblast (Johansen, 1950). After torpedo stage, the embryos were bipolar and consisted of an expanded leaf-like shoot zone with a central core of vascular tissue, surrounded by

cells rich in plastids, and a narrow region of elongated cells (Fig. 13), the whole structure resembling a miniature plantlet with a leaf and a root.

A coleoptile rather than a leaf is the first structure to be observed in somatic embryogenesis. Callus cultures of clone IJ76-316 showed that the regenerated plants originated predominantly through somatic embryogenesis. The presence of bipolar embryoids in the suspension cultures and of globular embryos in cultures on solid MSC_3 medium, and the development of scutellum-like structures with emerging coleoptiles, all strongly suggested the occurrence of somatic embryogenesis in sugarcane. Most plantlets obtained from callus cultures of clone IJ76-316 formed a coleoptile from which emerged the first leaf. In some cases, particularly when callus cultures were maintained on MSC_3 medium for 10 weeks, and transferred to half-MS medium supplemented with growth regulators (treatment 10), the embryos germinated precociously and coleoptile did not elongate sufficiently to be visible. However, in either case these plantlets, circa 4 to 10 mm long, were single entities, each with a complete shoot and root and were not joined at the base, which is usually true of plantlets originating from multiple shoot-primordia. Prolonged culture of calli undergoing somatic embryogenesis on high-auxin medium such as MSC_3 resulted in highly abnormal growth patterns, which were akin to several shoot-primordia or embryoids, all stuck to a single elongate root-like structure resembling deformed suspensors. These structures could be interpreted as either bipolar embryos or shoot zones but they showed the presence of coleoptiles in some cases (unpublished). It is likely that the presence of high auxin concentration in the post-embryogenic phase caused secondary proliferation and abnormal growth of the embryonic meristem zones following primary embryogenesis. The pattern of somatic embryo formation, i.e. the formation of scutellum surrounding a bipolar structure with a coleoptile and a root, observed in the present study was very similar to that reported in Pennisetum and Panicum species (Vasil and Vasil, 1981; Haydu and Vasil, 1981; Lu and Vasil, 1981), and to sexually produced embryos in gramineae (Johansen, 1950).

The formation of globular embryoids from single cells (Fig. 9-10) in suspension culture clearly demonstrated totipotency of individual sugarcane cells for the first time. Suspension cultures of this type would be valuable in obtaining pure mutants as opposed to chimeral variants which often originate from adventitious bud- and shoot-primordia. This study also confirms the previous hypothesis (Nadar et al., 1978) that embryoid-like structures can be initiated in sugarcane callus cultures on a medium containing relatively high quantities of 2,4-D. Transfer of calli with differentiated embryos to a high 2,4-D medium produced secondary callus from both the shoot and root zones, hence mature embryos would not be recovered from successive subcultures on medium with high 2,4-D.

While it is likely that the high frequency of somatic embryogenesis observed in the present study may be typical of only certain sugarcane clones, prolonged callus culture may be common to most clones for initiation of embryos. The subsequent growth and development of somatic embryos of sugarcane required removal of 2,4-D or a medium with a reduced level of 2,4-D. Once embryos were initiated, their maturation did not require 2,4-D. Instead, a reduction in the level of mineral salts was necessary. Addition of cytokinin (which was provided by either coconut water or zeatin) to the medium (Table I) promoted growth and proliferation of scutellum and somatic embryos. Amount of sucrose in the medium was critical to promote embryonic root-elongation. The relatively high (60 g l^{-1}) sucrose in the medium suppressed shoot formation and promoted rooting of the immature embryoids. Embryo maturation may thus require a critical range of osmotic pressure. Transfer of poorly rooted embryos to half-MS medium with 30 g l^{-1} sucrose also promoted root growth which suggests a differential requirement of osmotic pressure for shoot- and root-forming zones of embryos.

ACKNOWLEDGMENT

Technical help of Mrs. Peggy Hiraki in media preparation is gratefully acknowledged.

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