ANATOMICAL OBSERVATIONS ON THE HIGH FREQUENCY CALLUS FORMATION FROM ANTHER CULTURE OF CHRYSANTHEMUM

Watanabe, Kuniaki

Nishii, Yoshiaki

Tanaka, Ryuso

(Citation)
The Japanese journal of genetics,47(4):249-255

(Issue Date)
1972

(Resource Type)
journal article

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/90001246

JAPAN. J. GENETICS Vol. 47, No. 4: 249-255 (1972)

ANATOMICAL OBSERVATIONS ON THE HIGH FREQUENCY CALLUS FORMATION FROM ANTHER CULTURE OF *CHRYSANTHEMUM*

KUNIAKI WATANABE, YOSHIAKI NISHII AND RYUSO TANAKA

Botanical Institute, Faculty of Science, Hiroshima University, Hiroshima 730

Received May 11, 1972

In anther culture it has been reported that the rate of callus formation is very low (0-3% in *Oryza* by Niizeki and Oono 1968, 0-8% in *Brassica* by Kameya and Hinata 1970, very low in *Setaria* by Ban *et al.* 1971, 1% in *Aegilops* by Kimata and Sakamoto 1972). In order to maintain the genetically valuable strains of *Chrysanthemum*, we have attempted to induce clonal propagation from anther tissue. From 8 species and 2 varieties of *Chrysanthemum*, extremely high rates of callus formation have been obtained, and it has been easy to redifferentiate these calluses into roots and shoots. The present paper presents these results together with anatomical observations on the characteristics of callus formation.

MATERIALS AND METHODS

The following species have been used:
Diploids $2n=18$, *Ch. nipponicum* Matsumura and *Ch. boreale* Makino
Tetraploid $2n=36$, *Ch. indicum* L.
Hexaploids $2n=54$, *Ch. japonense* Nakai and *Ch. zawadskii* Herbich
Octoploids $2n=72$, *Ch. japonense* var. *octoploid*, *Ch. ornatum* Hemsl. and *Ch. shiwogiku* var. *kinokuniense* Shimotomai et Kitamura
Decaploid $2n=90$, *Ch. pacificum* Nakai
Hypo-decaploid $2n=89$, *Ch. japonense* var. *crassum* Kitamura

Young flower buds were sterilized in 70% ethanol for 30 seconds. Tube flowers were excised after removing the involucre bracts. Anthers with the cells at varied stages from tetrad meiocyte to young pollen grain were pushed out from the lower parts of tube flowers to the upper by a finger nail. They were then planted on the surface of the artificial medium in 300 ml Erlenmeyer flasks and were cultivated at 25°C under 200-500 lux inflorescent light. Some anthers were sterilized with 70% ethanol for 3 seconds in order to leave only microspore cells after the killing of the tissues of anthers. Miller's solution was used as the basic medium (Miller 1963). It was supplemented with 1.5 mg/*l* 2, 4-D (2, 4-dichlorophenoxyacetic acid), 1 mg/*l* IAA (indol-3-acetic acid) and 2 mg/*l* kinetin. The solution was adjusted to pH 6.0 and the medium was solidfied with 8 g/*l* agar.

In anatomical studies, the following species were used: Diploids, *Ch. nipponicum* and *Ch. boreale*, Decaploid, *Ch. pacificum*. After 4, 5, 8, 9, 10, 12 and 17 days from cultiva-

tion, anthers were fixed in Carnoy's fluid (95% ethanol 6: chloroform 3: acetic acid 1) for 1 hour. After embedding in paraffin, the sections were cut 10 microns thick and were stained with Heidenhain's iron haematoxylin solution.

RESULTS

Anther culture

The sterilization of anthers with 70% ethanol was unsuccessful, because the sterilized anthers turned black and died within 7 days. The results for anthers not sterilized are shown in Table 1. Callus formation occurred in all anthers of the 8 species and 2 varieties employed. The induction rates of callus was thus 100%.

