Faculty of Agriculture, Nagoya University, Nagoya, Japan

Relationships Between Anther Browning and Plantlet Formation in Anther Culture of *Nicotiana tabacum* L.

MASAHIRO MII

With 5 figures

Received November 5, 1975 · Accepted May 29, 1976

Summary

Anthers of *Nicotiana tabacum* cv. Wisconsin 38 containing pollen grains in various developmental stages were cultured for 55 days at 23 °C to investigate the relationship between the formation of plantlets from pollen and browning of the anther. Browning took place in anthers containing binucleate pollen grains during the first 10 days after inoculation onto NITSCH's basal medium (1969), whereas it was continuously observed for as long as a month in anthers containing microspores in the uninucleate stage and in those undergoing pollen mitosis. Plantlet formation in the first instance was observed during the first 3 to 5 weeks of culture and was never detected thereafter. There was no positive correlation between anther browning and plantlet formation. Plantlet induction was delayed for one week, but it was observed to be continuously effective until the cultures were 50 days old in the anthers containing uninucleate and mitotic microspores. The protraction of senescence in anthers appears to be of great importance for the induction of embryogenesis in microspores at this stage of development. There was a positive correlation between the duration of anther browning and the time required for plantlet production. That is, the percentage of plantlet formation in anthers having browned during the first 2 weeks was low, whereas the frequency of plantlet formation was high in long-lived anthers.

These results suggest that there is a critical period for the induction of pollen embryogenesis in the early binucleate stage. Most microspores not yet having undergone pollen mitosis can attain to this stage with the nutritional or hormonal aid of living anther tissue; the embryogenesis can never proceed if anther browning has occurred before this stage has been attained. In a more advanced stage the duration of such a situation seems to be shorter.

Key words: pollen embryogenesis, anther browning, Nicotiana tabacum.

Introduction

The changes in *Nicotiana tabacum* pollen grains in *in vitro* culture have been studied histochemically (BHOJWANI et al., 1973) and electronmicroscopically (DUNWELL and SUNDERLAND, 1974 a, b). According to these authors, in embryogenesis, the pollen grains proceeded through almost the same developmental

process as on intact plants up to the early binucleate phase. After this a change in programming from the normal gametophytic to the diverged sporophytic process, as manifested in embryogenesis, occurred.

PELLETIER and ILAMI (1972) studied the role of anther tissue using anthers containing predominantly pre-mitotic microspores and showed that the conditions required for pollen embryogenesis were different from anther to anther. They studied the possible role of the anther tissue in this difference and demonstrated the existence of two major periods of intensive anther browning; one within the first 10 days and the other after 25 days of culture. Only a few anthers browned between 10 to 25 days. The anthers browning in the later period were shown to possess a greater potential for embryo formation from pollen grains than those having browned earlier.

In the present investigation, the relationship between plantlet formation and anther browning was studied using anthers of *Nicotiana tabacum* in various developmental stages to make clear the role of anther tissue in pollen embryogenesis.

Material and Methods

Nicotiana tabacum cv. Wisconsin 38 was used as the experimental material. The anther culture technique used was very similar to that previosly reported (MII, 1973), NITSCH's medium (1969) without growth regulators was employed as a basal medium. Flower buds were classified into five stages according to their length; about 25 buds were used as replicates. The five stages roughly coincide with the developmental stages of pollen, as shown in Table 1. All anthers collected from 20 buds were inoculated onto a basal agar medium and the anthers from the remaining 5 buds were examined to check the pollen developmental states. Cultures were incubated at a temperature of 23 ± 1 °C in the continuous light of white fluorescent lamps producing an intensity of approximately 1,200 lux at the plant level.

Table 1: Relation between bud lengt and developmental state of pollen in *Nicotiana tabacum* cv. Wisconsin 38.

