SHORT COMMUNICATIONS

ZEA MAYS: METHODS FOR DIPLOID CALLUS CULTURE AND THE SUBSEQUENT DIFFERENTIATION OF VARIOUS PLANT STRUCTURES*

By P. M. GRESSHOFF† and C. H. DOY†

Abstract

A diploid callus line of *Z. mays* has been established on fully defined media, taking advantage of the callus-forming potential of squashed embryos. This method is general, and thereby not restrictive to one strain or mutant cell line. Methods for the differentiation of roots, shoot primordia, and ovule-bearing horns by transfer of callus to defined media with various hormonal constituents are discussed. The callus cell line has retained its growth characteristics and ploidy level for 12 months.

Botanical, genetical, and physiological studies of *Zea mays* have contributed substantially towards an understanding of the biology of higher plants. Further progress, especially at a molecular level, would be assisted by the availability of cell lines in tissue culture. Straus and LaRue (1954) established an endosperm callus culture, but we understand that this is now lost and that subsequent attempts by other workers failed to show the same success (A. Schwartz, personal communication). We now present a method which has proven successful for the induction of diploid callus of several varieties of *Z. mays*. The differentiation of plant structures from callus was only attempted with *Z. mays* variety B-48.

Seeds were surface-sterilized by washing with ethanol (100%) and sterile distilled water (each for 10 s), repeating the procedure three times. The seeds were then placed onto autoclaved, water-soaked Whatman filter paper in sterile Petri dishes. After 48 hr at 27°C in the dark, a swelling in the embryo area of the seeds was noticed. This indicated active growth of the embryo which was about to break through the seed coat. Embryos were removed aseptically and macerated with forceps and a scalpel blade, thereby removing any existing structural organization. The resulting "cell paste" (about 20 mg) was placed onto sterile, fully defined growth medium containing mineral salts (Gamborg and Eveleigh 1968; Gresshoff and Doy 1972*a*, 1972*b*) vitamins,‡ trace elements, iron-chelate (Gresshoff and Doy 1972*a*, 1972*b*), Difco agar (0·8%), glucose (2%), and growth hormones (growth medium 1, Table 1). The cultures were incubated at 27°C in the dark. Within 2 weeks the cell mass

* Manuscript received 12 July 1972.

† Department of Genetics, Research School of Biological Sciences, Australian National University, P.O. Box 475, Canberra City, A.C.T. 2601.

‡ *Note added in proof.*—The vitamin–glycine stock solution should be in 100 ml, not 1000 ml as stated in Gresshoff and Doy (1972*b*).

doubled as the result of callus growth. Subculture to growth medium 2 (Table 1) resulted in accelerated growth (doubling time 7–8 days). The resulting callus (Fig. 1) is very friable and without pigmentation. Cells are large, highly vacuolated, and only partially in contact with each other.

