Z. Pflanzenzüchtg. **72**, 89—94 (1974)

ISSN 0044-3298 / ASTM-Coden: ZEPZAD

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Projektgruppen Haploide in der Pflanzenzüchtung

Observations on the Growth in Culture of Anthers of *Secale cereale*

By

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With one plate

Received May 20, 1974

Introduction

The discovery that cultured pollen grains are able to by-pass their normal developmental pathway and give rise to haploid and/or homozygous tissue (Guha and Maheshwari 1966) has opened up new possibilities in plant breeding and plant cell genetics (Melchers 1972, Sunderland 1973). Anther culture experiments are currently being conducted in our laboratory with the objective of producing large numbers of haploid and/or homozygous plants from pollen of highly heterozygous F_1 hybrids of *Secale cereale*. If the parents of the F_1 hybrids contain characters of value to plant breeding programmes then success in inducing a high frequency of plantlet formation from pollen of the hybrids should rapidly uncover important gene combinations. The recent finding that pollen in anthers from tobacco F_1 hybrids can give rise to plants which are superior in yield and disease resistance to their parents suggests this aim to be feasible (Nakamura et al. 1974).

Material and Methods

Plants of *S. cereale* 'Somro' (obtained from F. von Lochow-Petkus GmbH, Bergen) and of a F_1 hybrid ('Pekuro' × Stamm MZ [short culm type], obtained from H. Kuckuck, Hannover) were grown to maturity. Flower spikes still enclosed within the flag leaves, were excised and sterilised by swabbing with 70 % aqueous ethanol. After discarding the flag leaves the florets were separated from the spike and the anthers removed and transferred to 6 cm plastic petri dishes containing a 0.8 % agar medium (12 anthers per dish). Only anthers with pollen between the tetrad and binucleate stage were selected for culture. The culture media tested included variations of the basal media of Blaydes (1966), Murashige and Skoog (1962),

NITSCH and NITSCH (1969), SCHENK and HILDEBRANDT (1972) and WHITE (1943), additionally containing various combinations and concentrations of growth compounds. Petri dishes were transferred to culture rooms maintained at 25 °C in the dark. After 6—10 weeks incubation, petri dishes containing calluses were transferred to similar rooms but with a 14 hour light period of 3000 lux (Osram Natura). Cytological staining was carried out by the acetocarmine squash method.

Results

The response of the cultured anthers on the different media combinations varied greatly and was dependent upon the physiological state of the anthers and upon the type and level of growth substances present in the culture media. Most anthers failed to give a growth response and rapidly became brown and necrotic. Others remained healthy and after 3—6 weeks incubation gave rise to macroscopic calluses. Generally the calluses were derived from the anther filaments, particularly on media containing high levels of auxins [$>$ 0.6 mg/l 2,4 dichlorphenoxyacetic acid (2,4-D) or $>$ 1.0 mg/l α-naphthalenacetic acid (NAA)]. In some experiments where low auxin levels were present, calluses have been obtained from the anther wall, from the connective and rarely from the pollen. For example plate 1 g, h shows a young pollen callus developing on NITSCH and NITSCH medium containing 0.6 mg/l 2,4-D and 0.6 mg/l indol-3-yl-acetic acid (IAA). Also on this medium containing 0.1; 0.25; or 0.5 mg/l 2,4-D we have frequently observed large numbers of multicellular and multinuclear pollen grains. Anthers have been examined after 10, 21, and 42 days culture on such 2,4-D containing media.

A small proportion of anthers examined after 10 days contain numerous pollen grains undergoing mitoses. Some of the mitoses appeared to represent normal stages in the formation of mature pollen grains but others showed clear deviations from the normal developmental pattern. For example the pollen grain shown in plate 1 a can be interpreted either as representing a bicellular grain in which mitosis is occurring in both the vegetative and generative nuclei or as a unicellular grain undergoing endomitosis. Other pollen grains were bicellular either with the nuclei of similar size and staining properties or with a clear difference between the nuclei. In the latter case either the generative or vegetative nucleus or both nuclei were undergoing further divisions. Plate 1 b is interpreted as representing a tricellular or trinuclear grain in which two of the cells are derived by division of the vegetative nucleus and where the generative nucleus has not yet divided. Certain grains, which were characterised by the failure of their cytoplasm to stain deeply and uniformly were clearly undergoing nuclear division without wall formation.

