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Haploid Culture and Double Haploid Induction in *Medicago sativa* L. cv. XinJiangDaYe

Bo Xu, Rina Wu, Fang Tang, Cuiping Gao, Xia Gao, Fengling Shi

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ABSTRACT

Background: Alfalfa (*Medicago Sativa*), a perennial cross-pollinated plant, is one of the most important forage crops in the world with commercial value and ecological significance. However, due to the complexity of its genome, varietal improvement is difficult. Therefore, generating genetically homozygous materials have greater significance for breeding. In the current study, we aimed to identify the best tissue culture conditions to obtain haploid plants and double haploid plants.

Methods: In this study, the haploid plants of alfalfa were obtained by combining tissue culture regeneration system with Flow cytometry. Different concentrations of colchicine were applied to the haploid plants using solid and liquid cultivation methods to determine the optimum conditions to obtain double haploid plants of *Medicago Sativa* L. cv. 'XinJiangDaYe'.

Result: Among the two colchicine cultivation methods tested, the doubling rate of regenerated plants obtained by liquid cultivation method was higher and the leaves developed under this system have the best doubling effect among the three explants tested. Optimal doubling conditions for alfalfa haploid (*Medicago Sativa* L. cv. 'XinJiangDaYe') were identified. The double haploid plant material generated from the current study could serve as a genetic resource for developing the hybrid combinations and for analyzing genetic linkage in alfalfa improvement programs.

Key words: Colchicine, Double haploid, Doubling rate, Haploid, *Medicago sativa.*

INTRODUCTION

Alfalfa (*Medicago sativa*), a perennial cross-pollinated plant, is one of the most important forage crops in semi-arid and arid areas (Rong *et al.*, 2017; Zhang *et al.*, 2017) with greater commercial and ecological significance (Yin *et al.*, 2018). However, cultivated alfalfa is a self-incompatible autotetraploid (2n = 4x = 32) plant with a very complex genome that hinders efforts to genetically improve the varieties (Chen *et al.*, 2020). Due to the large number of imported varieties and long-term artificial selection, the genetic base of alfalfa tends to be narrow. Research on generation and utilization of haploid and double haploid plants has important significance for widening the genetic base of alfalfa and also for accelerating the breeding of improved varieties. Crossing with double haploid plants could greatly improve the heterozygosity of F1 generation and could contribute to increased yield, quality and resistance (Riday *et al.*, 2002). The whole genome of alfalfa (*Medicago sativa* L. 'XinJiangDaYe') was published and this knowledge together with the availability of double haploids could be of greater genetic significance for research on genetic transformation and to determine genetic origin.

Haploid plants were obtained effectively by combining high-frequency induction regeneration system with Flow cytometry (Galbraith *et al.*, 2001). With the development of very efficient physical and chemical doubling techniques, double haploid (DH) plants of many species have been successfully developed (Sharma *et al.*, 2019). However, cell polyploidy is a complex process, which is influenced by culture methods (solid and liquid), reagent selection for doubling, explant selection, penetrant, treatment time and culture conditions. Moreover, suitable doubling conditions do vary by species. So far, haploid and double haploid plants have been successfully obtained by tissue culture with several plants including: maize (Jiang *et al.*, 2015), tobacco (Schedel *et al.*, 2016), rice (Joann *et al.*, 2017) and others, but there were only a few reports of progress from tissue culture of alfalfa.

In the previous research, we obtained the haploid plants of alfalfa (*Medicago sativa* L. 'XinJiangDaYe'), and we propagated the haploid plants by using cuttings from tissue culture (Xu *et al.*, 2019; Cui Nan, 2017). In the current study, we aimed to determine: 1) which of the two double culture methods (solid and liquid) is better?; 2) which of the three explants (leaves, shoot tips and root tips) has the best outcome? and 3) what is the optimal condition for generating double haploid of alfalfa (*Medicago sativa* L. 'XinJiangDaYe')?

MATERIALS AND METHODS

The anther tissue culture and regeneration system was established using *Medicago sativa* L. 'XinJiangDaYe', which

College of Grassland, Resources and Environment, Inner Mongolia Agricultural University, Hohhot, China.

Corresponding Author: Fengling Shi, College of Grassland, Resources and Environment, Inner Mongolia Agricultural University, Hohhot, China. Email: sfl0000@126.com

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was planted in 2015 in the forage experimental field of Inner Mongolia Agricultural University Hohhot (37°282 N, 97°202 E) in Inner Mongolia, P.R. of China. The experiments on anther tissue culture and ploidy identification of regenerated plants were conducted at Key Laboratory of Grassland Resources, Ministry of Education P.R. of China. The experiment began in July 2017 and ended in December 2019.