TABLE 1

HORMONE CONCENTRATIONS IN GROWTH MEDIA AND LIGHT CONDITIONS OF INCUBATION

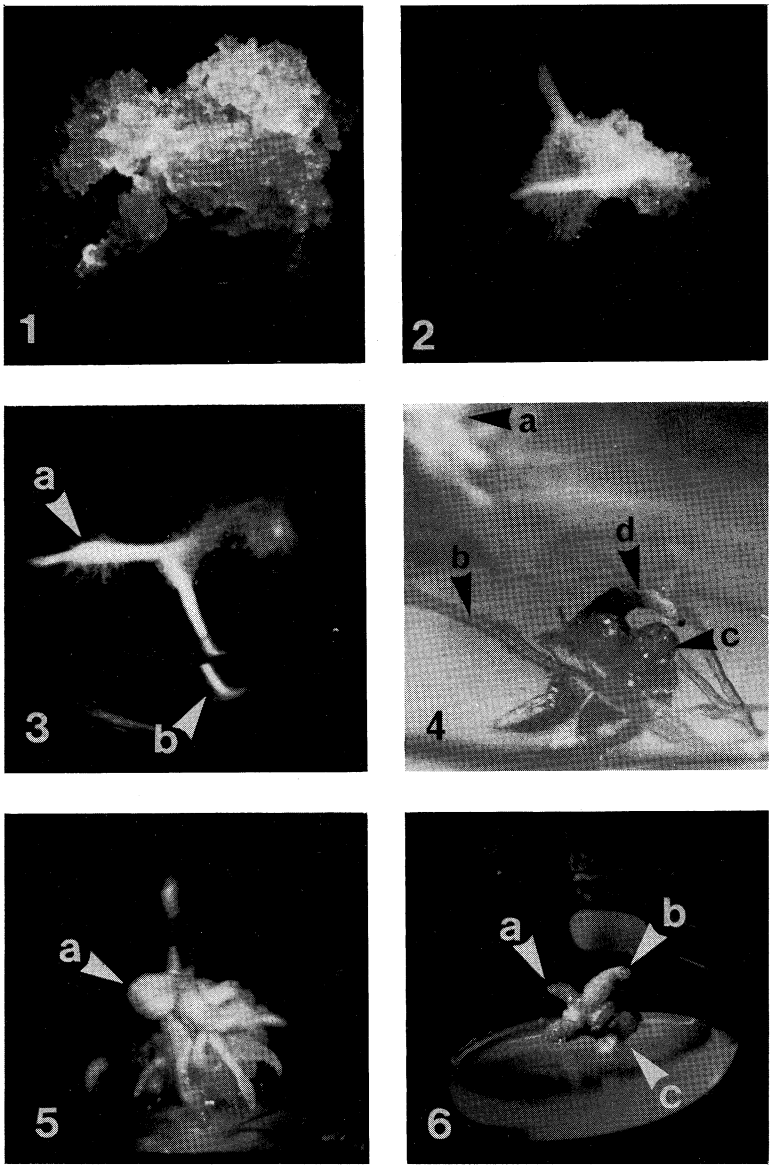
Medium No.	Naphthylacetic acid (mg/l)	Kinetin (mg/l)	Light condition
1	2·5	0·1	Dark
2	8·0	0·01	Dark
3	2·5	1·0	Dark
4	0·1	2·0	16 hr light–8 hr dark
5	8·0	0·1	Light

Extensive root formation (Figs. 2 and 3) followed the transfer of callus (about 50 mg) from the callus-supporting medium 2 (containing a high auxin to cytokinin ratio) to medium 3 (Table 1), which has a more balanced auxin to cytokinin ratio. The increase in kinetin resulted in a more compact callus of smaller closely packed cells resembling meristematic tissue. This change in the organization of callus has previously been shown (Gresshoff and Doy 1972*a*, 1972*b*) to be a prerequisite for organ differentiation. It is thought that increased contact, promoting cellular communication, is required for cells to proceed along specific developmental pathways terminating in various plant structures.

Two types of roots were produced initially, those with root hairs and those without, root hair formation being characteristic of aerial roots but not of roots penetrating the agar (Figs. 2 and 3). As the aerial roots continued to grow, the new tissue was hairless. Whenever these hairless portions came into contact with the agar a callus developed, therefore exposure to the high auxin–moderate kinetin of medium 3 provides the environment for callus induction and development from the meristematic region. When left undisturbed the new calluses served as centres for renewed root formation. All types of roots have a vascular system and anatomy similar to roots of normally grown *Z. mays*.

Feulgen staining of root tips from actively growing aerial roots showed that the cell line was diploid and demonstrated maintenance of this ploidy during differentiation. The diploid state was expected because of the embryo origin of the cell line. There are many reports regarding natural polyploidization of cultured cells or differentiating plant structures (Sacristan 1971), but in our studies with *Arabidopsis thaliana* and *Lycopersicon esculentum* (Gresshoff and Doy 1972*a*, 1972*b*), and now *Z. mays*, this has not occurred.