After 21 days in culture some anthers were shown to contain many multicellular pollen grains with cell numbers varying from 2 to approximately 16 cells. Some of the multicellular structures possessed equal-sized nuclei of similar staining properties (plate 1 d) and others nuclei of different size and staining properties (plate 1 f). Quite frequently multinuclear pollen grains could be found within the same anthers (containing up to 16 nuclei). Generally the cytoplasm in such multinuclear grains stained very poorly with acetocarmine but occasionally some were observed which stained so deeply that it was difficult to distinguish them from multicellular grains (plate 1 e). After 42 days incubation

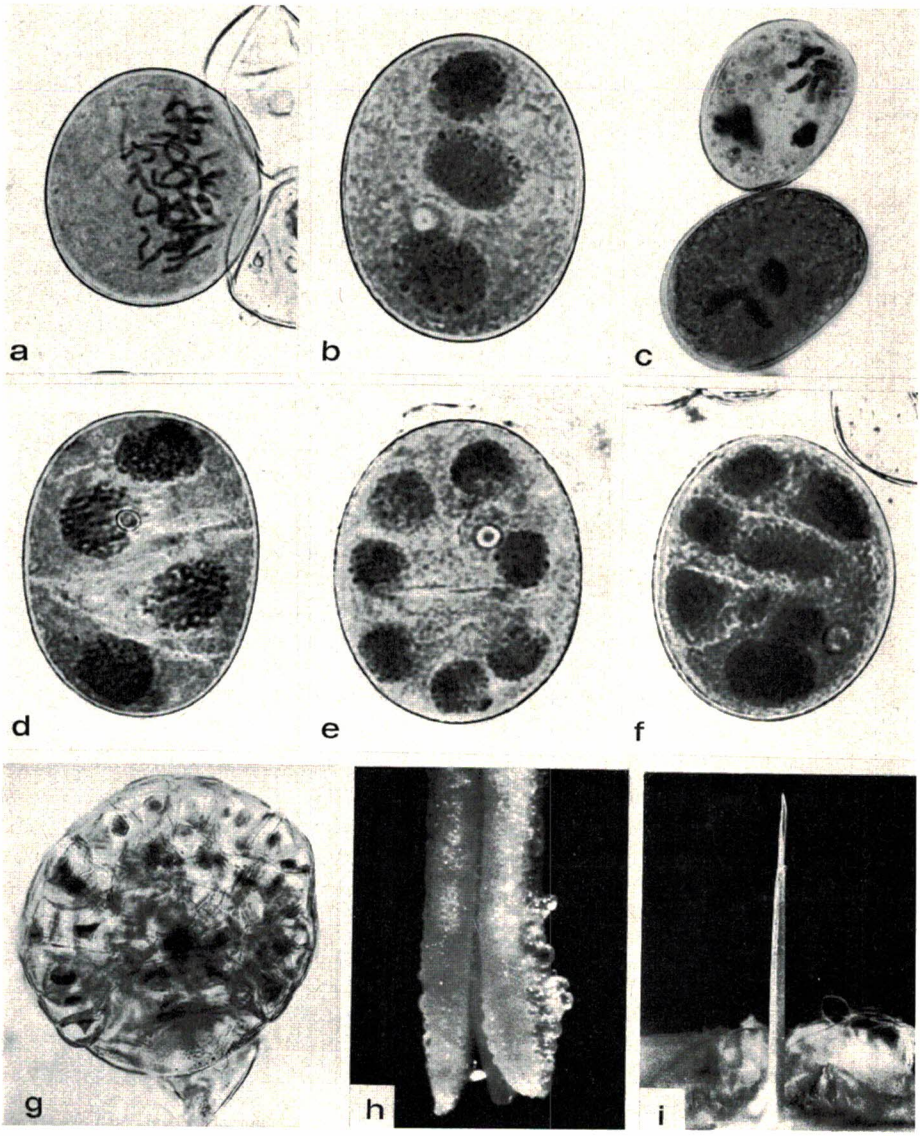

Plate 1. a) Pollen grain undergoing abnormal mitosis; b) Trinucleate or tricellular grain in which one nucleus (generative derivative) is smaller and stained more deeply than the other two (vegetative derivatives); c) Normal mature pollen grain (lower grain) with an abnormal grain undergoing divisions (both are present *in vivo*); d) Multicellular grain and e) Multinuclear grain, both containing nuclei of similar size and staining properties; f) Multicellular grain containing nuclei of different sizes and staining properties; g) Large multicellular grain isolated from a cultured anther after 42 days; h) Cultured anther where large numbers of multicellular grains have ruptured the anther wall; i) Plantlet formed from somatic tissue of a cultured anther on MURASHIGE and SKOOG medium containing 2 mg/l NAA, 2 mg/l 6-BAP and 2 mg/l GA_3

on medium containing 2,4-D small numbers of cultured anthers (< 1 %) split open to reveal the presence of callus-like masses (plate 1 h). Examination of the masses showed them to consist of numerous large multicellular pollen grains (plate 1 g). This development occurred in anthers from both plant types studied.

Cytological study of mature anthers prior to anthesis has shown that some of the pollen grains fail to form the typical vegetative cell and sperm nuclei, but undergo abnormal mitoses. Pollen grains have been found containing three or four nuclei and where the nuclei were undergoing further divisions (plate 1c). In common with many of the multicellular structures observable in cultured anthers the cytoplasm of such grains stained only weakly.

