Competition among Pollen Grains for Haploid Tobacco Plant Formation by Anther Culture

I. Analysis with Leaf Color Character

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Synopsis. To determine if any competition exists among pollen grains for haploid tobacco plant formation by anther culture, observed ratio in types for leaf color in plantlets emerged from anthers was compared with theoretical ratio.

Obtaining of haploid plants has become quite easy by anther culture in tobacco. Tobacco plants emerged from anthers were found to be haploid (TANAKA and NAKATA, 1967; BOURGIN and NITSCH, 1967; NAKATA and TANAKA, 1968; NITSCH, NITSCH and HAMON, 1968; TANAKA and NAKATA, 1969; NITSCH and NITSCH, 1969; SUNDERLAND and WICKS, 1969; NITSCH, 1969; CARLSON, 1970). In other crops haploid plants have been obtained from *Oryza sativa* (NIZEKI and OONO, 1968) and *Brassica oleracea* (KAMEYA and HINATA, 1970) by anther culture.

In application of this technique to basic studies of plant breeding as well as of genetics, it would be desirable to ascertain the possibility of competition among pollen grains. It would be very useful to observe frequencies of types of characters of haploid tobacco plants developed from pollen grains of the F_1 hybrid, in which certain characters are known to be segregated in an expected ratio. The nature of the deviation of observed ratio from expected ratio may be tested by calculating χ^2 for goodness of fit to an expected ratio.

This paper described the results on the frequencies in green and albino plantlets obtained by anther culture of the F_1 hybrid between *Nicotiana tabacum* and *N. tabacum* bearing Ws(pbg) gene. A brief statement already has appeared (NAKATA, 1969).

Materials and Methods

The F_1 hybrid between *N. tabacum* cv: Hicks 2 and *N. tabacum* bearing Ws(pbg) gene from *N. plumbaginifolia* was used for anther

culture and this was kindly supplied by Sato, Iwata Tobacco Experiment Station, Iwata, Japan. The latter parent of this cross was the same material as that handled by NIWA (1966). The segregation in F_2 of this hybrid was expected on a 15 green: 1 albino ratio for leaf color character. Green used in this paper referred to normal green and variegated leaves were classified as albino.

The F_1 hybrid plants were grown in a greenhouse kept at approximately 25°C. The uni-nucleate stage of pollen grains were used for anther culture. Haploid plants were obtained when anthers in the uni-nucleate stage were placed on the medium containing KNO_3 200, $Ca(NO_3)_2 \cdot 4H_2O$ 600, $MgSO_4 \cdot 7H_2O$ 100, KH_2PO_4 100, $MnSO_4 \cdot 4H_2O$ 10, Na_2EDTA 37.35, $FeSO_4 \cdot 7H_2O$ 27.85, IAA 1.5, kinetin 1.5, adenine sulphate 10, thimidine 5, thiamine hydrochloride 0.4, myo-inositol 100, sucrose 30,000, agar 6,000 mg/l, respectively.

The number of green and albino plants was counted at the following stages of plantlet formation: 1) when maximum size of a leaf reached approximately 2 mm in length (20–25 days after placing of anthers), 2) when maximum size of a leaf reached approximately 5 mm (30–50 days after placing of anthers), 3) when maximum size of a leaf reached 20–50 mm (50–60 days after placing of anthers), and furthermore 4) when albino plantlets were secondarily arisen from callus which had been formed from albino plantlets in culture. Differentiated plantlets in culture were directly transferred to soil in pots and allowed to grow further. Before flowering, chromosome behaviour at meiotic division of PMC's was

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observed for verification of haploid.

Results

Normal development of pollen grains into plantlets

Plantlets developed from approximately 70 percent of anthers, in which appreciable numbers of multicellular bodies varying from two-cell stage to globular stage in the ontogeny of tobacco embryo were observed. The number of multicellular bodies increased with incubation time in culture. It could be mentioned that the pattern of the development of

pollen grains into plantlets conformed to those of zygotic embryo.

