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Full Length Research Paper

Establishment of plant regeneration system from anther culture of *Tagetes patula*

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Plant regeneration of *Tagetes patula* was achieved from anther explants via adventitious shoot differentiation from callus. The effects of genotype, temperature pretreatment, plant growth regulators, light regimes and sucrose concentration were studied. Eight of ten genotypes tested were successfully regenerated. The highest callus induction rate and regeneration frequency of line 21605 was obtained when inflorescence buds were stored at 4°C for 4 days, and anthers with microspores at the mid to late uninucleate stage were cultured on MS basal medium containing BA (2.2 µM) and NAA (1.82 or 2.7 µM). Frequencies of callus induction and shoot regeneration were 100 and 70.5%, respectively with the whole regeneration procedure completed in 40 days under light. This highly efficient, rapid regeneration system can be applied for both genetic transformation and doubled haploid plant induction.

Key words: *Tagetes patula,* genotype, anther culture, plant regeneration.

INTRODUCTION

The *in vitro* production of haploids is extremely valuable in plant breeding and genetics (Custódioa et al., 2005). Anther or microspore cultures have been found to be the most efficient techniques for obtaining a large number of haploid plants (De Buyser and Henry, 1980). With haploids, the establishment of homozygous lines of new varieties is possible in a short period of time, hence doubled haploid plants (DHs) provide a useful tool with which to accelerate plant breeding cycles (Devaux and Li, 2001; Zhang et al., 2011). In addition, anther culture could also be an effective vehicle for producing variation as it allows early expression of recessive genes (Chen et al., 2001). Some cultivars have been successfully released by anther culture technique, such as, rice (Afza et al., 2000; Guzmán and Zapata-Arias, 2000), wheat (Patel et al., 2004), barley (Lazaridou et al., 2005) and banana (Assani et al., 2003) etc. Moreover, while the anther culture technique is widely used for practical breeding, its application is still limited by many factors which influence culture efficiency, such as the genotype of the explants, the growing conditions of the donor plants, the developmental stage of the microspores, light conditions, media and the temperature pretreatment (Arzate-Fernandez et al., 1997; Afza et al., 2000; Zhang et al., 2009). In this paper, we studied the effects of genotype, temperature pretreatments, plant growth regulators and light on callus induction and shoot regeneration from anthers of *Tagetes patula* L.

T. patula is native to Mexico. Most studies on *T. patula* have focused on applied aspects, such as medicinal (Vasudevan et al., 1997), industrial (Tereschuk et al., 2003) and commercial (Margl et al., 1985; Bii et al., 2003; Tereschuk et al., 1997; Hitmi et al, 2000; Naranjo et al., 2000), and *T. patula* is also used for ornamental purposes as garden flower and cut flower (Sreekala and Raghava, 2003). *T. patula* is used extensively in China as an ornamental plant and has strong adaptability to adverse environments as it is resistance to pests and disease. To our knowledge however, there are no reports of plant regeneration from anther culture of plants in the genus *Tagetes*, although plant regeneration has been obtained

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Abbreviations: MS, Murashige and Skoog medium; **NAA,** naphthaleneacetic acid; **IAA,** indole 3-acetic acid; **BA,** 6-benzyladenine; **GA_3**, gibberellic acid; **PGR,** plant growth regulator.

Table 1. Effect of genotypes on callus induction and shoot regeneration from anthers of *Tagetes patula*.

