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An improved method for dihaploid production in *Nicotiana rustica* through anther culture

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Summary. The efficiency of dihaploid production from anther culture in *N. rustica* has been improved by studying the effects of pretreatment temperature, pretreatment duration and initial anther stage on anther response, anther productivity and time to first plantlet production. Pretreatment was most effective on anthers at or around the stage of pollen mitosis. Pollen mitosis stage anthers pretreated at 9 °C for 15 days gave the best results. Both spontaneous and induced dihaploids were obtained. Small plantlets treated with 0.4% colchicine and 2% DMS solution for 5 h produced the maximum number of dihaploids (more than 50%). These considerable improvements in the efficiency of the techniques have made dihaploidy an attractive method for producing inbred lines in *N. rustica*. This will permit a large scale comparison of dihaploids with more conventional methods of inbreeding such as single seed descent and pedigree breeding.

Key words: *Nicotiana rustica* – Temperature-pretreatment – Anther culture – Haploids – Dihaploids

Introduction

Although it is believed that the genus *Nicotiana* has a high androgenic potential for haploid production, success has been reported in only 16 species (Nitsch 1969; Nakamura et al. 1974; Tomes and Collins 1976; Hlasnikova 1977) and successful production of adequate numbers of dihaploids for comparison with conventionally produced inbreds is limited to just two species, *N. tabacum* (Nakamura et al. 1974; Oinuma and Yoshida 1974; Burk and Matzinger 1976; Brown and Wernsman 1982) and *N. sylvestris* (De Paepe et al. 1981). Failure to obtain a high success rate in most species probably indicates that the optimum conditions for haploid production have not been achieved.

Haploid production in *N. rustica* was first reported by Nitsch (1969) and further work has been reported by Nakamura et al. (1974); Tomes and Collins (1976) and Hlasnikova (1977). These investigations were, however, part of a general survey of the *Nicotiana* genus and a detailed study of *N. rustica* has not been attempted. Fresh anthers, taken from the flower buds immediately after their excision from the plant were cultured by Nitsch (1969); Nakamura et al. (1974) and Hlasnikova (1977), showed anther response was less than 10%. A similar result (12%) was also reported by Tomes and Collins (1976), using anthers from both fresh and pretreated (48 h at 4 °C) flower buds. However, Sunderland and Roberts (1979) working on *N. tabacum* reported that pretreatment was much more effective when the temperature was raised to 7 °C or 9 °C and the duration of pretreatment was extended beyond 4 days.

Burk et al. (1972) reported a rapid, efficient and simple technique for diploidizing large numbers of anther derived haploids by immersing plantlets of approximately 1 cm in length in 0.4% colchicine solution for 3–4 h. Jensen (1974), however, obtained a better rate of dihaploid production in Barley by treating them with 0.1% colchicine plus 2 or 4% Dimethyl sulphoxide (DMS) rather than with 0.1% colchicine alone.

To produce large numbers of haploids and dihaploids for breeding purposes it is necessary to develop methods more efficient than those currently available. The effects of pretreatment temperature, duration of pretreatment and initial anther stage in *N. rustica* on anther response, anther productivity and days to first plantlet production have, therefore, been investigated. A comparison of 10 different colchicine treatments on dihaploid production from anther derived plantlets has also been made. The results of these investigations are reported here.

Materials and methods

F_1 seeds obtained from a cross between varieties 2 and 12 of *N. rustica* (for further information about the material see Mather and Vines 1952) were sown in plastic petridishes and the seedlings transplanted into 4 inch plastic pots containing John Innes seed compost. The material was raised in the glasshouse under a 16 h day length at 20 °C. Consecutive sowings were made at 2 week intervals to ensure a continuous supply of flower buds.