The number of plantlets emerged from an anther ranged from 2 to 30, mostly 20. Plantlets did not emerge from an anther simultaneously but successively. Most of plantlets emerged from anthers after 20 to 25 days in culture regardless of whether they were green or albino plantlets (Fig. 1). However, albino plantlet could not grow on soil owing to low viability although they grew well and developed roots in culture (Figs 2 and 3). In some cultures variegated leaves were observed:

Fig. 1~4. Tobacco plantlets emerged from anthers of the F_1 hybrid [*Nicotina tabacum* cv: Hicks $2 \times N. tabacum$ bearing Ws(pbg) gene from *N. plumbaginifolia*] in culture. Fig. 1. Young green (g) and albino (a) tobacco plantlets emerged from anther. Fig. 2. Green (g) and albino (a) tobacco plantlets growing on medium. Note the presence of roots. Fig. 3. Variegated (v) tobacco leaves. Fig. 4. Albino (a) tobacco plantlets differentiated from albino-originated callus. Note lack of root.

whitish leaf having green spots (Fig. 3). These variegated plantlets developed many more roots than did completely whitish albino plantlets.

Callus formation from multicellular bodies were not encountered in this study. However, albino plantlets were easily dedifferentiated into callus from which in turn albino plantlets were secondarily developed (Fig. 4). These albino plantlets failed to develop roots on medium as shown in Fig. 4. In green plantlets, on callus formation was observed.

Color of plantlets could not be distinguished at early stages of plantlet formation. Gradually a few whitish or greenish structures became visible in culture. When maximum size of a leaf reached approximately 2 mm in length, color of the leaf could be clearly distinguished.

Segregation of leaf color in plantlets emerged from anthers

The F_1 plants from the cross between *N. tabacum* cv: Hicks 2 and *N. tabacum* bearing $Ws(pbg)$ gene were all green. The F_2 plants from the same cross were classified 893 green to 70 albino plants which was a good fit to a 15:1 ratio. Therefore, when the genotype of the F_1 hybrid was designated as Ws_1ws_1 , Ws_2ws_2 , the gametic genotype would be classified into Ws_1Ws_2 , Ws_1ws_2 , ws_1Ws_2 , and ws_1ws_2 , respectively. Thus, 3 green: 1 albino would be expected in plantlets developed from pollen grains of F_1 hybrid.

The frequencies of green and albino in plantlets emerged from anthers are given in Table 1. The segregation of leaf color character showed good fitness to an expected 3:1 ratio except for dedifferentiation of

albino plants into callus. The deviation from expected ratio in this culture was due to excess numbers of albino plantlets, most of which were arisen from callus. As mentioned above, any dedifferentiation into callus was not observed from green plantlets.

Verification of haploid in plantlets emerged from anthers

Forty six green plantlets growing vigorously were selected and transferred to soil. Of forty six plants five died before flowering. Forty one of surviving plants flowered normally. Twenty four univalent chromosomes were observed in all of these green plants whose meiotic division showed the same chromosome behaviour as previously reported by several workers (TAKENAKA and TANAKA, 1956; TANAKA and NAKATA, 1969). Albino plantlets could not be subjected to observation of meiotic division of PMC's. Also, chromosome counts in roots were difficult due to low frequencies of cell division.

Discussion

It remained uncertain whether so-called competition among pollen grains exists for plantlet formation; i.e., whether each pollen grain undergoes division to form a whole plant, independently, whether division of pollen grain is promoted or inhibited by other ones, and furthermore whether plantlet formation is confined to special genotypic pollen grains.

This paper showed that the observed ratio of green and albino in plantlets emerged from anthers was a good fit to an expected 3:1 ratio, especially at early stage of plantlet formation as shown in Table 1. On the basis

of this result it seems unlikely that competition among pollen grains exists for plantlets formation. It should be noted, however, that the deviation from the expected ratio was highly significant when whitish albino plantlets were secondarily differentiated from albino-originated callus. Thus, it would be desirable to prevent plantlets from dedifferentiation into callus in anther culture.

Table 1. Observed ratio of green and albino in plantlets emerged from anthers and goodness of fit to a 3:1

	Green	Albino	χ^2 (3:1)	P
1) when maximum size of a leaf reached approx. 2 mm in length	48	14	0.1035	.95—.50
2) when maximum size of a leaf reached approx. 5 mm in length	29	15	0.939	.50—.20
3) when maximum size of a leaf reached 20-50 mm in length	89	35	0.688	.50—.20
4) when albino plantlets were dedifferentiated into callus from which in turn whitish albino plantlets were secondarily developed	21	16	6.567	.02—0.1

