

Colchicine Induced Embryogenesis in Date Palm (*Phoenix dactylifera* L.) Anther CultureFatemeh Farhadi Kolahkaj¹, Payam Pour Mohammadi^{2*}, Mohammad Farkhari²,Khalil Alami-Saeid¹

Abstract

An efficient method to produce doubled haploid plants of date palm (*Phoenix dactylifera* L.) was established using *in vitro* colchicine treatment of anthers. For this study end male in florescences were harvested in the appropriate developmental stage. Separated anthers after one day cold (4°C) pretreatment, in uninucleate stage of microspore were transferred to the modified Y3 medium containing different concentrations of colchicines (125- 250-500- 1000 mg/L) for one of four treatment durations (12- 24- 36- 48 hours) and were compared to control anthers (without colchicine treatment). Based on results, different concentrations of colchicine, different exposure times and their interaction had a significant different effect on callus induction. No callus and embryo were produced from control anthers; however, the callus and embryo were induced from colchicine-treated anthers. Among used concentration in the 1000 mg/L and 125 mg/L colchicine, the rate of callus induction was very low. In contrast concentration of 500 mg/L and duration of 12 hours had the highest rate of callus induction. In concentration of 500 mg/L and duration of 36 hours some direct embryos were observed. These results indicate that the colchicine treatment of date palm anthers can induce callus and embryo for the production of doubled haploid lines.

Keywords: Anther Culture, Date Palm, Colchicine Treatment, Haploids, Somatic Embryogenesis

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Introduction

The date palm (*Phoenix dactylifera* L.) is a monocotyledonous woody diploid ($2n = 2x = 36$) and dioeciously species, i.e. the male and the female flowers are on separate plants [1]. The date palm belongs to the *Arecaceae* family and it is considered as a symbol of life in the desert, because it tolerates high temperatures, drought and salinity more than many other fruit crops [2].

Haploid plants are highly valuable in breeding programs, genetic and mutation analysis. Great achievements have been obtained in the production of haploid plants in monocots by androgenesis, interspecific or intergeneric crosses and gynogenesis during the last 20 years [3]. Androgenesis has been reported in more than 250 plant species, belonging to 100 genera and 40 families [4]. However, in woody species, androgenesis has had only limited success [5]. The developmental stage of the microspores is a critical factor that determines the success of another culture [5, 6]. Various stress treatments have been necessary to block gametophytic development and trigger pollen embryogenesis in microspores [7]. Few studies have been conducted on coconut anther culture. Koor [7] observed callus formation in cultured coconut anthers at a low frequency, while Iyer [8] obtained many celled anther derived pre-embryos that, however, failed to develop. Thanh-Tuyen and de Guzman [9] reported the development of embryos from pollen in

cultured anthers at less than 1%. Likewise, these embryos showed no further development.

Among the different anti-microtubule agents used successfully, colchicine has been the most widely used *in vivo* and *in vitro*. Herbicides such as amiprofos-methyl (APM), oryzalin, trifluralin and pronamide, have also been used *in vitro*. All these compounds, with the exception of pronamide, inhibit spindle formation by binding to tubulin, disrupting the normal polar segregation of sister chromatids and result in doubling chromosome number (C-mitosis) [10]. Different actions of colchicine have been proposed to explain the positive effects on embryogenesis, as an increase of symmetrical divisions, the depression in the synthesis of pollen specific tubulins, and cytoskeletal restructuration [11]. The identification of tubulin and actin-associated proteins in the microspores would explain the diverse behavior of microtubules and microfilaments to antimitotic drugs [12]. Colchicine could also cause a reduction of chloroplast DNA abnormalities [13] or a selective elimination of microspores having abnormalities [14]. In several researches the effect of incorporating colchicine was investigated on anther culture derived plants in several cereals: wheat [15], maize [16-18], rice [19] oilseed rape [20] and triticale [21].

The present study is the first experiment on the anther culture and simultaneously determines in systematic manner the effect of the colchicine concentration and the



duration of colchicine treatment on the induction of callus and embryo in date palm anther culture.