Pretreatment of flower buds

Flower buds with corolla lengths of between 3 and 6 mm were collected from the F_1 plants on each of 10 consecutive days, classified into anther stages 2 to 6 as defined by Sunderland (1974) and placed in plastic petri dishes. A drop of water was placed in each dish without touching the buds, and the dishes were sealed with nescofilm. To minimise water loss each dish was wrapped in thin aluminium foil and stored in either a refrigerator (5 ± 0.5 °C) or a refrigerated incubator (7 ± 0.5 °C, 9 ± 0.5 °C and 15 ± 0.5 °C). Buds were pretreated at 5 ± 0.5 °C for 2, 7, 15 and 21 days; at 7 ± 0.5 °C for 2, 5, 7, 10, 15 and 21 days; at 9 ± 0.5 °C for 2, 5, 7, 12, 15 and 21 days; and at 15 ± 0.5 °C for 2, 5, 8, 12 and 15 days.

Culture procedure

Five combinations of fresh and 105 combinations of pretreated anthers were used and in each combination 120 anthers in 3 batches were cultured. Flower buds were surface sterilized in 70% ethanol and then immediately transferred into 1% sodium hypochlorite solution (from a source of 10% available chlorine) containing 0.1% 'Tween 20' as a wetting agent for 5 min. Anthers were removed aseptically. Four anthers from each bud were placed on 5 ml of Nitsch (1969) +0.1 mg/l NAA medium in a plastic petridish and the remainder used to assess the developmental stage of its pollen by staining in aceto-carmine. On each occasion 100 pollen grains were scored. The dishes were sealed with nescofilm and incubated in a growth cabinet regulated to give 18 h continuous light of 3,000 lux. The temperature was maintained at 24 °C during the light period and 20 °C during the dark period with 80% relative humidity.

Colchicine treatments of plantlets

A total of 1,138 anther derived plantlets were subjected to 10 combinations of colchicine treatment. When the emerging plantlets from anthers were approximately 1 cm in length they were removed from the dishes, separated and immersed in 0.4% colchicine solution (Burk et al. 1972) for 4.0 and 5.0 h; 0.4% colchicine + 2% DMS for 3.0, 4.0, 5.0 and 5.5 h; 2% colchicine for 4.0 and 5.0 h; and 0.2% colchicine + 2% DMS for 4.0 and 5.0 h. Plantlets were then rinsed with tap water and transferred to seed compost. The very small ones (approximately 0.5 cm in length) were planted in deep plastic petri dishes containing seed compost and incubated in the growth cabinet. After incubation for 15 days, plantlets from petri dishes were transferred to seed compost and after approximately 20 days the plants were transplanted into 4 inch plastic pots of soil and kept in the glasshouse for flowering. Similarly, 250 anther derived plantlets were raised to maturity without colchicine treatment.

Recording of data

After 15 days in culture, each anther was checked daily for plantlet production and the number of days to first plantlet formation was recorded. At the time of colchicine treatment the number of plantlets in each anther was scored. Anther response was recorded as the percentage of the total number of anthers in each combination that produced plantlets. Anther productivity was estimated as the total number of plantlets divided by the total number of cultured anthers.

Anthers failing to produce any plantlets were discarded after 60 days in culture. The ploidy level of 20 untreated plantlets was determined from root tip mitosis using the standard squash procedure. Both colchicine treated and untreated plants were checked for their pollen fertility. Fertile plants were self-pollinated.

Results

Effects of initial anther stage

Figure 1 shows the productivity of anthers of different stages in cultures derived from buds pretreated for 12 and 15 days at 9 °C. Pretreatment of stage 2 anthers (mid uninucleate microspores, mean corolla length – 3.2 mm) was almost ineffective and only an occasional plantlet was produced. A rapid increase in yield from anthers at stage 3 (late uninucleate microspores, mean corolla length – 3.7 mm) to stage 4 (first mitotic pollen,