Four days after cultivation, the anthers became slightly inflated as compared with the control (Fig. 1-A). After 8 days they became extremely inflated at the central parts. The epidermis became broken, and cell clusters were forced out to proliferate from the inside of the anthers. Callus formation was then clearly visible (Fig. 1-B). Twelve days after cultivation, the calluses had attained a volume 3-4 times that of the original anthers. The outside of each anther was embedded with callus and the original form of anther had become obscure (Fig. 1-C).

In *Ch. nipponicum* and *Ch. japonense* var. *crassum* callus formation occurred at 4-5 days after cultivation, while in *Ch. pacificum* the time taken was 6-8 days. The calluses obtained from *Ch. zawadskii* were light brown and soft but those from all other species were solid.

Thirty to fifty days after cultivation, roots were observed on most calluses of *Ch. boreale* and *Ch. japonense* var. *crassum* and these roots had many root hairs (Fig. 1-D). Some calluses of *Ch. pacificum*, *Ch. ornatum* and *Ch. japonense* also differentiated roots.

Table 1. Results of anther culture

Species	Chromosome number (2n)	No. of implanted anthers	No. of anthers formed callus (%)
Data in 1970			
Ch. boreale	18	240	240(100)
Ch. indicum	36	107	107(100)
Ch. japonense	54	31	31(100)
Ch. japonense var. *octoploid*	72	58	58(100)
Ch. japonense var. *crassum*	89	114	114(100)
Ch. shiwogiku var. *kinokuniense*	72	22	22(100)
Ch. pacificum	90	46	46(100)
Data in 1971			
Ch. boreale	18	35	35(100)
Ch. nipponicum	18	64	64(100)
Ch. zawadskii	54	93	93(100)
Ch. ornatum	72	216	216(100)
Ch. japonense var. *crassum*	89	233	233(100)
Ch. pacificum	90	257	257(100)

The calluses which had differentiated roots were transplanted to the medium without 2, 4-D and IAA. Fourty to fifty days later numerous shoots differentiated from all calluses of *Ch. japonense* var. *crassum* (Fig. 1-E) and some calluses of *Ch. japonense*. The calluses of *Ch. nipponicum* differentiated shoots about 50 days after transplanting to the same medium. The young plants were potted off at the three-leaf stage, and