Materials and Methods

Plant material

Excised anthers from male flowers of 15 to 20 years old date palms were used as the explants. The collection of date inflorescence rachillae at desirable stages of microspore development was based on spadix age in the number of weeks before split opening (WBS). Collected anthers from inflorescences of 3 weeks before split opening of the spadix (Fig. 1A) were used. The rachilla (Fig. 1B) was collected and they were subjected to cold (4°C) pre-treatments for 1 day.

Anther culture and colchicine pretreatment

Male florets on the excised rachilla were wrapped in a wet tissue paper and aluminum foil, respectively, and placed in a refrigerator at 4°C for 1 day in the dark for cold pre-treatments. The nanthers were excised from the fila-

ments and surface sterilized by immersion in 2% (w/v) sodium hypochlorite with a few drops of liquid detergent, for 15 min followed by three rinses with sterilized water under aseptic conditions. An induction medium (Y3 medium) containing 0.1% colchicine was prepared and filter sterilized using a 0.2 µm filter. Anthers were treated with one of four colchicine concentrations (125, 250, 500 and 1,000 mg/L) in one of four treatment durations (12, 24, 36 and 48 h) based on completely randomized designs. After the colchicine treatments, the anthers were transferred into colchicine-free Eeuwens Y3 [22] semi-solid medium, supplemented with 9% (w/v) sucrose and 0.1% (w/v) activated charcoal (BDH acid washed). Each treatment was consisted of 3 replications, each containing 25 anthers per Petri dish (120×20 mm), within 15 ml of semi-solid culture medium (Fig. 1-C). The petri dishes were sealed with Para film and the cultures were maintained under dark at 25°C for 3 months. The number of calli and embryoids in each treatment was record.

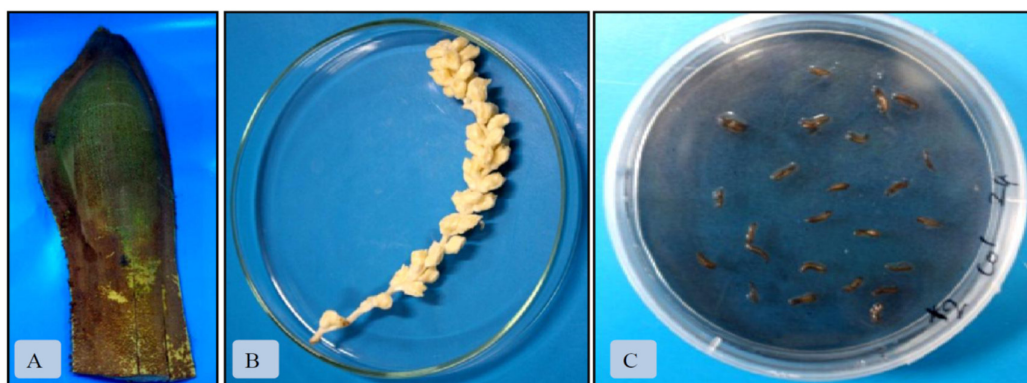


Figure 1. Spadix (A), Rachilla (B), Anthers cultured in Y3 medium (C).

Results

In this study the effects of different levels of colchicines (125, 250, 500 and 1,000 mg/Lm) and different periods of exposing (12, 24, 36 and 48 h) of anther date palm to colchicine and their interaction was studied using factorial experiment based on completely randomized design. In concentrations of 125mg/L and 1000 mg/L colchicine no callus were produced, so variance analysis (ANOVA) was done using two concentrations of 250 mg/L and 500 mg/L (table 1) for number of calluses. The direct embryogenesis was obtained using treatment with 500 mg/L colchicine for 36 h (Fig. 2A). According to the ANOVA results in table

1, all of effects including interaction effects were highly significant. So, just mean comparisons were done for interaction effect by Duncan test (Figure 3).