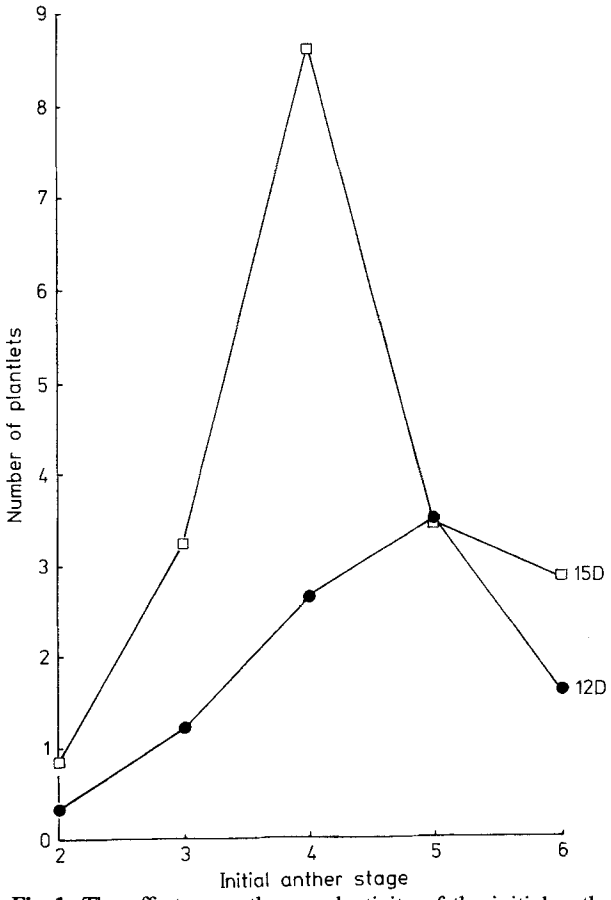

Fig. 1. The effect on anther productivity of the initial anther stage following 12 and 15 days of pretreatment at 9 °C

mean corolla length – 4.1 mm) was found but yield declined sharply from anthers at stage 5 (early binucleate pollen, mean corolla length – 4.6 mm) and at stage 6 (bicellular pollen, mean corolla length – 5.4 mm). The maximum yield (8.5 plantlets/anther) was attained from anthers at stage 4 and the minimum (0.8 plantlet/anther) from anthers at stage 2.

The productivity at different pretreatment durations showed little variation in yield between stages after 2, 5 and 7 days of pretreatment, whereas after 15 days the differences were large (Fig. 2). The superior productivity of anthers at stages 3, 4 and 5 relative to 2 and 6 was apparent.

Effects of pretreatment temperature

The effect of 15 days of pretreatment at 5, 7, 9 and 15 °C on the productivity of anthers at stages 2 to 6 is shown in Fig. 3. The results clearly point to the overall superiority of the intermediate temperature (9 °C). The yield was dramatically decreased at 15 °C. Stage 4 anthers gave the best response and stage 2 anthers a consistently poor response. The superiority of the 9 °C pretreatment temperature and 15 days pretreatment duration on anther productivity is also shown in Fig. 4. After more than 15 days of pretreatment at 9 °C the yield declined.

Effects of pretreatment duration

Anthers from fresh buds (without pretreatment) gave an 8% anther response, produced 0.25 plantlet/anther and required 26 days to first plantlet formation. Anthers at stage 4 pretreated for 15 days at 9 °C showed 96% anther response, produced 8.6 plantlet/anther and required 22 days for first plantlet formation (Table 1). Productivity increased with the increase of pretreatment duration at all pretreatment temperatures and an inverse relationship between pretreatment temperature and duration on the peak attainment (Fig. 4) was observed. Stages 3, 4 and 5 showed a linear response in anther productivity with the increasing pretreatment duration, stage 2 gave consistently poorer yield and stage 6 showed a decline after 15 days (Fig. 2).