Stage	Bud length (nm)	Developmental state of pollen
1	13–16	early uninucleate microspore
2	17–20	late uninucleate-mitosis
3	21–24	early binucleate pollen grain
4	25–28	binucleate pollen grain
5	29–32	binucleate pollen grain

The percentage of anthers showing browning, the time course of plantlet formation and the relation between the time having passed until the start of anther browning and the time required for plantlet formation were investigated. These data were calculated from about 100 anthers in each stage.

Results

Anther browning

When the anthers in each of the different stages were inoculated onto nutrient media, the time required until the anther browning begun was clearly affected by the anther stage at excision (Fig. 1). The number of browned anthers increased roughly linearly after inoculation at stages 1 and 2 (uninucleate to mitotic microspore), whereas the browning took place more rapidly in anthers of stages 3 to 5 (binucleate pollen grain). Two types of browning processes were observed in both uninucleate to mitotic and binucleate stages.

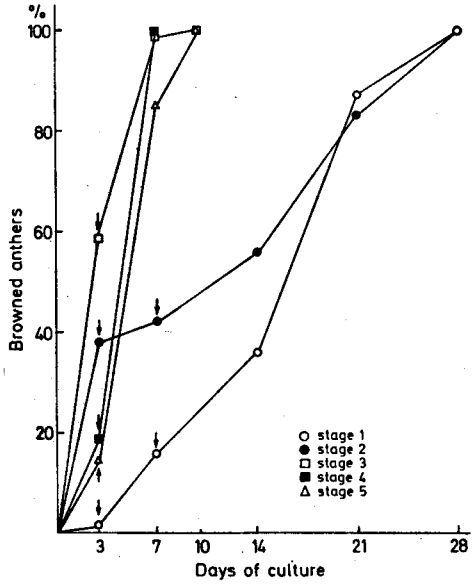

Fig. 1: Rate of anther browning. ○: stage 1, ●: stage 2, □: stage 3, ■: stage 4, △: stage 5. Arrows indicate anthers of the drastic-browning type.

One type was a drastic change in color from green to dark brown which occurred during the first 7 days of culture in the uninucleate to mitotic stage and during the first 3 days in the binucleate stage. The color change in these anthers took place over the whole surface of the anther, but in a few cases certain regions or one locule of the anther tissue remained green. These anthers never enlarged and dehisced except for the green-remaining regions. The other type was a gradual change from green to dark brown via yellow and yellowish brown coloration. Anthers of this gradual-browning type enlarged at stages 1 and 2 to almost the same size as did those on intact flowers at anthesis and some of them dehisced before plantlet emergence. Anthers in stages 3 to 5 which had already reached an almost mature size at excision swelled only slightly, and most of them dehisced during the first 2 weeks of culture. The more

advanced the stage at excision, the more frequent was the occurrence of dehisced anthers. It was also noted that half of the cultured anthers showed drastic browning in the stages 2 and 3 (arrows in Fig. 1).

Plantlet inductions

The percentage of occurrence of anthers having plantlets is shown in Fig. 2. A difference was found between the frequencies of anthers containing microspores and those containing binucleate pollen grains. The anthers in stages 1 and 2 showed similar time courses of plantlet formation; plantlet emergence began after about one month of culture and increased linearly until 50 days. The anthers containing binucleate pollen grains (stages 3 to 5) produced plantlets about one week earlier than those in stages 1 and 2. Plantlets were never seen to emerge after more than 5 weeks of culture.