Genotype	Petal shape	Petal color	Percentage of anthers forming calli (%)	Percentage of calli forming shoots (%)
T. patula 21608	Single	Light red	42.9 ± 0.03^{b}	10.5 ± 0.01^{cd}
T. patula 21611	Single	Yellow margin red heart	0.0 ± 0^{g}	0.0 ± 0^{d}
T. patula 21614	Single	Red golden	22.5 ± 0.02^{de}	0.0 ± 0^{d}
T. patula 21613	Layer	Orange	54.3 ± 0.01^{ef}	59.6 ± 0.11^{b}
T. patula 'Little Hero Golden'	Layer	Golden	16.0 ± 0.09^{ef}	2.8 ± 0.03^{d}
T. patula 'Safari Yellow'	Layer	Yellow	37.5 ± 0.03^{bc}	0.0 ± 0^{d}
T. patula 'Safari Red'	Layer	Red	55.8 ± 0.03^{a}	34.2 ± 0.04^{c}
T. patula 'nana petite Gold'	Layer	Golden	31.6 ± 0.02^{ef}	25.8 ± 0.11^{cd}
T. patula 21605	Layer	Crimson	87.1 ± 0.01^{fg}	79.2 ± 0.13^{a}
T. patula ' nana'	Layer	Crimson	56.2 ± 0.03^{fg}	6.3 ± 0.06^{cd}

In each column, means followed by the same letters are not significantly different according to LSD (α = 0.05).

from leaf explants (Kothari and Chandra, 1984; Qi et al., 2005), cotyledons (Bespalhok and Hattori, 1998; Mohamed et al., 1999), hypocotyls (Mohamed et al., 1999) and stem segments (Pablo et al., 2002), and whole plant regeneration of *T. patula* has been reported by Bespalhok and Hattori, (1998). This work therefore aimed to establish an efficient plant regeneration protocol and thereafter provide a fundamental system for genetic manipulation and haploid induction.

MATERIALS AND METHODS

Plant material and sterilization procedure

Different lines and cultivars of *T. patula* (Table 1) were obtained from seeds companies namely Gansu Jiuquan Golden Autumn Horticulture Seeds Company and Zhejiang Hongyue Seeds Company, and planted in an experimental field at Huazhong Agricultural University, China. In a preliminary experiment in order to decide the developmental stage of microspores, we observed florets with length from 0.5~2.5 mm under a light microscope, and found that when florets were 1 to 1.5 mm in length, microspores in anther were at the mid to late uninucleate stage. So, in this experiment, florets of 1 to 1.5 mm long were chosen.

Prior to inoculating the anthers, the inflorescence buds were surface sterilized with 70% ethanol for 1 min, in 0.8% sodium hypochlorite for 20 min, and subsequently rinsed with sterile distill water three times. After sterilization, the buds were cut in half and the florets could be easily observed. Five anthers were picked from each floret with needles and placed onto induction media.

Anther culture media and culture conditions

Effect of genotypes

The inflorescences buds were stored at 4°C for 4 days and the anthers of ten lines and cultivars of *T. patula* were cultured on induction medium (MS + 2.7 µM NAA + 4.4 µM BA) based on preliminary experiments designed to test the effect of genotype on callus induction and shoot regeneration.

Effect of temperature pretreatment

Line 21605 was chosen for the temperature pretreatment test, and inflorescence buds of this line were put on a piece of wet filter paper in a Petri dish and kept in the dark at 4°C for 0, 2, 4, 7 and 9 days and at 31°C for 1 day. After temperature pretreatment, anthers were inoculated onto induction medium.

Effect of plant growth regulators in the medium

The inflorescence buds of line 21605 were stored at 4°C for 4 days and anther were inoculated on MS basal medium supplemented with 3% sucrose, 0.8% (w/v) agar and different combinations of NAA and BA or IAA and BA to establish the best combination of plant growth regulators.

Effect of light condition during culture

Anthers of line 21605 were cultured on induction medium (MS + 2.7 µM NAA + 2.2 µM BA), which was decided according to the results of experiment on plant growth regulators. Two kinds of light condition, under continuous light and under light after twenty-day darkness of culture, were tested to check the effect of light on callus induction and shoot regeneration.