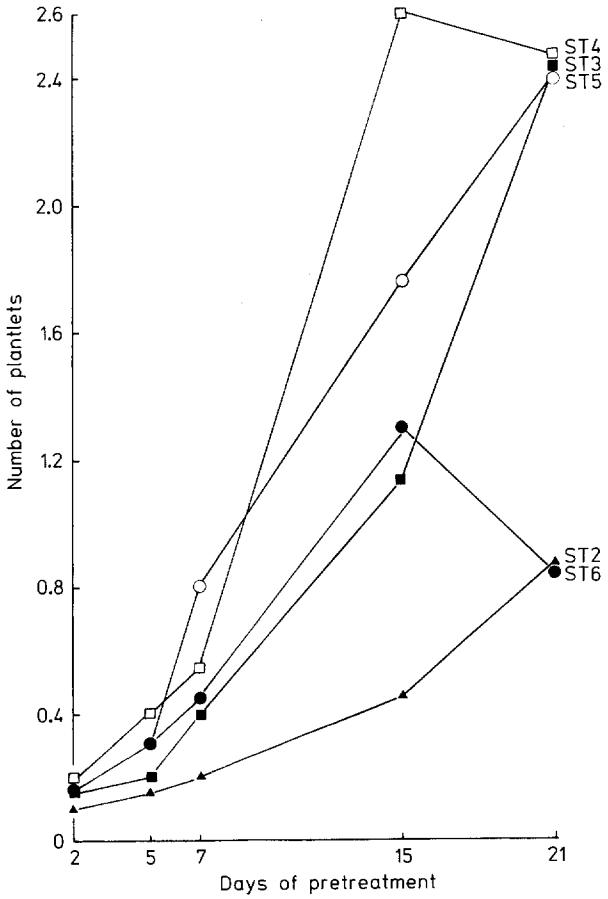

Fig. 2. Changes in productivity (over all temperatures) of anthers at stage 2–6 with increasing pretreatment duration

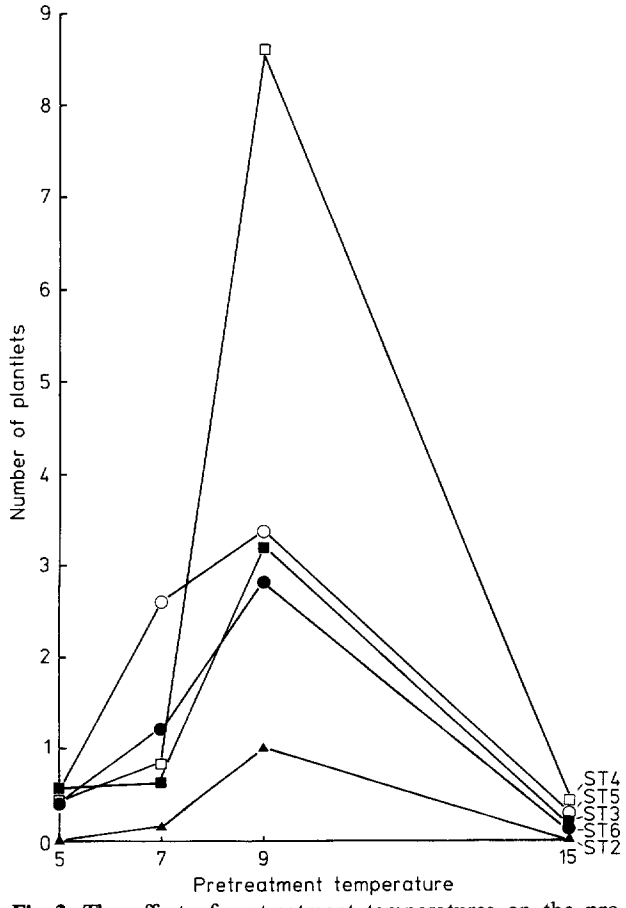

Fig. 3. The effect of pretreatment temperatures on the productivity of anthers at stages 2–6 pretreated for 15 days

Table 1. A comparison of the performance of fresh anthers with anthers pretreated at 9°C for 15 days (all anthers were initially at stage 4)

Treatment	No. of anthers cultured	Total no. of anthers responded	Total no. of plantlets obtained from cultured anthers	Mean no. of days of first plantlet formation
Fresh	120	9.0	30	26
Pretreated	120	115.0	1032	22