Induction of chloroplast development in specific areas just beneath the callus surface and shoot induction (Fig. 5) followed the transfer of rapidly growing callus from medium 2 to the low auxin–high kinetin medium 4 (Table 1) and incubation at 27°C with a 16 hr/8 hr light–dark cycle for 4–6 weeks. The reversal of the auxin



Figs. 1–6.—*Zea mays:* Diploid callus and the differentiation of various plant structures.
Fig. 1.—Diploid callus.
Fig. 2.—The development from callus of a primary aerial root with root hairs.
Fig. 3.—Simultaneous development of an aerial root (*a*) and an agar root (*b*). Note that the aerial root has entered a second stage of development where the new growth at the tip is hairless. The agar root is typically hairless.
Fig. 4.—Mini-callus formation (*c*) from a secondary aerial root (*b*). The position of the original callus is indicated (*a*). The mini-callus is producing an aerial root (*d*) which is partially pigmented red.
Fig. 5.—Induction of a plantlet which has shoots (*a*) and roots.
Fig. 6.—Development of phallic horns (*a* and *b*). *a* is partly red-pigmented and represents an early stage (cells with red pigmentation later slough off) and the mature horn (*b*) resembles a natural cob in miniature. An area of internal greening is indicated (*c*). This figure was produced by the re-photography of a Kodachrome II transparency onto Panatomic X.

to kinetin ratio to initiate plantlet differentiation is well documented (Pillai and Hildebrandt 1969; Gresshoff and Doy 1972*a*, 1972*b*). Meristematic regions produced high populations of chloroplasts before the development of shoot primordia. As yet we have been unable to induce a complete plant.

Phallic horns developed (Fig. 6) when differentiating regions of callus, growing on medium 4 for 4 weeks, were transferred to a medium with a higher auxin to kinetin ratio (growth medium 5, Table 1), held at 27°C in continuous light. Medium 5 is closely related to a root-induction medium and the shape of the horn resembles a root initial (see Fig. 4). However, the horns were partly red-pigmented and developed ordered rows of small protrusions. Microscopic examination of these protrusions lead us to propose that these are minute ovules arranged on the horn in a fashion similar to the pattern found on the familiar corn cob. These structures may only be properly illustrated by colour photography.

In this laboratory pseudo fruits of tomato have been induced directly onto a callus of haploid tomato tissue (Gresshoff and Doy 1972*b*). The pattern of hormonal transfers was similar in the tomato to the one described here for *Z. mays*. In both cases, meristematic tissue of callus was induced to differentiate further. However, the normal developmental sequence towards plants was interrupted by a transfer to a higher auxin medium. This clearly simulated the hormonal environment experienced by ovule and other fruit-determining cell primordia found in normal fruit development.

The processes described here have been observed on a number of occasions over a period of one year. Little change has occurred in the cell line. Ploidy, callus morphology, growth rate, and differentiation potential have remained unchanged.

Acknowledgments

We thank Dr. A. Pryor, Division of Plant Industry, CSIRO, for supplying *Z. mays* seeds. Mr. N. Gowen and Mr. B. Parr are thanked for technical and photographic assistance.

References

GAMBORG, O. L., and EVELEIGH, D. (1968).—Culture methods and detection of glucanases in suspension cultures of wheat and barley. *Can. J. Biochem.* **46**, 417.

GRESSHOFF, P. M., and DOY, C. H. (1972*a*).—Haploid *Arabidopsis thaliana* callus and plants from anther culture. *Aust. J. biol. Sci.* **25**, 259.

GRESSHOFF, P. M., and DOY, C. H. (1972*b*).—Development and differentiation of haploid *Lycopersicon esculentum* (tomato). *Planta* **107**, 161.

PILLAI, S. K., and HILDEBRANDT, A. C. (1969).—Induced differentiation of geranium plants from undifferentiated callus *in vitro*. *Am. J. Bot.* **56**, 52.

SACRISTAN, M. D. (1971).—Karotypic changes in callus cultures from haploid and diploid plants of *Crepis capillaris* (L.) Wallr. *Chromosoma* **33**, 273.

STRAUS, J., and LARUE, C. D. (1954).—Maize endosperm tissue grown *in vitro*. I. Cultural requirements. *Am. J. Bot.* **41**, 687.