Effect of different sucrose concentration

In order to ascertain if the sucrose concentration can affect the regeneration way on anther of *T. patula*, anthers of line 21605 were cultured on induction solid medium (MS + 2.7 µM NAA + 2.2 µM BA) which was supplemented different sucrose concentration including 3, 5, 6, 7, 8, 9 and 13%. The other cultured condition was same as the previous experience. The pH of each medium was also adjusted to 5.8 ± 0.2 prior to autoclaving at 121°C for 20 min, and then anthers were incubated at 23 ± 1°C under 14 photoperiod of white fluorescent light (40 µmol m^{-2} s^{-1}) in a culture room.

The experimental design was a randomized complete block with three replications. A replication consisted of two Petri dishes with 15 to 21 anthers in each of 7.5 cm Petri dish, so there were 90 to 126 anthers in three replications. The percentage of callus induction (total calli per 100 anthers) was calculated 25 days after inoculation and the percentage of shoot bud induction (calli regenerated buds per 100 total calli) was calculated 40 days after inoculation. The significant difference in the frequency of anthers forming callus and the production of shoots was calculated using least significant difference (α = 0.05).

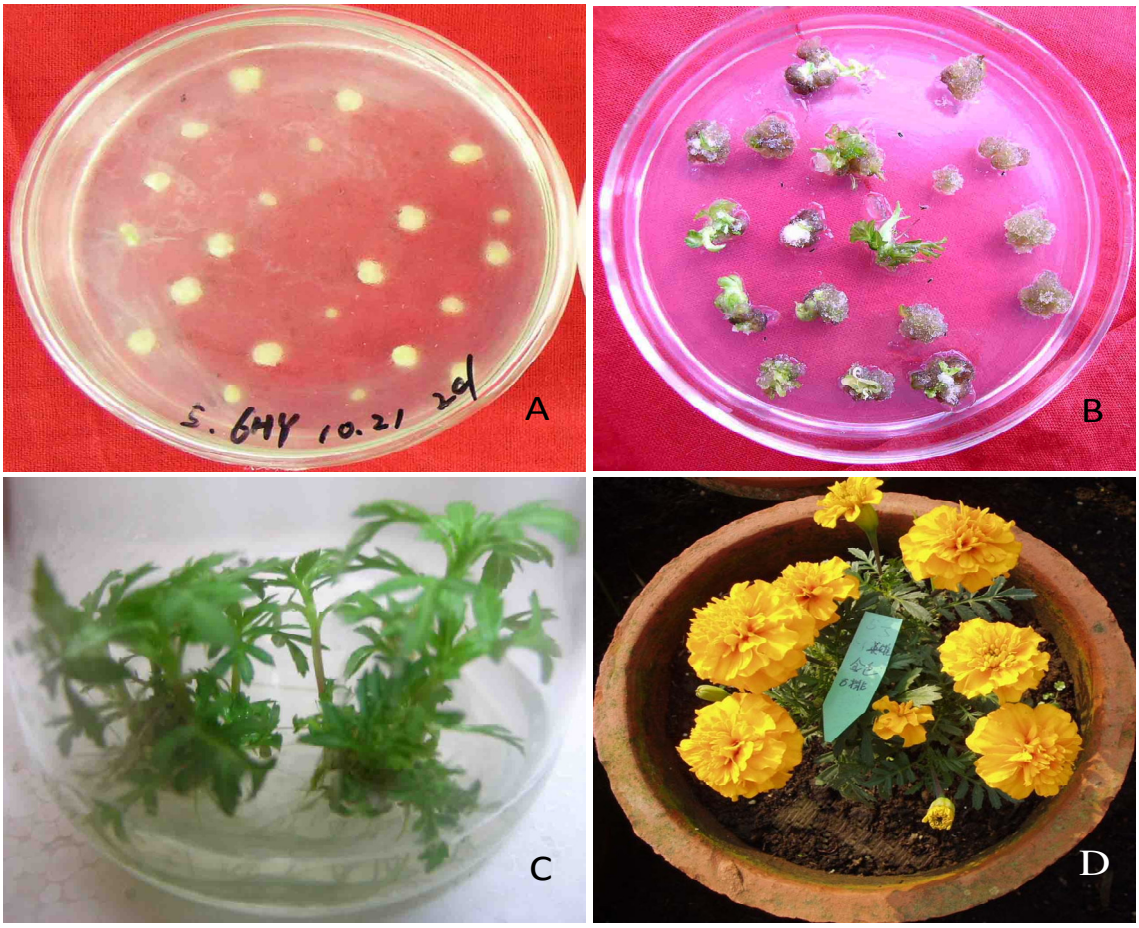

Figure 1. Callus induction and plant regeneration from anthers of *Tagetes patula* line 21605. (A) Callus formation, (B) shoot differentiation, (C) shoot elongation and rooting (D) potted plant.

Shoot elongation and rooting

After 30 to 40 days of culture, the shoot buds plus some callus were transferred to the following 4 media: hormone free MS medium, hormone free 1/2 MS medium and MS medium with GA_3 (0.5 or 1.0 mg/L) for plant elongation and rooting. The well developed plantlets were acclimatized for five to seven days. Subsequently, the plantlets were washed with tap water to remove traces of the medium and transferred to pots containing a mixture of vermiculite, peat and garden soil (1:3:6) and grew in the greenhouse.