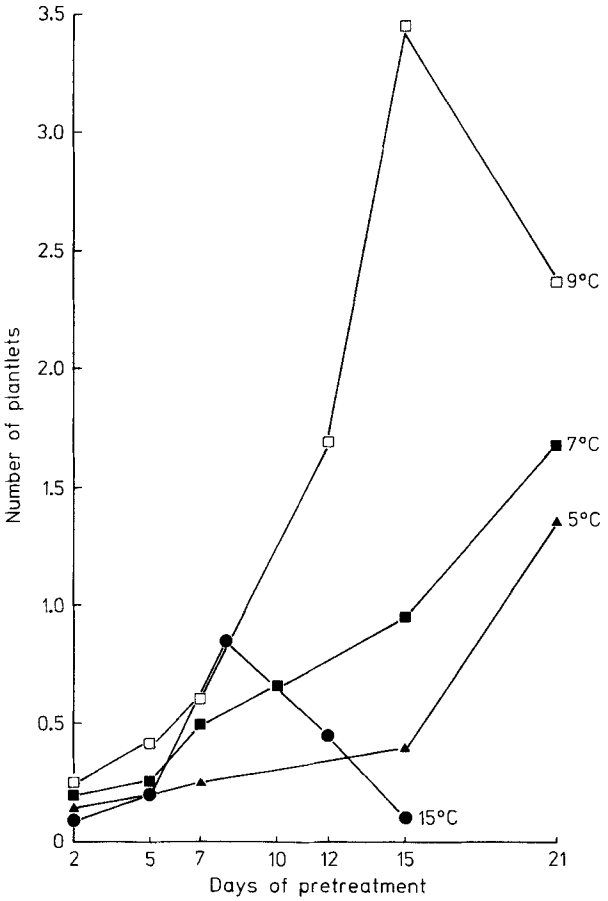

Fig. 4. Changes in the productivity of anthers with increasing pretreatment duration at four pretreatment temperatures

Dihaploid production from anther derived plantlets

Haploids differed clearly from dihaploids both in their reduced vigour and their sterility. Their ploidy level was confirmed by cytological examination. Four hundred and seventeen dihaploids were produced by colchicine treatment of 1,138 anther derived plantlets (36.64%). The success rate increased slightly with an increase in both the colchicine concentration and the duration of the treatment, although a decline was observed after 5 h of treatment. The addition of 2% DMS to both 0.2% and 0.4% colchicine also induced an increased response (Table 2).

Table 2. Dihaploid production through colchicine treatments of anther derived plantlets

Treatments	No. of plantlets treated	No. of dihaploids produced	% of dihaploids
0.2% colchicine			
4.0 h	94	28	29.8
5.0 h	87	30	34.5
0.2% colchicine + 2% DMS			
4.0 h	99, 26[a]	36, 17[a]	36.4, 65.4[a]
5.0 h	82	38	46.3
0.4% colchicine			
4.0 h	139	44	31.7
5.0 h	84	36	42.9
0.4% colchicine + 2% DMS			
3.0 h	88	22	25
4.0 h	193, 24[a]	54, 15[a]	28, 62.5[a]
5.0 h	87	44	50.6
5.5 h	135	53	39.3
Total	1138	417	36.64

[a] Plantlets of approximately 0.5 cm in length
DMS – Dimethy sulphoxide

Small plantlets (approximately 0.5 cm in length) were found to be twice as responsive as larger plantlets (approximately 1 cm in length). Seven spontaneous dihaploids were obtained from 250 untreated anther derived plantlets.