Normally calluses growing on media containing auxin as the sole growth compound initiate roots. In certain cases, the calluses can after prolonged incubation (2—5 months) spontaneously initiate shoots. Where shoot formation does occur up to 50 % are albinos and the green ones only develop further with difficulty. In an attempt to increase the frequency of plantlet formation, actively growing calluses have been transferred to media containing various combinations and concentrations of gibberellic acid (GA_3), 6-benzylaminopurine (6-BAP), kinetin, 2,4-D, NAA, and IAA. In the presence of cytokinins (2 mg/l 6-BAP or 2 mg/l kinetin) many of the calluses became black and necrotic, whereas others, especially those with roots, initiated new calluses which later formed green plantlets. The presence of GA_3 in the medium was beneficial and shoots once formed elongated rapidly. In certain cases the shoots formed on media containing GA_3 resembled very closely the shoots formed following seed germination (plate 1 i). Shoot-forming calluses are capable of further proliferation and morphogenesis so that numerous shoots can be obtained. They can be rooted by transfer to a basal medium lacking growth substances and then transferred to potting compost and grown to maturity. Most plants so far examined possess the diploid chromosome number ($2n = 14$) but occasionally higher ploidy levels can be found.

Discussion

Our observations of developing pollen in cultured anthers of *S. cereale* indicate that pollen in the uninucleate condition is most likely able to by-pass its normal developmental pathway and form multicellular or multinuclear structures. The observations suggest that when a generative and vegetative nucleus are formed, either derivatives of the latter or derivatives of both can contribute to the bulk of the developing structure. Further, our finding that bicellular or multicellular structures can possess morphologically identical nuclei suggests that the formation of a distinct vegetative and generative nucleus may not always occur and in such a case both nuclei can contribute to the developing structure. This view is not in agreement with that of SUN et al. (1974) who demonstrated that although morphologically identical nuclei are formed after the first pollen grain mitosis in *Triticale* the nuclei are physiologically different in that one always behaves in the manner of a generative nucleus. However, our observations do not eliminate the possibility that developments similar to those observed in *Triticale* can also occur in *Secale*. The evidence suggests a close resemblance of our system to the *Nicotiana* type (NITSCH and NORREEL 1973,

SUNDERLAND 1973) where the critical stage for multicellular pollen grain formation seems to be immediately prior to, during, or immediately after the first pollen grain division.