Ploidy analysis

In order to define the kind of ploidy of regenerated plantlets, before they were transferred to pots, we randomly chose 20 plantlets from each experiment and leaves were taken for ploidy analysis by flow cytometer (PA-II, PATEC, Germany).

RESULTS

Effect of genotypes of *T. patula*

Anthers from 10 genotypes were inoculated on MS medium containing 2.7 µM NAA and 4.4 µM BA post cold pretreatment. The results indicate that the frequency of callus induction and shoot regeneration were significantly different among different genotypes. The highest percentage of callus and shoot induction were obtained in line 21605 (Figure 1A) at 87.1 and 79.2%, respectively (Table 1). According to this observation, *T. patula* 21605 was chosen as material for the experiment on temperature pretreatment.

Effect of temperature pretreatment

Temperature pretreatment at 4°C for day 0, 2, 4, 7 and 9 and at 31°C for 1 day was tested. Ten days after inoculation the anthers exhibited obvious expansion from both ends of the anthers and callus developed within 20 days independent of the different temperature pretreatments. However, the percentages of callus induction in treatments of day 0, 2 and 7 were significantly higher than those in treatments of 4 and 9 days (Table 2). Statistical analysis also documented that there was no

Table 2. Effect of temperature pretreatment on callus induction and shoot regeneration from *Tagetes patula* 21605[a].

Storing time (days)	Percentage of anthers forming calli (%)[b]	Percentage of calli forming shoots (%)
0	87.5 ± 0.08[a]	10.0 ± 0.03[c]
2	95.0 ± 0.02[a]	31.3 ± 0.02[b]
4	50.0 ± 0.04[b]	62.0 ± 0.06[a]
7	91.3 ± 0.04[a]	0.0 ± 0[c]
9	26.3 ± 0.18[b]	11.6 ± 0.08[c]

[a]Anthers were cultured on medium MS + NAA (2.7 µM) + BA (4.4 µM). [b]In each column, means followed by the same letters are not significantly different according to LSD (α = 0.05).

Table 3. Effect of plant growth regulators on callus induction and shoot regeneration from anthers of *Tagetes patula* 21605.