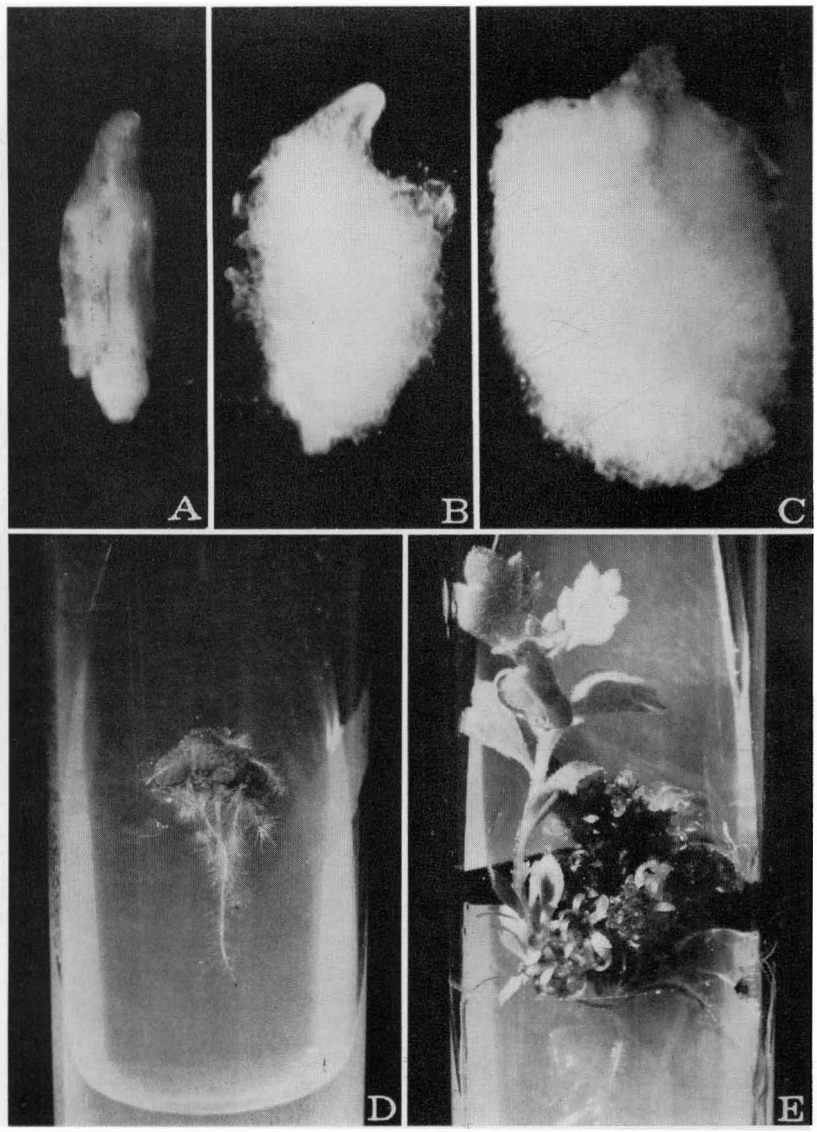

Fig. 1. A, B and C, callus formation in anther culture of *Chrysanthemum nipponicum*, $2n=18$. Four days(A), 8 days(B) and 12 days(C) after the beginning of cultivation. D, root formation from the callus of *Ch. japonense* var. *crassum*, $2n=89$, 40 days cultivation. E, shoot formation from the callus of *Ch. japonense* var. *crassum*, 70 days after transplanting to the medium without the auxin hormones. A-C, ×50. D, E, ×1.4.

their chromosome numbers were examined. The observed 6 plants derived from *Ch. japonense* var. *crassum* $2n=89$ had the chromosome number $2n=89$. The observed 2 plants derived from *Ch. japonense* $2n=54$ had the chromosome number $2n=54$. Thus, the plants derived from calluses were found to have chromosome constitutions identical with those of their mother plants.

Anatomical observations on the formation

The structure of the anthers was found to be similar in all of the three species studied. Each anther contains 4 loculi which are paired in 2 anther thecae. The 2 thecae are separated by connective tissue comprising many cells riched in cytoplasm. Three cell layers surround each loculus. The cells of outermost layer are epidermis and those of the innermost are tapetum. The epidermis breaks down before dehiscence during normal development of the anther, so that only membranous fragments of it remain. The cells of middle layer, the endothecium, thus become the functional outer layer. The tapetum cells, which are full of dense cytoplasm and are often multinucleate, degenerate during the maturation of the pollen (Figs. 2-A, 3-A).

The anthers of *Ch. nipponicum* showed following morphological changes under culture condition. Four days after cultivation, the anthers had become inflated to about 1.2 times (in cross section), as compared with the control. The cells of the endothecium surrounded the tapetum, while the cells of connective tissue enlarged 1-3 times as com-

