Turkish Journal of Botany

Volume 44 | Number 1 Article 7

1-1-2020

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TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2020) 44: 76-84

doi:10.3906/bot-1903-34

Induction of pollen embryo and chromosome doubling in tobacco (*Nicotiana tabacum* L.)

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Received: 24.03.2019 • **Accepted/Published Online:** 04.10.2019 • **Final Version:** 07.01.2020

Abstract: Anther culture is an effective breeding method for improving desirable traits, since it allows fast achievement of genetically homozygous lines. The present studies were carried out to evaluate the response of flue-cured tobacco anthers for high frequency pollen embryo induction in different culture media, and the effects of colchicine treatment on tobacco chromosome doubling. The results showed that different media caused significant changes in pollen embryo induction, in which the most obvious change was found in B medium with induction rates of 23.28%, 26.64%, and 28.07% for TMK-12 × Jiyan 5, K326 × Shangkang 70, and NC71 × Jingyehuang, respectively. In addition, shoot tips of the haploid aseptic seedlings cultivated from anthers were treated with 4 g/L colchicine for 48 h, and the ploidy levels were then checked by using flow cytometry and the number of chloroplasts in stomatal guard cells. The results suggested that colchicine treatment not only doubled the chromosomes of sterile haploid seedlings, but also caused some sterile haploid seedlings to die. The survival rate of TMK-12 × Jiyan 5, K326 × Shuangkang 70, and NC71 × Jingyehuang seedlings were 33.29%, 35.11%, and 17.61%, respectively. Similarly, TMK-12 × Jiyan 5 and K326 × Shuangkang 70 also showed a relatively high chromosome doubling rate compared with NC71 × Jingyehuang; their chromosome doubling rates were 21.53%, 18.54%, and 7.98%, respectively. In general, our work provides a theoretical basis for accelerating the breeding and improvement of new flue-cured tobacco varieties.

Key words: Tobacco, pollen embryo, colchicine, chromosome doubling

1. Introduction

Nicotiana belongs to the family Solanaceae with more than 64 species, among which *N. tabaccum* is one of the most cultivated species. It is an allopolyploid species (2n = 48) with a basic chromosome number of x = 12 (Clarkson et al., 2004). Tobacco is one of the most important cash crops worldwide. Apart from the economically-important leaves, the seeds, which contain 38% nonedible oil, can be used as an appropriate substitute for diesel fuel (Giannelos et al., 2002). Tobacco is usually divided into many types according to the processing methods for the tobacco leaves, including flue-cured tobacco, air-cured tobacco, sun-cured tobacco, and smoked tobacco (Hamid et al., 2013). The role of tobacco production in increasing fiscal revenue has been very prominent, having made great contributions to the development of various countries. The world's major tobacco producers are China, the United States, India, Brazil, and Turkey. Among them, China is the world's largest flue-cured tobacco grower, and Turkey is the world's largest producer of Oriental tobacco. However, in recent years, with global climate change, pests, and diseases, the quality and yield of tobacco production have been seriously affected. Therefore, cultivation of new high-quality tobacco varieties is important. Genetic and variety improvement are important foundations for tobacco production (Peng et al., 2017). Owing to recent advancements in cell and molecular biology, induction of haploid tobacco by anther culture has become an important method for building the tobacco double haploid (DH) population and performing cell engineering and breeding (Germanà, 2006; Zhao et al., 2015).

In recent years, studies on the molecular mechanism of microspore embryogenesis have been beneficial to the development of advanced genomics, transcriptomics, and proteomics (Seguı´-Simarro and Nuez, 2008). Application of these tools is likely to improve the identification of many interesting genes involved in microspore reprogramming and embryogenesis (Soriano et al., 2013). This will lead to a better understanding of cellular processes and development of more efficient protocols, all of which will lead to the effective application of gametic embryogenesis and haploid technology to improve all plant species. Although tobacco has been used as a model plant for plant tissue culture, the frequency of inducing pollen embryos

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through anther culture has been very low. In addition to the genotype and developmental stage of pollen (Lu et al., 2016), various factors influence the production of pollen embryos, such as the growing conditions of the donor plants (Kumar and Murthy, 2004), the culture medium, the pretreatment of the anthers, and culture conditions (Wetherell and Dougall, 2006; (Seguí-Simarro and Nuez, 2008).