Concentration (µM)		Percentage of anthers forming calli (%)	Percentage of calli forming shoots (%)
NAA	BA		
0.0	0.0	-	-
1.82	2.2	93.68 ± 0.05[ab]	67.83 ± 0.07[ab]
	4.4	63.88 ± 0.22[bcd]	36.7 ± 0.16[bcd]
	6.6	100 ± 0[a]	69.4 ± 0.05[ab]
2.7	2.2	100 ± 0[a]	70.5 ± 0.15[a]
	4.4	54.69 ± 0.05[d]	56.1 ± 0.05[abc]
	6.6	48.28 ± 0.11[d]	0 ± 0[e]
	2.2	83.43 ± 0.1[abc]	60.7 ± 0.1[ab]
5.4	4.4	59.08 ± 0.05[d]	15.7 ± 0.05[de]
	6.6	61.77 ± 0.11[cd]	24.6 ± 0.11[cde]

In each column, means followed by the same letters are not significantly different according to LSD (α= 0.05).

significant correlation between callus induction and bud regeneration. The highest frequency of shoot regeneration was obtained in the treatment of 4 days, although, the frequency of callus induction in this treatment was not the highest observed. Anthers stored at 31°C for 1 day turned brown and eventually died and neither callus formation nor plant regeneration was obtained (data not shown). In summary, the treatment of being stored for 4 days at 4°C was the best and thus this pretreatment was adopted for the following experiments.

Effect of plant growth regulators

The anthers of *T. patula* 21605 were cultured on MS medium with different plant growth regulators. In combinations of NAA and BA, the best callus formation was obtained with 1.82 µM NAA + 6.6 µM BA (100%) and 2.7 µM NAA + 2.2 µM BA (100%) (Table 3). The highest frequency of shoot differentiation was observed with 2.7 µM NAA + 2.2 µM BA (70.5%) (Table 3, Figure 1B). Combinations of IAA and BA were also tested, although shoot differentiation frequency was higher than expected, callus induction frequency was very low (data not shown). Thus, 1.82 µM NAA and 6.6 µM BA or 2.7 µM NAA and 2.2 µM BA were the best combinations for both callus induction and shoot differentiation.

Effect of light during cultivation

Although, calli were formed under light and in the dark in 20 days cultures, calli produced under light were green and large with adventitious buds that could be seen in medium MS+ 2.7 µM NAA + 2.2 µM BA after a week contrasted to those produced in the dark which were pale yellow and could not differentiate. Moreover, when the dark grown cultured was transferred to the light, the calli became green and obtained differentiation ability just like those continuously cultured under light (data not shown).

Effect of different sucrose concentration

The effect result of sucrose concentration on anther cultured is shown in Figure 2. The highest callus