Discussion

The results show that the pretreatment of excised flower buds of *N. rustica* considerably increases the response (approximately 10 fold) and productivity (approximately 35 fold) of the anthers compared to fresh anthers. The relationships between initial anther stage, pretreatment temperature and pretreatment duration on anther response and anther productivity (see Figs. 1–4) are in general similar to those obtained by Sunderland and Roberts (1979) using *N. tabacum* and they

have already discussed the possible explanations in detail. The results of the present investigation, however, differ from those of Sunderland and Roberts (1979) in the effect of pretreatment temperature on peak attainment. The peak productivity of the anthers pretreated at 7 and 9 °C in their investigation appears to be the same whereas in the present investigation it was almost double at 9 °C compared to that at 7 °C. This variation may be due to genetic differences between the two species. However, the inferiority of the stage 2 anther is undeniable. The pretreatment is found to be effective if given between stages 3 and 5. The optimum stage of anther induction with or without pretreatment coincides with pollen mitosis. This suggests that the pretreatment does little to broaden the critical period, its effect is mainly to increase the number of responding anthers and their productivity. Tomes and Collins (1976) also found an increase in anther productivity from pretreated anthers but anther response remained unchanged in their investigation. This may be due to their use of a relatively low temperature (4 °C) and a short pretreatment period (48 h).

The low yield from the fresh anthers compared to that of pretreated ones was found by microscopical examination to be due to the rapid deterioration of the anthers in culture. Pretreatment prevents the deterioration of anthers and thus allows the continued development of a large number of embryogenic pollen grains to the embryo stage. Based on the results of the present investigation two pretreatment conditions can be recommended for this species for all anthers between stages 3 to 5, these are 1) 15 days of pretreatment at 9 °C and 2) 21 days at 7 °C or 5 °C.

Microscopic determination of the stage of development has been replaced by some workers by morphological measurement (Burk et al. 1972; Hlasnikova 1977). Observations made during the present investigation, however, showed that the relationship between microspore stage and bud size varied with the genotype, the environment and the age of the plant. Morphological measurement is not, therefore, an entirely reliable method of determining microspore stage in this material, although if induction response is high this may not be important.

Dihaploids were obtained both spontaneously and through induction although the frequency from a spontaneous origin was very low. There are three probable causes of a spontaneous origin: 1) fusion of two haploid cells during androgenesis; 2) formation of an unreduced microspore that undergoes embryogenesis; 3) embryogenesis from a somatic cell. The first mechanism will lead to complete homozygosity and is therefore equivalent to induction. The second and third types, however, will lead to heterozygosity and will not therefore be desirable for producing pure breeding lines. This heterozygosity will be apparent from segregants in the next generation. The presence of haplo/diploid chimeric plants amongst the spontaneous dihaploids indicates that at least some of them were produced by the fusion of two haploid cells. Even if all the spontaneous dihaploids are of the homozygous type, their frequency is too low for use in a breeding programme. This very low frequency does, however, indicate that the risk of contamination in the induced dihaploids by spontaneous ones retaining heterozygosity is probably negligible.

There are a number of ways to induce diploidization of anther derived haploids:
a) colchicine treatment of flower buds before anther culture (De Paepe et al. 1981);
b) colchicine treatments of anther derived plantlets (Burk et al. 1972);
c) colchicine treatments of the auxillary buds of haploids (Chowdhury 1982);
d) colchicine treatments of the inflorescence of haploids (Nakamura et al. 1974);
e) from haploid tissue culture (Kasperbauer and Collins 1972).

From a comparison of the methods b), c) and d) on the haploids derived from the same source of *N. tabacum* (Chowdhury 1982) method b) gave the highest response. This method permits large numbers of haploids to be handled. In this study plantlets treated in this way for 4 h with 0.4% colchicine solution gave a success rate of 31.7%, close to that obtained by Burk et al. (1972) on *N. tabacum*. The improvement in success as a result of the addition of DMS solution was also found by Jensen (1974). This chemical may act by facilitating the entry of colchicine into the meristematic tissues of the treated plantlets. Small plantlets were found to be more susceptible to colchicine.

N. rustica has been used for the last 40 years as a model organism for the study of plant breeding methods. The development of the techniques described here for the rapid and efficient production of homozygous diploid lines in this species are, therefore, important since these will permit a large scale assessment of this novel plant breeding approach in comparison with more conventional methods.

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