In tobacco anther culture, haploid plants develop directly from microspores; the frequency of spontaneous chromosome doubling is very low (Burk et al., 1972). To produce dihaploid tobacco plants, colchicine is usually applied to the plantlets derived from anther culture (Nakamura et al., 2008). Colchicine is the most commonly used chromosome doubling reagent for plant doubling. It triggers chromosome doubling by inhibiting the formation of spindles during mitosis (Ramulu et al., 1991). At present, the experimental protocol for doubling tobacco chromosomes using colchicine is well-established. The method of chromosome doubling mainly includes dipping anther (Nakamura et al., 2008), soaking seedlings (Zhao et al., 2015), and embryoid body treatment (Takashima et al., 1995). With the development of haploid breeding technology, the DH populations of many plants have been successfully established. Compared with other plants such as strawberry (Zhang et al., 2009), more efforts are required to increase the production of DH population for tobacco. Although much progress has been achieved with regard to research on anther and pollen culture, there are still many theoretical and application challenges. Specifically, the induction frequency is still low and the regeneration rate of the embryoid body is not sufficient (Soriano et al., 2007; Lu et al., 2016). A perfect high-frequency anther culture regeneration system is far from being established, which limits the construction of the DH population. Therefore, it is important to optimize the induction of haploidy in tobacco anther culture and to establish a method of inducing tobacco pollen embryos with low mutation frequency.

In this study, we investigated the effects of 3 kinds of culture media on pollen embryo induction in flue-cured tobacco anther culture by setting different basic media and hormone concentrations. We also compared the efficiency of using colchicine for chromosome doubling in different tobacco materials to provide a deeper understanding of the tobacco chromosome doubling process.

2. Materials and methods

2.1. Plant material

The anthers of the F_1 generation were obtained from plants derived from the crosses between flue-cured tobacco cultivars TMK-12 × Jiyan 5, K326 × Shuangkang 70, and NC71 × Jingyehuang. Tobacco hybrids were planted in the field of the Fufeng Research Base of Shaanxi Province Tobacco Company of the China National Tobacco Company. During the flowering period, the flower buds were collected when the corolla was as long as the calyx. The flower buds were put in a culture flask and taken to the lab at a temperature of –4 °C.

2.2 Culture media preparation

2.2.1. Pollen embryoids induction medium

To determine the best medium for the induction of tobacco pollen embryos, 3 different media were tested: CK, A, B (Table 1).

2.2.2. Haploid growth medium

To increase the growth rate of anther cultured seedlings, haploid growth medium was used (Table 1).

After autoclaving the prepared medium at 121 °C and 1.1 kg/cm^2 pressure for 20 min, 50 mL of pollen embryo induction medium was poured into 100-mL sterile Erlenmeyer flasks, and 50 mL of haploid growth medium was poured into 240-mL sterile tissue culture flasks. The media were stored in a refrigerator at 4 °C.

2.3. Pollen embryo induction

The flower buds were subjected to a low temperature (4 °C) for 48 h, and sterilized with 70% ethanol for 30 s, followed by 0.1% mercuric chloride ($HgCl_2$) for about 8

Table. Culture media used in this study.

Medium		Components
Pollen embryoids induction	CK	1/2 MS (macronutrients + micronutrients + Fe salt) + MS organic compound + 0.1 mg/L IAA + 30.0 g/L sucrose + 6.0 g/L agar, pH 5.6
	A	Nitsch H + 15.0% (v/v) coconut juice + 0.1 mg/L IAA + 30.0 g/L sucrose + 6.0 g/L agar, pH 5.6
	B	Nitsch H + 15.0% (v/v) coconut juice + 0.1 mg/L IAA + 0.1 mg/L 6-BA + 30.0 g/L sucrose + 6.0 g/L agar, pH 5.6
Haploid growth		1/2 MS + 0.5 mg/L IAA + 20.0 g/L sucrose + 6.5 g/L agar, pH 5.8

MS: Murashige and Skoog (1962) medium.
Nitsch H: Bourgin and Nitsch (1967) medium.

min, and then rinsed 3 times with sterile distilled water under aseptic conditions. The anthers were carefully peeled off with sterile forceps on a clean bench and inoculated with pollen embryo induction medium. One hundred forty-four Erlenmeyer flasks, each containing 45 anthers, were placed in the dark at 28 ± 1 °C for 3 weeks and then transferred to 28 ± 1 °C, 3000–4000 lx, for a 16 h light/8 h dark photoperiod, to promote embryo germination.