In our studies the very low frequency of pollen callus formation suggests that many of the observed multicellular structures may be abortive. This phenomenon may be due in part to inadequate cultural conditions or to competition between grains for compounds essential to their development. On the other hand it is possible that some pollen grains contain imbalanced chromosome numbers or lethal gene combinations which can affect their prolonged development in culture. The fact that certain pollen grains of *S. cereale in vivo* are abnormal, and are presumably sterile, has been demonstrated in our studies. Further the resemblance of these grains to multinuclear grains observed *in vitro*, particularly in the staining response of their cytoplasm, suggests that at least some abnormal grains are able to develop in culture. BENNETT and HUGHES (1972) have recorded similar observations in sterile pollen from ethrel treated anthers of *Triticum*. Although at present we cannot decide with certainty whether the abnormal grains observed *in vivo* are genetically imbalanced there is a large body of evidence indicating that pollen sterility in *Secale* can be caused by imbalanced chromosome numbers (WILLIAMS 1964).

One of the difficulties encountered in our studies was that somatic tissues of the anther as well as the pollen grains undergo further development. In such cases it is difficult to distinguish between the calluses of different origin or to decide whether growth of the pollen derived tissue is suppressed at the expense of the somatic tissue. Progress in this field demands that conditions are determined which may allow the observed multicellular pollen grains to undergo embryogenesis without prior callus formation.

Summary

Experiments have been undertaken with the objective of inducing pollen grain growth in cultured anthers of *Secale cereale*. During these studies the ability of pollen and of somatic anther tissue to form callus has been exposed and examined. Under appropriate conditions the somatic calluses can be induced to form plantlets. It has been possible to observe many different stages in the formation of multicellular pollen grain structures and our evidence suggests that the stimulus for their formation must be given either before, during or just after the first pollen grain mitosis. Multinuclear pollen grains which failed to form cell walls have also been observed in our cultures and comparison of these grains with mature pollen *in vivo* suggests that they arise from sterile pollen grains. The findings are discussed in relation to current views on the origin of multicellular pollen structures.

Zusammenfassung

Beobachtung von Entwicklungen in *Secale cereale*-Antherenkulturen

In Antherenkulturen von *S. cereale* konnten bei somatischem und gametophytischem Gewebe Callusbildungen induziert und aus dem somatischen Callus

auf bestimmten Kulturmedien ganze Pflanzen regeneriert werden. In Pollenkörnern, die aus auf Agar kultivierten Antheren isoliert waren, wurden bei cytologischen Untersuchungen vielkernige und vielzellige Strukturen gefunden. Aus den Beobachtungen wird geschlossen, daß der Anstoß für die Umstimulierung der normalen Pollenentwicklung zur Bildung haploider Calli kurz vor, während oder unmittelbar nach der ersten Pollenmitose erfolgen muß. Der Vergleich vielkerniger Pollen mit reifen, nicht kultivierten Pollenkörnern zeigt, daß sich diese vielkernigen Strukturen wahrscheinlich aus sterilen Pollen entwickelt haben. Unter Einbeziehung anderer Befunde über die Entstehung vielzelliger Pollenstrukturen werden die Ergebnisse diskutiert.

Acknowledgements

The authors gratefully acknowledge the assistance of all technicians of the haploid group particularly I. RICKER and W. LEHMANN. We are also grateful to KWS, Einbeck and Prof. H. KUCKUCK, Hannover for their useful discussion and for providing the plant material. We are indebted to the M.P.G. and to Prof. E. KNAPP and his staff for providing facilities which are making these studies possible. Finally we extend our gratitude to Prof. G. MELCHERS, Tübingen, for his advice during the course of this work and during the preparation of the manuscript.

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