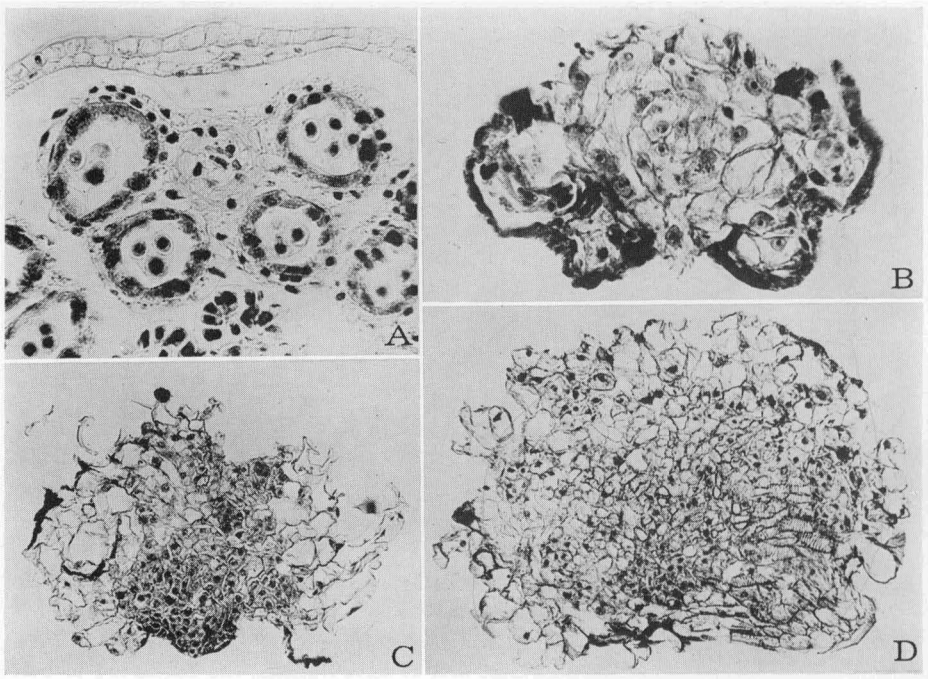

Fig. 2. Photomicrographs of the cross sections of the anther and its calluses of *Chrysanthemum nipponicum*, $2n=18$. A, the anther having tetrad meiocytes before the cultivation. B, C and D, deforming anthers by callus formation at 4 days, 8 days and 12 days after the cultivation, respectively. A, B, ×300. C, D, ×100.

pared with the control and their cell walls became thickned. The number of cells in connective tissue also increased to about twice and the volume of each cell showed an increase by as much as 4 times. Mitotic divisions with varied orientation were observed in the connective tissue (Figs. 2-B, 3-B). The $2n=18$ chromosomes were counted in these mitotic cells. In the loculi either tetrads and young pollen grains or mature pollen grains were present, but there was no cell proliferation from them. They were observed to be flattend due to the pressure from the growth of connective tissue.

After 8 days, the anthers had enlarged about 2.4 times in cross section. The cells of endothecium and tapetum were no longer observable. In the loculi no trace of microspores was detectable. The callus of the connective tissue had enlarged to 10-12 times its original volume. In this some clusters of small cells were observed. These small cells possessed a dense cytoplasm and a nucleus with some nucleoli. Differentiating vascular bundles were observed in the central region of the callus (Figs. 2-C, 3-D). Twelve days after cultivation, anthers increased to about 4 times its original size. The original tissues of the anther were no longer visible, and the whole callus appeared to be derived from connective tissue. The number of cells in the callus increased 7-20 times that of the original connective tissue. The volume of cells in the callus showed increase in volume of about 1-6 times. Vascular bundles could be observed conspicuously (Fig. 2-D).

Callus formation from anther cultures of *Ch. boreale* $2n=18$ and *Ch. pacificum* $2n=90$ was similar to that of *Ch. nipponicum*. In *Ch. pacificum* some of the cells of the in-