After 20 days of culture, the number of anthers that produced regenerated plants (embryoid bodies) in each medium was counted.

2.4. Plant regeneration and colchicine treatment

The seedlings that developed from the pollen embryo that grew 2–3 leaves were transferred onto haploid growth medium and further cultured at 25 ± 2 °C, 3000–4000 lx, for a 16 h light/8 h dark photoperiod. When the regenerated shoots grew to 2–3 cm, they were soaked in 4.0 g/L colchicine for 48 h and then rinsed 3 times with sterile distilled water under aseptic conditions. Thereafter, the seedlings were transferred to the haploid growth medium and further cultured at 25 ± 2 °C, 3000–4000 lx, for a 16 h light/8 h dark photoperiod.

2.5. Ploidy identification

We used the following 2 methods for identification of chromosome ploidy.

2.5.1. Number of chloroplasts in the stomatal guard cells

The first identification of chromosome ploidy was based on the number of chloroplasts in stomatal guard cells when the regenerated shoots grew to the phase of having 5–8 leaves. Leaves were carefully peeled off the lower epidermis by hand and placed on a glass slide; a drop of 1.0% I-KI solution was then added to stain it before a cover slide was placed. Eight stomata were randomly selected to observe the average number of chloroplasts in these stomata under the microscope at 10 × 40 magnification. Fewer than 15 chloroplasts were recorded as haploid, 15–25 chloroplasts as double haploid, and more than 25 as polyploid (Beck et al., 2005). On the basis of this evaluation, the putative double-haploid plants were transplanted in the greenhouse.

2.5.2. Identification of ploidy by flow cytometry

To reconfirm the ploidy of the plants that had already been screened out on the basis of chloroplast counts, flow cytometric analysis was performed using Attune®N×T (Thermo Fisher Scientific, Waltham, MA, USA) flow cytometer. Measurement of the DNA content of colchicine-treated plants was performed according to the two-step procedure described by Dolezel (2007). Leaf tissue (1.0 cm^2) from each of the selected plants was chopped with a sharp razor blade in a 50 × 12-mm plastic petri dish with 1 mL nuclear extraction buffer (Partec). The resultant suspension was filtered through a Cell-Trics filter with a mesh diameter of 35 μm and stained with 4 μg/mL 4',6-diamidino-2-phenylindole (DAPI, Sigma Aldrich, St. Louis, MO, USA) (Jaskani et al., 2005; Ojiewo et al., 2006). After 2 minutes of incubation, the fluorescence intensity of the nuclear mixture was measured using an Attune®N×T Flow cytometer. To determine the standard peak of double haploid cells, at least 10 leaves were collected from cultivated tobacco plants (K326, 2n = 4x = 48) grown in the greenhouse. The standard peak was programmed to appear at about channel 200 of relative fluorescent intensity. This setting was kept constant and readjustments were made frequently.

2.6. Statistical analysis

The data were analyzed by the analysis of variance (ANOVA). Duncan's test was used to examine the differences in the survival rate and chromosome doubling rate of sterile tobacco seedlings.

3. Results

3.1. Effect of different media on tobacco pollen embryos induction

After the anthers were inoculated with pollen embryoid induction medium (Figure 1a), they were placed in darkness at 28 ± 1 °C for 3 weeks. It was observed that the anther tissue was dehiscent and produced many milky embryoid bodies (Figure 1b). When the anther tissue was transferred to 28 ± 1 °C, 3000–4000 lx, for 16 h light/8 h dark photoperiod culture for 3 weeks, many embryoid bodies germinated to form seedlings (Figure 1c). The pollen plants were then cut down and transferred to growth medium to grow into robust haploids for later use (Figure 1d).