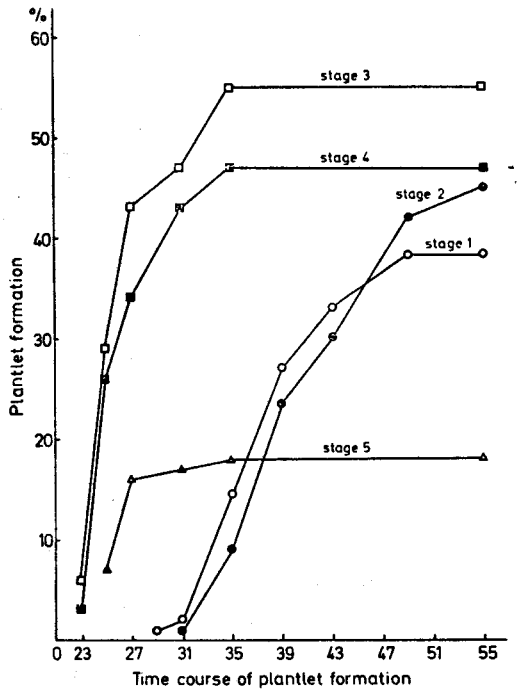

Fig. 2: Percentage of anthers having plantlets. ○: stage 1, ●: stage 2, □: stage 3, ■: stage 4, △: stage 5.

The percentage of plantlet formation was higher in anthers in the early binucleate stages (3 and 4) than in those uninucleate and mitotic stages (1 and 2). In mature anthers (stage 5) the rate of plantlet production was only 15 per cent.

The number of plantlets per anther was great in stages 1 to 3. The anthers which produced more than ten plantlets comprised half or more of the productive anthers.

However, in more advanced stages (4 to 5) the percentage of occurrence of such anthers decreased drastically (Fig. 3).

Relationship between anther browning and plantlet formation

Since the processes of anther browning and plantlet emergence are easily distinguishable in the period between the uninucleate and pollen-mitotic stages, the relationship between the time required for anther browning and plantlet formation was studied using flower buds of 15 to 20 mm in length. The results of three

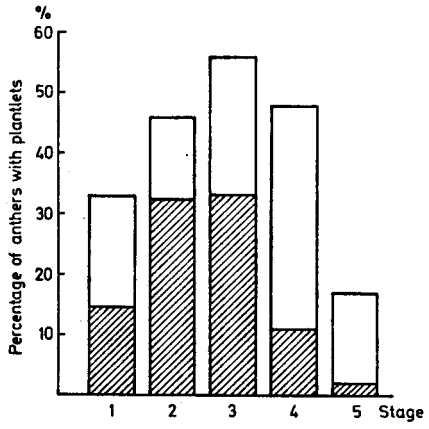

Fig. 3: Comparison of the percentages of anthers having plantlets. The dashed column represents the percentage of occurrence of anthers which produced more than ten plantlets per anther.

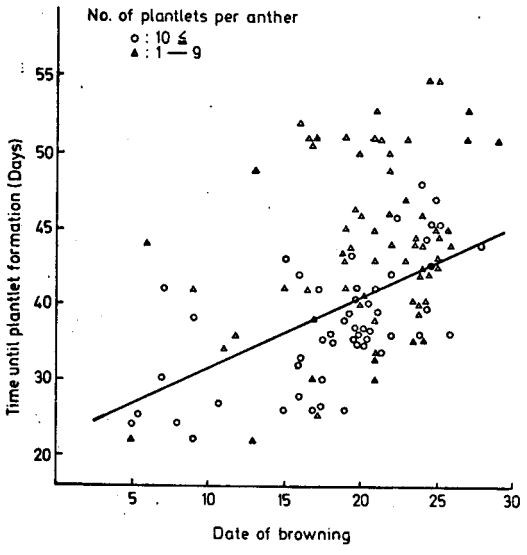

Fig. 4: Correlation between the times until anther browning and plantlet formation in stages 1 and 2. 0–50 days: $r = 0.557$; $Y = 0.47\,X + 31.12$.