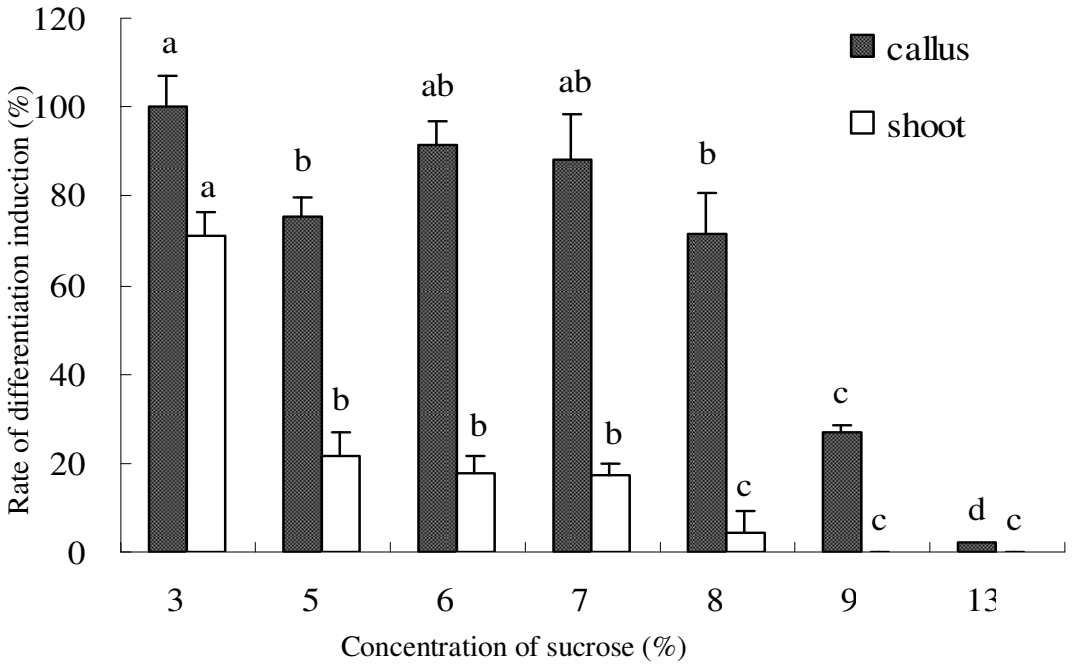

Figure 2. Influence of different sucrose concentration on anther callus induced and adventitious shoot differentiation.

induction (100%) was obtained at sucrose level of 3%. With increasing concentration, the rate of callus induction reduced slowly, although the frequency of callus production was a little higher at 71.52% when the concentration was up to 8%. The frequency of callus production was not significantly different at sucrose concentration of 3 to 8% according to LSD. The rate of callus production was reduced sharply when sucrose concentration was added up to 9%. The frequency of shoot regeneration was affected by different sucrose concentration. As the concentration was low, the frequency was a litter higher (71.24%). The frequency of shoot regeneration was reduced obviously at a concentration of 5%, the frequency of shoot regeneration reduced slowly while at level of 6 and 7%, there was no significantly difference among the three levels. When the sucrose concentration was added up to 8, 9 and 13%, the frequency of shoot differentiation reduced sharply with no differentiation and no significant difference among the three concentrations.

Shoot elongation and rooting

Shoots plus some calli were transferred to different media for elongation and rooting. On MS media without PGR, normal plantlets with well-developed root and shoot systems were obtained in 20 days (Figure 1C). After one month of culture on elongation and rooting medium, the plantlets were transferred to soil in the greenhouse for two weeks, and subsequently moved outdoors where they completed their life cycle normally (Figure 1D). In the other 3 media, roots could be induced but the plants could not elongate and continued to grow in clusters.

Ploidy analysis

All the 20 regenerated plantlets chosen for ploidy analysis by flow cytometer had the same peaks as the control plant at 100, which meant there was no haploid obtained.

DISCUSSION

Many authors have reported that the developmental stage of anthers can drastically affect plant regeneration because microspores respond only at a specific development stage, which ranges from the tetrad, early and mid-uninucleate to the early binucleate stage. As such anther of the Lilium × 'Enchantment', excised at the uninucleate microspores stage could form callus and regenerate bulblets (Niimi et al., 2001). Again, according to Afza et al. (2000), the callusing abilities of anthers of rice from different panicle positions were significantly different. Within one panicle, microspore in anthers from the top part is the oldest, while that in the basal part is the youngest. In local climate, it is easier to obtain appropriate inflorescence buds if seeds are sown in February. If seeds are sown later, the plants grow faster because of the increasing temperature, and reduced periods are available to select inflorescence buds at the

proper stage. In the present study, florets that were 1.0 to 1.5 mm in length contained microspores at mid to late uninucleate stage, and anthers at this time were suitable for callus induction. However, inflorescence of *T. patula* is capitulum, anthers can only be found in bisexual florets, and microspore in anthers from outside part are oldest and in the inside part youngest. The appearance of florets with microspores at mid to late uninucleate stage changes with season (spring and autumn); growing condition (under stress or not) and positions of inflorescence (on main shoots or on side shoots), hence it is not easy to select the appropriate material without practice and experience. If anthers at mid to late uninucleate stage are chosen, ideal regeneration can be guaranteed under the culture condition we optimized in this experiment.