The response of the anthers to the 3 media varied significantly, especially for the B medium. As showed in Figure 2, in B medium, the induction rates for pollen embryos of TMK-12 × Jiyan 5, K326 × Shuangkang 70, and NC71 × Jingyehuang were 23.28%, 26.64%, and 28.07%, respectively. Compared with the CK medium, the induction rate for pollen embryos of B medium were increased by 15.48%, 19.27%, 13.96% for TMK-12 × Jiyan 5, K326 × Shuangkang 70, and NC71 × Jingyehuang, respectively. However, compared to the A medium without 6-BA, the frequency of pollen embryos was increased by 12.32%, 15.62%, and 10.23% when 0.1 mg/L 6-BA was incorporated into the media, respectively. This indicates that B medium was more efficient in inducing tobacco pollen embryos.

3.2 Survival of tobacco seedlings after colchicine treatment

Treatment with colchicine caused phytotoxic effects in all tobacco haploid seedlings. Initially, treated haploid seedlings showed slow growth and some of them yellowed

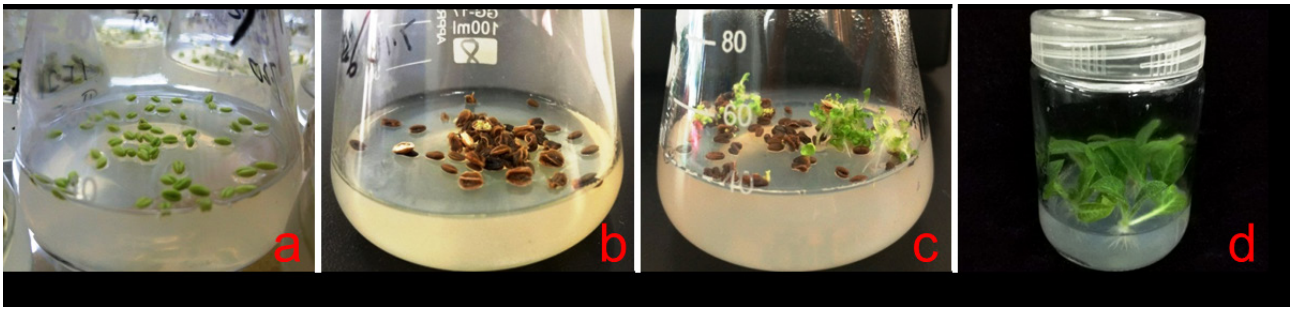

Figure 1. Induction of pollen embryo. a. Freshly inoculated anthers, b. Anther dehiscence and appearance of white embryos, c. Embryoids germinating to form small seedlings, d. Robust haploid seedlings.

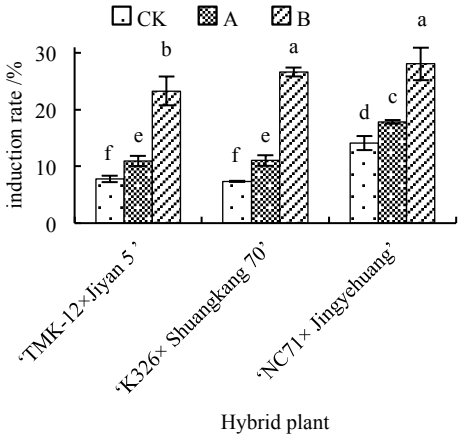

Figure 2. Induction rate of tobacco pollen embryos by different media. Letters shown above the bars are significantly different by Duncan's multiple range test at $P < 0.05$. The error bars represent the standard error of 3 replicates.

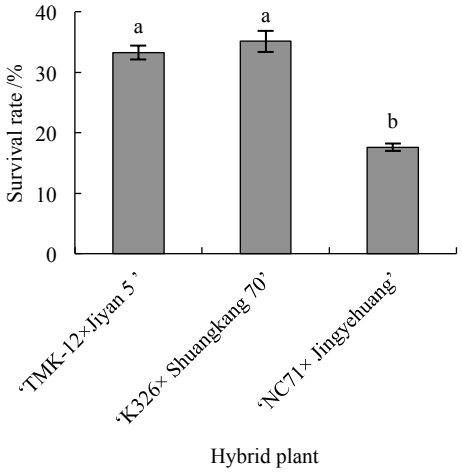

Figure 3. Effects of using colchicine on survival rate. Letters shown above the bars are significantly different by Duncan's multiple range test at $P < 0.05$. The error bars represent the standard error of 3 replicates.

and died. In the comprehensive analysis of the survival rate after colchicine treatment, the survival rate ranged from 17.61% to 35.11% and was affected by the hybrid type of the plant (Figure 3). The survival rate of TMK-12 × Jiyan 5, K326 × Shuangkang 70, and NC71 × Jingyehuang seedlings were 33.29%, 35.11%, and 17.61%, respectively.