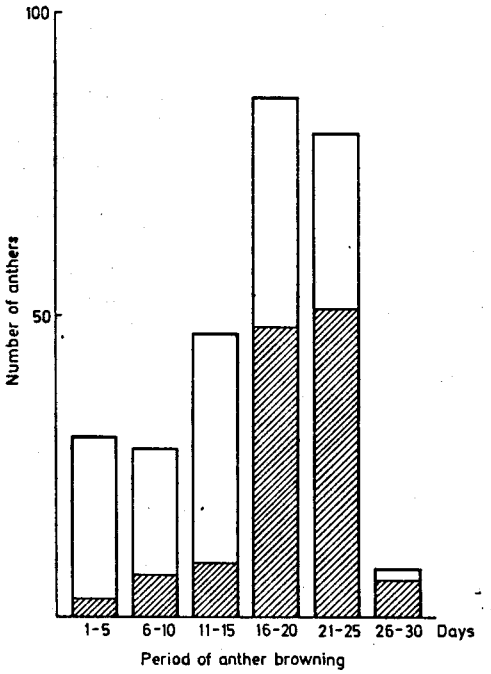

Fig. 5: Relation between the period of anther browning and plantlet formation in the uninucleate to mitotic microspore stages. Anthers were investigated within five-day intervals and were classified into six groups. The dashed column represents the anthers which produced plantlets and the open column the anthers which did not produce plantlets.

experiments were summarized in Fig. 4. There was a significant positive correlation between the variables. After 50 days of culture the anthers produced few plantlets, i.e. no positive correlation was observed.

Fig. 5 shows the relation between the period of anther browning and plantlet formation. Anthers which browned in the early period of culture formed only a few plantlets, whereas the anthers which browned during a period of 16–25 days produced a great number of plantlets.

Discussion

As shown by PELLETIER and ILAMI (1972), anther browning is essentially classified into two types; one is a drastic and the other a gradual change in color. Drastic browning in an early phase of the culture may result from some wound or physiological changes stemming from the excision from the mother plant, and gradual browning is considered to be an expression of the process of senescence of the anther itself.

Anther browning, except in the cases when it was drastic, took place during the first 10 days of culture in the stage of binucleate pollen grains and was observed continuously until one month of culture in anthers containing uninucleate to mitotic

microspores. The developmental state of the anther tissue at excision whether it was excised before or after the stage of pollen mitosis, clearly affected the anther browning. The rate of senescence in the anther may be accelerated after pollen mitosis because of the difference in longevity between the binucleate and uninucleate to mitotic stage of anther tissue after inoculation. On the other hand, a high percentage of anthers showing drastic browning are observed during a period lasting from the mitotic to the early binucleate stages. This suggests that anther tissues are very sensitive to excision, sterilization and the composition of medium, etc., at these stages. In the stages from microspore to mitosis, the longer the anther lives, the greater its chance to produce plantlets (Fig. 5). The results presented here essentially coincide with those of PELLETIER and ILAMI (1972). In the anthers which produced plantlets in stages 1 and 2, there is a positive correlation between the time required for anther browning and that which passed until the onset of plantlet formation (Fig. 4). That is, the browning at the end of the culture period results in retardation of the plantlet formation. On the contrary, such clear relation was not observed between anther browning and plantlet formation in the post-mitotic stage.

PELLETIER and ILAMI (1972) infered from the results of their transplantation experiments that anther tissue may attain a condition which favours pollen embryogenesis during the first day(s) and that it is necessary that the anthers maintain this state until the embryoids can attain an autonomous function. From our results, in relation to the above hypothesis, it is possible to consider that the browning itself has harmful effects on pollen grains which have not yet attained the critical stage of development. This may be when the switching of the program for the gametophytic to that for the sporophytic process occurs, or at some further stage of embryogenesis. However, after these changes have been reached, nutritional requirements may become less strict and living anther tissue may begin to play a competitive role in nutrient uptake, rather than the role of a nurse for the embryoidal grain. Thus the browning of the anther may cause no harmful effect, but may rather tend to accelerate further embryoidal development. The positive correlation between the time elapse until anther browning and that until plantlet formation may be interpreted as a reflection of such a situation.