Microspores need a signal to switch from the gametophyte to the sporophyte pathway. Cold or heat shock pretreatment has been applied to a variety of plant species. In some species, 4°C for about one week is beneficial to induced regeneration such as in *Cucumis sativus* (Ashok et al., 2003), *Lilium longiflorum* (Arzate-Fernandez et al., 1997), *Lolium temulentum* (Wang et al., 2005), wheat (Patel et al., 2004), barley (Wojnarowiez et al., 2004; Lazaridou et al., 2005) and rye (Tenhola-Roininenl et al., 2005). In other species, the cold shock at about 10°C was better, like *Bupleurum falcatum* (Shon et al., 2004), recalcitrant barley (Devaux and Li, 2001) and basmati rice (Bishnoi et al., 2000). In addition, the proper heat pretreatment temperature is usually above 30°C as shown in papaya (Rimberia, 2005) and in Tronchuda cabbage (Dias et al., 2002). In *T. patula*, we found that a 4-day cold shock at 4°C was beneficial as it significantly improved shoot regeneration that may have postponed rate of degradation of epidermis and endothelium membrane at low temperature, so it has a positive effect on its callus induction (Zhang et al., 2010), although, heat shock was not beneficial in line *T. patula* 21605. More also, light conditions appeared to be the most important factor on anther culture in some species. Arzate-Fernandez et al. (1997) reported that under darkness the callus was observed for *L. longiflorum* anther culture, while no callus formed under light. In our study, although callus could form under darkness, adventitious buds could not be induced when the callus were transferred to light, so continuous light was necessary for anther culture of *T. patula.*

Medium is another important factor affecting anther culture. Custódio et al. (2005) reported that the best medium for calli induction of carob tree was MS supplemented with 2.3 µM 2,4-D + 8.2 µM TDZ. Plant regeneration from anther of rye (Teija et al., 2005) and banana (Assani et al., 2003) was obtained in MS + 4.4 µM BAP + 2.3 µM IAA and MS + 9.0 µM 2, 4-D + 2.3 µM kinetin medium, respectively. MS basal medium supplanted with different combination of plant growth regulators was reported to be used in tissue culture of *T. patula* (Margl et al.,1985; Bespalhok and Hattori, 1998), our results are similar to those in which MS basal medium containing BA (2.2 µM) and NAA (1.82 and 2.7 µM) was shown to be the best for plant regeneration. It indicated that there is a significant differentiation on plant regeneration from anther in different species.

It was also reported that the microspore cell was much easy to be induced with increasing sucrose concentration (Lin, 2000; Zhang, 1998). However, in our study, the shoots all came from somatic cell and the microspores were still not be induced although the sucrose concentration was added up to 8%. Moreover, tomato and sugarcane need 13% and 20% sucrose concentration respectively to induce calli of anthers (Xie and Liu, 2004); therefore it may be necessary to increase sucrose concentration for further experiment in *T. patula*.

In summary, this is the first report of establishing an efficient and rapid plant regeneration system from anther explants of *T. patula*. In this work, the effects of genotype, temperature pretreatment, plant growth regulators, light and sucrose concentration were studied and the result showed that when *T. patula* 21605 anthers with microspores at the mid to late uninucleate stage were cultured on MS basal medium containing BA (2.2 µM) and NAA (1.82 µM or 2.7 µM) and 3% sucrose concentration under light, optimum regeneration could be obtained, and frequencies of callus induction and shoot regeneration were 100 and 70.5%, respectively. In addition, the haploid plants were not obtained, may be the callus were obtained from the anthers wall. The results also demonstrated that frequency of plant regeneration from anther culture were highest when microspores were at the mid to late uninucleate stage. This system was advantageous in that the calli could be maintained or induced to differentiate by light control, and plants could be regenerated as needed. This regeneration system is therefore useful for developing an efficient genetic transformation system, and an efficient doubled haploid plant induction system could be established after further modification of the culture parameters.

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