3.3. Results of ploidy identification

3.3.1. Number of chloroplasts in stomatal guard cells

The first screening of colchicine-treated seedlings was made using the number of chloroplasts in the stomatal guard cell. Figure 4 presents the stomatal guard cell chloroplast numbers. Fewer than 15 chloroplasts were haploid (Figure 4a). For double haploid, the number of chloroplasts ranged from 15 to 25 (Figure 4b), and more than 25 chloroplasts were recorded as polyploid (Figure 4c).

3.3.2 Flow cytometric analysis

The putative double-haploid tobacco seedlings selected on the basis of the number of chloroplasts were then transplanted to the greenhouse for further investigations and seed production. Ploidy level of these plants was reconfirmed by flow cytometric analysis. The histograms of different ploidy levels of the plants are shown in Figure 5. The control cultivated tobacco appeared at channel 200, as shown in Figure 5a. With the same detection parameters, the haploid peaks were at the 100 position (Figure 5b), and plantlets with double haploid (Figure 5c) peaked at channel 200.

3.4 Effects of colchicine on tobacco chromosome doubling

The chromosome doubling rate is used as a measure of doubling efficiency. In the comprehensive analysis of the chromosome doubling rate after colchicine treatment, the result showed that chromosome doubling rate ranged from 7.98% to 21.53% (Figure 6). The chromosome doubling rate of TMK-12 × Jiyan 5 and K326 × Shuangkang 70 seedlings was significantly higher than that of NC71 × Jingyehuang, which were 21.53% and 18.54%, respectively.

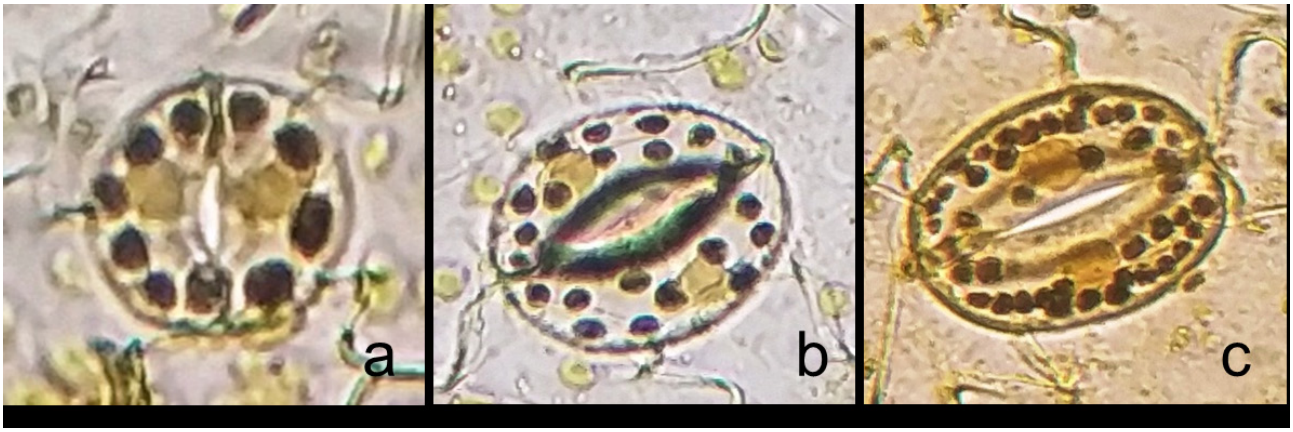

Figure 4. Number of chloroplasts in stomatal guard cells of a. Haploid, b. Doubled haploid, and c. Polyploid leaf of tobacco.