Based on Figure 3, treatment combination of 500 mg/L colchicine with exposure time of 12 hours had the best result (Fig. 2B). Amount of callus severely was decreased by increasing of colchicine to 1000 mg/L that is probably because of the colchicine toxic effects on the microspores. Also callus was not occurred in concentration of 125 mg/L colchicine inducing that the induction in this concentration is little.



Figure 2. Haploid embryo (A), Haploid callus as a sample (B).

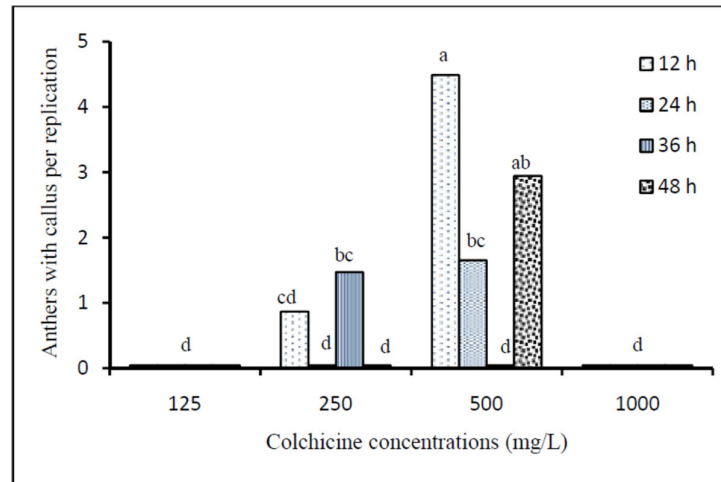


Figure 3. Comparing the means of treatment combination by Duncantest at the level of 1% probability.

Discussion

The present results show that an efficient callus and embryo induction from anther date palm can be achieved by treating anthers with colchicine. The duration of colchicine treatment is a critical parameter for callus and embryo induction. The duration of colchicine exposure had a considerable effect on chromosome doubling induction and the survival rate. The present experiment showed that the treatment duration of 48 h had fewer positive effects on the callus and embryo induction, especially at 125, 250 and 1000 mg/L colchicine concentration.

Table 1. Analysis of variance for callus number of date anther culture.

Source of variation (S.O.V)	D.F.	Mean of squares (MS)
Colchicine treatment	1	1.94**
Times duration	3	0.53**
Colchicine × time	3	1.12**
Experimental error	16	0.102

** Significant difference at 1% probability level.

The pollen developmental stage is known to be critical for androgenesis and thus it is very important to determine the most suitable developmental stage of anthers for culture initiation. It is believed that the early, during or immediately after the first pollen mitosis is ideal for the induction of coconut androgenesis [23]. In other studies reported on coconut anther culture, anthers at first pollen mitosis [9], tetrad stage [7] and uninucleate stage [8], have given rise embryos or calli at a low frequency. Anthers bearing late uninucleate microspores have been reported to be optimal for induction of androgenesis in many crop species [9]. It has been suggested that any microtubule

disrupting agent exhibiting a symmetric first mitotic division in microspores would also lead to both embryo induction and spontaneous chromosome doubling [24]. In Brassica, the application of colchicine produced high frequency of embryogenesis and chromosome doubling [25], and could even substitute a heat stress pretreatment [26]. Performed studies in maize, with a wide range of colchicine concentrations, indicated that although colchicine can induce embryogenesis, but was different optimal concentration for embryogenesis and chromosomal doubling [16, 27]. Also in maize (*Zea mays* L.) anther culture, colchicine at a concentration of 125 mg/L produced significantly the highest number of embryo like structures (ELSs), while the control (colchicine-free pretreatment medium) and 400 mg/L of colchicine produced the least ELSs [18]. The results presented here indicate that the duration of colchicine treatment is a critical parameter for embryo and callus production from date palm anthers. The duration of colchicine exposure has a considerable effect on chromosome doubling induction and the survival rate. The present experiment showed that the treatment duration of 12 h had fewer positive effects, especially at the 125 mg/L colchicine concentrations and 500 mg/L colchicine for 12 h can be used for producing callus from date palm anthers.

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