The time required for plantlet formation was also influenced by the developmental state of the anther tissue at the time of excision as well as by the rate of anther browning. That is, the plantlet formation in anthers containing binucleate pollen grains was more simultaneous and took place one week earlier than in those containing microspore to mitotic pollen grains. A similar result was obtained with *Datura innoxia* by SUNDERLAND et al. (1974). It is presumed that pollen grains in young anthers need a long period of time to attain this critical condition and that, during this inductive phase, the anthers must live for a long time in a healthy state to be able to play a role as the nurse tissue. In the early binucleate stage (stages 3 and 4), on the other hand, they seem to have attained the critical condition necessary for switching from the gametophytic to the sporophytic process at the beginning of the

culture as shown by BHOJWANI et al. (1973). In fact, our preliminary cytological observation on pollen embryogenesis suggests that the difference in days between uninucleate-mitotic microspore and binucleate pollen stages required for plantlet formation is due to the difference in the induction period of the subsequent mitosis. In the binucleate stage, the indispensable period required by the anther tissue for pollen embryogenesis is inevitably shorter than that required in the uninucleate to mitotic stage. However, the longevity of anther tissue in the binucleate stage is much less than that in the microspore stage, so that the duration of plantlet emergence is restricted to shorter periods than it is in the microspore stage.

Although there are a few reports concerning the successful establishment of embryoid or callus tissue in direct pollen culture with angiosperm species (KAMEYA and HINATA, 1969; BINDING, 1972) or in nurse culture of isolated pollen grains (SHARP et al., 1972), direct culture of pollen grains themselves have still not been made with *Nicotiana tabacum*. Moreover, the cutting of the anther resulted in the formation of few plantlets or none at all (MII, unpublished data). A similar observation has been reported for *Datura innoxia* by SOPORY and MAHESHWARI (1972).

These results suggest that the anther tissue mainly plays the role of nurse for the pollen grains it contains. Furthermore, it may have other important roles, such as the isolation of pollen grains from the circumstances outside the anther wall.

The authors wishes to express his thanks to Drs. H. TORIKATA, E. MAEDA and S. KAKO for their helpful discussion and to Dr. Y. KATO for his critical reading of the manuscript. Thanks are also due to Mr. K. SAKAKIBARA and Mr. S. TORII for their aid in culturing plant materials.

References

BHOJWANI, S. S., J. M. DUNWELL and N. SUNDERLAND: Nucleic-acid and protein contents of embryogenic tobacco pollen. J. Exp. Bot. *24*, 863–871 (1973).

BINDING, H.: Nuclear and cell divisions in isolated pollen of *Petunia hybrida* in agar suspension cultures. Nature, New Biol. *237*, 283–285 (1972).

NITSCH, J. P.: Experimental androgenesis in Nicotiana. Phytomorphology *19*, 389–404 (1969).

DUNWELL, J. M., and N. SUNDERLAND: Pollen ultrastructure in anther culture of *Nicotiana tabacum*. I. Early stage of culture. J. Exp. Bot. *25*, 352–361 (1974).

– – Pollen ultrastructure in anther culture of *Nicotiana tabacum*. II. Changes associatet with embryogenesis. J. Exp. Bot. *25*, 363–373 (1974).

KAMEYA, T., and K. HINATA: Induction of haploid plants from pollen grains of *Brassica*. Japan. J. Breed. *20*, 82–87 (1970).

MII, M.: Studies of the haploid formation from immature pollen grain. I. Relation between pollen stage and the plantlet formation and the process of embryoid formation in *Nicotiana tabacum*. Japan. J. Breed. *23*, 27–34 (1973).

PELLETIER, G., and M. ILAMI: Les facteures de l'androgénèse *in vitro* chez *Nicotiana tabacum*. Z. Pflanzenphysiol. *68*, 97–114 (1972).

SHARP, W. R., R. S. RASKIN and H. E. SOMMER: The use of nurse culture in the development of haploid clones in tomato. Planta *104*, 357–361 (1972).

Sopory, S. K., and S. C. Maheshwari: Production of haploid embryos by anther culture technique in *Datura innoxia* – A further study. Phytomorphology *22*, 87–90 (1972).
Sunderland, N., G. B. Collins and J. M. Dunwell: The role of nuclear fusion in pollen embryogenesis of *Datura innoxia* Mill. Planta *117*, 227–241 (1974).

Dr. M. Mii, Faculty of Agriculture, Nagoya University, Nagoya 464, Japan.