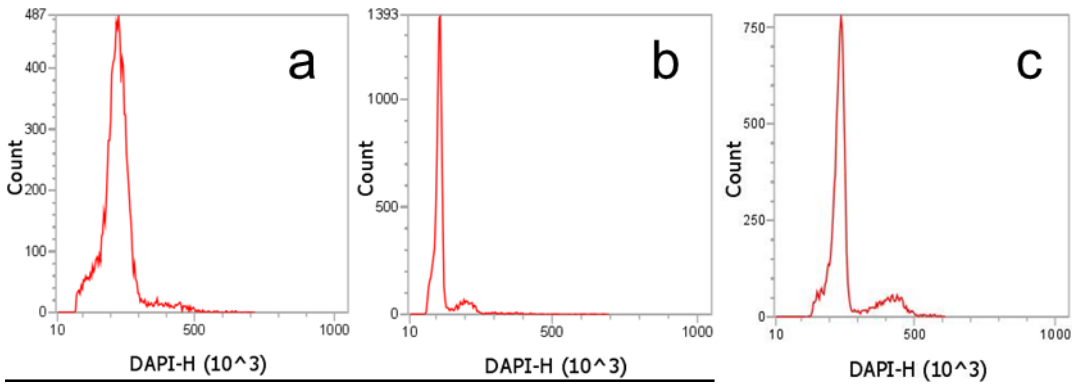

Figure 5. Flow cytometric histograms of the nuclear DNA content in tobacco plants. a. Cultivated tobacco (K326), b. Haploid, c. Double haploid.

4. Discussion

4.1. The induction of tobacco pollen embryos

Although pollen embryogenesis is a widely applied technique and many species respond well to anther culture, other species do not respond well. The cellular and molecular mechanisms involved in the transformation of microspores into pollen embryoids are poorly understood (Germanà, 2011). Major advances in technology have been achieved by empirical, time-consuming, and costly testing of protocols (particularly media and environmental conditions). Although this has achieved significant success, there is still no universally recommended method for studies on new species (Dunwell, 2010).

To improve the induction rate of anther pollen embryoids, it is necessary to pretreat the anthers before inoculation (Sato et al., 2002). For different crops, the optimal temperature for pretreatment also varied (Redha et al., 1998; Herath et al., 2009; Salas et al., 2011). The pretreatment of tobacco anthers is mainly conducted at low temperatures of 0–9 °C (Sunderland and Roberts, 1979). Low-temperature pretreatment not only delays the degradation of tobacco pollen, it also reduces the aging of anther drug wall tissue, allowing more pollen cells to begin a new round of reproductive growth and the development of pollen embryos (Nitsch and Nitsch, 1969). Following the research of predecessors, this experiment treated the tobacco anthers at 4 °C for 48 h before inoculation of tobacco anthers and achieved good results.

The genotype of donor plants is an important factor affecting the rate of embryogenesis. Differences in genotypes affect production ability and the number of embryoid bodies produced during anther culture (Mitykó et al., 2010). Meanwhile, the genotype of the donor plant not only affects the probability of pollen embryogenesis, but also affects its quality. Genotype-dependent phenomena have been reported in microspore culture of plants such as radish (Zhou et al., 2007) and wheat (Labbani et al., 2010). In this experiment, 3 genotypes of tobacco were successfully induced to produce embryoid bodies, but the embryo yields were different. Although the molecular mechanism controlling pollen embryogenesis remains unclear, it is clear that genetic factors interact with other factors to affect pollen embryogenesis.

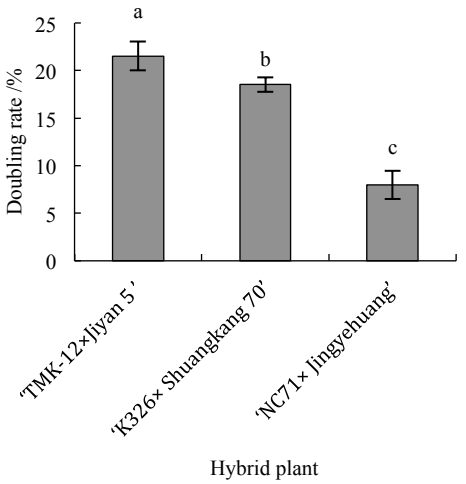

Figure 6. Effects of using colchicine on chromosome doubling. Letters shown above the bars are significantly different by Duncan's multiple range test at $P < 0.05$. The error bars represent the standard error of 3 replicates.

The basic medium is an important condition affecting pollen initiation and redifferentiation in anther culture. Therefore, it is important to select the appropriate medium for tobacco anther culture. Nitsch H and MS are the most commonly used media for tobacco anther culture (Tazawa and Reinert, 1969). At the same time, Nitsch (1973) and Li (2013) reported the superiority of H medium for the anther culture of dicots. The present results are in agreement with those of earlier studies stating that the high efficiency of Nitsch H medium in inducing tobacco pollen embryos is evident when compared to MS medium (Chen et al., 2011). Similar conclusions were reported in chili pepper (*Capsicum annuum*) (Zhao et al., 2010) and *Atropa belladonna* (Heberle-Bors 1980), both belonging to Solanaceae.