In this paper 20 to 25 plantlets were easily obtained from each anther. Plantlets emerged from approximately 70 percent of anthers. However, the number of plantlets from an anther was very few as compared with those of pollen grains within an anther. More recently, SUNDERLAND and WICKS (1969) reported that only 5 percent of pollen grains developed into multicellular structures in their best preparations. Although this problem also remained unresolved, the following interpretation seems pertinent to them. As shown in microchamber culture by JONES *et al.* (1960), division of a single cell occurs only when high densities of cell suspension were spread on the surface of the medium. In conditioned medium of chick embryo cells, about 50 times as many as cells are required to support the maximum growth of another population containing small numbers of chick embryo cells (RUBIN, 1966). This may be true for early stages of anther culture. It seems reasonable to suppose, thus, that division of pollen grains may be promoted rather in relation to other ones which are present within the same anther. Plantlets emerged from an anther at early stages of culture grow well because of greater available nutrition than that at later stages. And then they may inhibit or disturb remaining undivided pollen grains in their subsequent development. Accordingly the frequencies with which pollen grains develop into plantlets would be lowered. It would be assumed that only plantlets emerged from an anther at early stage of culture are allowed to grow in the restricted space of an anther.

In this connection, the problem mentioned above could be overcome by pollen grain culture. By pollen grain culture, tissue-like structure was obtained from *Ginkgo biloba* by TULECKE (1957). More recently, KAMEYA and HINATA (1970) have reported that mature pollen grains from the F_1 hybrid between *Brassica oleracea* and *B. alboglabra* were induced to divide on medium supplemented with coconut milk. However, there are no data indicating the percentage of divided pollen grains. It seems, by improvement of medium, that pollen grains could be successfully cultured in future.

The question arises of how many haploid plants should be produced to make this

technique available for basic studies of plant breeding as well as of genetics. On the other hand, however, monoploid technique was first reported by CHASE (1949). The possibility of utilizing haploid plants for improvement of tobacco variety was investigated by OKA (personal communication) on the basis of formula presented by NEI (1963) and is being examined in the field using haploid-derived diploid by KADOTANI (1969).

This paper gave additional support to the evidence that each pollen grain gives rise to plantlet, individually. This was supported by the fact that the deviation from an expected ratio was slight in anther culture.

With regard to characters other than leaf color character, leafbase shape was taken up to observe with the same materials as used for leaf color character. It was reported by VAN der Veen (1957) that the inheritance of leaf shape is controlled by three pairs of genes: Br, Pd, and Pt. Thus, segregation of petioled leaf and sessile leaf in plants emerged from anthers was expected to be 1 petioled leaf : 3 sessile leaf (EGUCHI, personal communication). However, large deviation from an expected ratio was observed in this study. It requires more experiments to conclude whether it can be assumed that they could not have arisen from a 3 : 1 of population ratio.

All of forty one green plants on soil were haploid. Therefore, it seems unlikely that spontaneous doubling of chromosome number may easily occur in anther culture.

Summary

This study was conducted to determine whether any competition exists among pollen grains for haploid plant formation by anther culture.

Segregating ratio for green and albino in plantlets, emerged from anthers of the F_1 hybrid between *Nicotiana tabacum* cv: Hicks 2 and *N. tabacum* bearing Ws(pbg) gene from *N. plumbaginifolia*, was compared with an expected ratio of 3 green : 1 albino at the following stages: 1) when maximum size of a leaf reached approx. 2 mm in length, 2) when maximum size of a leaf reached approx. 5 mm, 3) when maximum size of a leaf reached 20–50 mm, and furthermore 4) when albino plantlets were dedifferentiated into callus from

which in turn albino plantlets were secondarily developed.

In the stage of 1) to 3), green and albino segregated in good agreement with a 3 : 1 ratio. However, in 4) the deviation from an expected ratio was highly significant. Failure of 4) to segregate in an expected ratio was due to excess numbers of albino plantlets differentiated secondarily from albino-originated callus.

These results demonstrated that no competition could be observed among pollen grains for haploid plant formation as to leaf color character in these cultures unless dedifferentiation of plantlets into callus occurs.