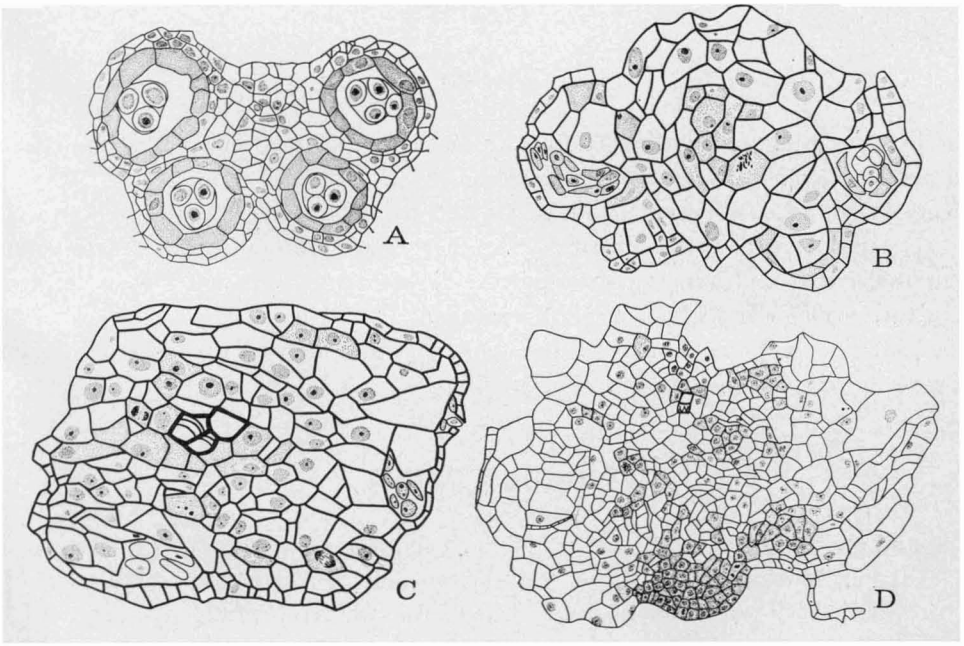

Fig. 3. Cross sections of the anther and its calluses of *Chrysanthemum nipponicum*, $2n=18$. A, B and D, text-figures of A, B and C in Fig. 2, respectively. C, deforming anther by callus formation at 5 days after the cultivation. A, B and C, ×300. D, ×125.

duced calluses were found to be derived from the middle layer, the endothecium, but most were derived from the connective tissue.

DISCUSSION

In *Chrysanthemum*, using young anthers, calluses were readily obtained with very high frequency. These calluses were found to have derived from the cells of the connective tissue. Therefore, the cells of the connective tissue can be regarded as undifferentiated or scarecely differentiated. In our experiments, it is of interest that the cells of the connective tissue were selectively induced.

It is emphasised that the anther culture technique is very simple. The florets inside the young inflorescence buds are already sterile, and callus formation is readily obtained.

Plants redifferentiated from the calluses derived from the anther of an aneuploid *Ch. japonense* var. *crassum* with $2n=89$ showed the same chromosome number, indicating that the induced plants were derived from somatic tissue of the anthers. Similarly, plants induced from anther calluses of *Ch. japonense* $2n=54$ showed the same chromosome number as the mother plant. The present authors have also obtained calluses at the 100% rate from anther cultures of *Haplopappus gracilis* $2n=4$, an annual member of Compositae (unpublished data).

From the present findings on anther culture, it can be said that it is possible to maintain genetically valuable lines through clonal propagation.

SUMMARY

Calluses were induced with 100% rate from anthers of 8 species and 2 varieties of diploid and polyploid *Chrysanthemum* species.

Most of the calluses were found to be derived from the cells of the connective tissue between the thecae of the anthers. Within 40 days from cultivation, these calluses differentiated roots, and numerous shoots were differentiated within 40 days after transplanting to the medium without auxin hormones.

The induced plants examined in some genetic strains showed the same chromosome number as the respective mother strains.

ACKNOWLEDGMENTS

The authors are indebted to Dr. H. Kobayashi, Chugoku Agri. Exp. Station and Dr. S. Takami, Hiroshima Univ., for their helpful advice in the preparation of culture media, to Mr. M. Kinoshita, Izumi High School, Kagoshima Pref., Japan, and Mr. S. Kurizono, the Attached High School of the Hiroshima Univ., for their cooperation of the collection of materials. In the preparation of the English manuscript, we have been helped from Prof. S. Smith-White, Univ. of Sydney, Australia, a Leverhulme Visiting Fellow at the Hiroshima Univ. We wish to express him grateful acknowledgments.

LITERATURE CITED

Ban, Y., T. Kokubo, and Y. Miyaji, 1971 Production of haploid plant by anther-culture of *Setaria intalica*. Bull. Fac. Agr., Kagoshima Univ. **21**: 77-81.

Kameya, T., and K. Hinata, 1970 Induction of haploid plants from pollen grains of *Brassica*. Japan. J. Breeding **20**: 82-87.

Kimata, M., and S. Sakamoto, 1972 Production of haploid plants of *Aegilops* by anther culture. Japan. J. Genetics **47**: 61-33.

Miller, C. O., 1963 Kinetin and Kinetin-like compounds. In "Moderne Methoden der Planzen-analyse" Band **6**: 194-202. Spring-Verlag, Berlin.

Niizeki, H., and K. Oono, 1968 Induction of haploid rice plant from anther culture. Proc. Japan Acad. **44**: 554-557.