The type and concentration ratio of hormones play an important role in the induction and differentiation of anther embryoid bodies. Different hormone mixtures may influence the development of pollen either to embryoid body or callus (Sopory and Maheshwari, 1976). Although exogenous hormones are not necessarily essential for the induction of embryoid bodies in all crop anther cultures, proper addition is beneficial to increase embryogenesis. Song et al. (2007) reported that H medium supplemented with 6-BA in combination with IAA was best for embryogenic induction in cucumber. It was speculated that 6-BA had positive effects on embryogenesis by altering microspore development pathway. In this experiment, the frequency of pollen embryo production was significantly greater when 6-BA at 0.1 mg/L was incorporated into the androgenesis induction medium containing 0.1 mg/L IAA. Overall, B proved to be the superior medium, followed by A; CK medium produced the lowest frequency of pollen embryo induction. Further studies are important for development of an efficient protocol for pollen embryo induction in order to establish androgenesis as part of a breeding program to generate genetically homozygous lines.

4.2. The colchicine treatment of doubling tobacco chromosomes

Breeders have long recognized the advantages of DH technologies derived from the theoretical and practical aspects of plant biology and genetics of haploidy technology (Forster and Thomas, 2005). The benefits of applying haploidy, doubled haploidy, and pollen embryos in breeding are evident. Haploids and doubled haploids are derived from the in vitro culture of gametophytic cells, particularly anther culture (Forster et al., 2007). The construction of a tobacco DH population mainly involves using anther culture to obtain haploid plants and chromosome doubling to obtain DH plants (Takashima et al., 1995). So far, double haploids have mainly been obtained through treatment with colchicine (Zhou et al., 2017).

Previous studies have found that the doubling rate and the concentration of colchicine have a quadratic polynomial regression relationship. At a certain concentration range, the doubling rate increases with the increase of colchicine concentration (Liu et al., 2009). However, colchicine is a highly toxic substance as excessive concentrations can cause phytotoxicity to plants, and inhibit or even stop plant growth (Soriano et al., 2007). Vardar et al.'s (2017) research results demonstrated that 0.4% colchicine application induced toxic effects on growth and development of sunflower seedlings. In this study, we used the same concentration of colchicine for different hybrid plants to treat tobacco haploid seedlings; ploidy identification was then performed by using the number of chloroplasts in stomatal guard cells and flow cytometry analysis. However, it is interesting to note that the results of our research showed that the concentration of 4.0 g/L colchicine was too high, as it caused serious damage to the test seedlings; furthermore, a large number of tobacco seedlings died, especially those of NC71 × Jingyehuang. The adverse effect of higher doses of colchicine treatment on the survival rate of treated plants has also been reported in other chromosome doubling studies (Abdoli et al., 2013; Bagheri and Mansouri, 2015; Tavan et al., 2015). Therefore, an appropriate colchicine concentration should be applied to ensure successful chromosome doubling. Moreover, we used the same colchicine treatment method, concentration, and time, and found that there was a significant difference in chromosome doubling rate among different tobacco hybrid plants. Similar observations have been made for potatoes (Choudhuri and Banerjee, 1961) and gerbera (Li

et al., 2009). The reason for this difference may be related to the genotype of the test material and the physiological state and growth status of the seedlings, but the specific mechanism of influence needs further research.

Overall, our study provided an effective pollen embryo induction system in tobacco anther culture and the pollen embryo induction rate was over 23.0% in the 3 tobacco hybrid plants, which demonstrates significant utility for tobacco breeding. This study shows that using a solution with a concentration of 4.0 g/L colchicine is not only effective and economical for chromosome doubling in tobacco seedlings, but also causes serious toxic effects on tobacco seedlings. In addition, the efficiency of colchicine on the doubling of tobacco haploid chromosomes is highly influenced by the genotype of the test material.

Acknowledgments
This work was supported by Shaanxi Province Tobacco Company of China National Tobacco Company (project No. KJ-2016-01).

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