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Literature Cited

- BOURGIN, J. P. and J. P. NITSCH 1967. Obtention de *Nicotiana* haploïdes à partir d'étamines cultivée in vitro. *Annls Physiol. veg.* (Paris) 9 : 377~382.
- CARLSON, P. S. 1970. Induction and isolation of auxotrophic mutants in somatic cell culture of *Nicotiana tabacum*. *Science* 168 : 487~489.
- CHASE, S. S. 1949. The monoploid method of developing inbred lines. *Proc. 6th Annual Hybrid Corn Industry-Research Conf.* p. 29~34.
- JONES, L. E., A. C. HILDEBRANDT, A. J. RIKER and J. H. WU 1960. Growth of somatic tobacco cells in microculture. *Amer. J. Bot.* 47 : 468~475.
- KADOTANI, N. 1969. Studies on the haploid method of breeding by anther culture in tobacco. 1. Production of diploid plants from haploid by pith and root tissue culture. *Bull. Iwata Tobac. Exptl. Sta.* 2 : 73~77.
- KAMEYA, T. and K. HINATA 1970. Induction of haploid plants from pollen grains of *Brassica*. *Japan. J. Breed.* 20 : 82~87.
- NAKATA, K. 1969. The effects of the inter-pollen-grain competition on the differentiation of haploid by the anther culture of tobacco. *Japan. J. Breed.* 19. Suppl. 2 : 123~124.
- NAKATA, K. and M. TANAKA 1968. Differentiation of embryos from developing germ cells in anther culture of tobacco. *Japan. J. Genetics* 43 : 65~71.
- NEI, M. 1963. The efficiency of haploid method of plant breeding. *Heredity* 18 : 95~100.
- NIIZEKI, H. and K. OONO 1968. Induction of haploid rice plant from anther culture. *Proc. Japan Acad.* 44 : 554~557.
- NITSCH, J. P. 1969. Experimental androgenesis in *Nicotiana*. *Phytomorphology* 19 : 389~404.
- NITSCH, J. P. and C. NITSCH 1969. Haploid plants from pollen grains. *Science* 163 : 85~87.
- NITSCH, J. P., C. NITSCH and S. HAMON 1968. Réalisation expérimentale de l' "androgénèse" chez divers *Nicotiana*. *C. r. Seanc. Soc. Biol.* 162 : 369~372.
- NIWA, M. 1966. Radiation-induced interspecific transfer of Ws(pbg) gene from *Nicotiana plumbaginifolia* to *N. tabacum*. 1. Some observations on the irradiated hybrid plants. *Japan. J. Breed.* 16 : 260~266.
- RUBIN, H. 1966. A substance in conditioned medium which enhance the growth of small numbers of chick embryo cells. *Exptl. Cell Res.* 41 : 138~148.
- SUNDERLAND, N. and F. M. WICKS 1970. Cultivation of haploid plants from tobacco pollen. *Nature* 224 : 1227~1229.
- TAKENAKA, Y. and M. TANAKA 1956. Cytogenetic studies in *Nicotiana*. XIII. Haploid plants of *Nicotiana tabacum*. *Bot. Mag. (Tokyo)* 69 : 193~198.
- TANAKA, M. and K. NAKATA 1967. On the formation of haploid plants. (Speech), Symp. Oct. Meeting, Japan. Soc. Breed. (9th Symp. Japan. Soc. Breed. Abstr. 1 : 11~15).
- TANAKA, M. and K. NAKATA 1969. Tobacco plants obtained by anther culture and the experiment to get diploid seeds from haploid. *Japan. J. Genetics* 44 : 47~54.
- TULECKE, W. 1957. The pollen of *Ginkgo biloba* : In vitro culture and tissue formation. *Amer. J. Bot.* 44 : 602~608.
- VAN DER VEEN J. H. 1957. Studies on the inheritance of leaf shape in *Nicotiana tabacum* L. Thesis, Agric. Univ. Wageningen, The Netherlands.

葯培養によるタバコ半数体形成における花粉間競合

1. 葉色形質による解析

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本実験は葯培養により半数体を形成させる場合、花粉間に競合が存在するかどうかを明らかにするためにおこなった。

Nicotiana tabacum cv: Hicks 2 および *N. plumbaginifolia* からの Ws(pbg) 遺伝子を有する *N. tabacum* 間の F₁ 雑種の葯培養により生じた植物体について、緑色タバコとアルビノの出現比を期待値 (3 緑色 : 1 アルビノ) と比較した。

この比較は、1) 最大葉の葉長が約 2 mm に達したと

き、2) 最大葉の葉長が約 5 mm に達したとき、3) 最大葉の葉長が 20~50 mm に達したとき、ならびに 4) アルビノが脱分化し、アルビノ由来カルスより二次的にアルビノが分化したときにおこなった。1)~3) では、観測値と期待値とは一致したが、4) では両者間のずれは大であつた。このずれは、アルビノが脱分化したことによる。したがつて、培養中脱分化が起らない限り、本実験での葉の色については、花粉間に競合が見られないことが判明した。