Developing regenerated plants by anther tissue culture

In June 2017, the buds at the uninucleate eccentric stage (the bud length: 2~3mm) were selected according to Zagorska and Dimitrov (1995). The anothers were removed by dissection needle and placed in the callus induction medium (MS basal salts and vitamins, 1.0 mg/L 2,4-D, 0.5 mg/L 6-BA, 0.2 mg/L NAA, 3.0 mg/L kinetin, 30 g/L sucrose, 0.01 g/L activated carbon, pH 5.8). Dark culture for 60 days in the shaking bed at 25°C with a rotating speed of 110 rpm. After subculture for 20 days, all callus tissues were transferred to shoot induction medium (MS basal salts and vitamins, 1.5 mg/L 2,4-D, 1.0 mg/L 6-BA, 0.2 mg/L NAA, 20 g/L sucrose, 7 g/L agar, pH 5.8). After 30 days, the regenerated shoots were transferred to root induction medium (MS basal salts and vitamins, 0.1 mg/L NAA, 20 g/L sucrose, 7g/L agar, pH 5.8).

Ploidy identification of regenerated plants

The ploidy of regenerated plants from another culture was identified by Beckman coulter cytoFLEX at 488 nm (Loureiro *et al.*, 2006). The external standard control materials were the known ploidy seedlings of *M. ruthenica* (2n=2x=16) and *M.varia Martin* 'Caoyuan No.1' (2n=4x=32). In addition, the nuclear suspensions of the regenerated plants were prepared using LB01 according to Galbraith *et al* (1983).

Solid and liquid colchicine treatment medium for doubling

The colchicine treatment for doubling experiment started in January 2019. Using MS as the base medium, orthogonal tests were conducted on the three factors and four levels of colchicine concentration (Cc), osmotic Dimethyl sulfoxide (DMSO) concentration and treatment time (Tt) according to Wang *et al*. (2013). The gradient was set according to $L_{16}(4^3)$. 20 g/L sucrose was added into the liquid medium (pH 5.8) and dark culture was conducted in a shaking bed at 25°C with a rotating speed of 110 rpm. The solid medium had the same composition as the liquid, but 7g/L agar was added to solidify it. Explants identified from haploid plants were excised and placed in solid and liquid colchicine doubling treatment medium.

Improved culture of callus and regeneration culture of explants after doubling treatment

After colchicine treatment for doubling, the explants were rinsed with sterile water, dried with filter paper, and placed in the callus induction medium by the positive insert, and 10 explants per bottle were placed and this process was repeated 3 times. Most of the callus induced from the explants treated by colchicine were non-embryogenic. The two-factor random combination of 2,4-D (0.5, 1.0mg/L) and 6-BA (0, 0.5mg/L) was used to improve culture conditions (Li *et al.*, 2010). The improved callus underwent the same differentiation and rooting culture as above. Regenerated seedlings were used to identify the ploidy by using Beckman coulter cytoFLEX as described above.

Callus induction rate (%) =

$$\frac{\text{Number of callus produced}}{\text{Number of explants inoculated}} \times 100$$

Improvement rate (%) =

$$\frac{\text{Number of embryonic callus}}{\text{Number of callus inoculated}} \times 100$$

Statistical analyses

The callus induction rates of each orthogonal experiment were evaluated via analysis of variance (ANOVA, $P < 0.05$). The ploidy ratio of regenerated plants was calculated after doubling treatment.

RESULTS AND DISCUSSION

Ploidy identification of regenerated plants from another culture of alfalfa

The regeneration process of anther tissue culture is shown in Fig 1 (A to D). As indicated in Fig 2, there are two types of ploidy, tetraploids and diploids, in the regenerated plants. The main peak value of diploid plants is located in the linear graph of PE Channel 7 to 9 and the main peak value of tetraploid is located in 15 to 18. Among all the 76 regenerated plants, 18 haploids (23.7%) and 56 tetraploids (76.3%) were identified by Flow cytometry.

Effect of colchicine treatment on callus rate

Compared with the CK, the callus induction rate of shoot tip, leaf and root tip after solid treatment decreased by 43.46% to 81.24% (Table 1). The callus induction rate of the three explants decreased with the increase of colchicine concentration, DMSO concentration and treatment time. These results are consistent with the results from studies on *Miscanthus* and *Limonium bellidifolium* (Mori *et al.*, 2016; Katarzyna *et al.*, 2010). After liquid treatment, the callus induction rate of the three explants was reduced by 51.99 to 88.90%. Moreover, the callus induction rate of three explants treated with colchicine was compared, and the highest was obtained from leaf, followed by shoot tip and root tip. The callus induction rate of shoot tip or leaf was significantly higher than that of root tip ($P < 0.05$). This may be due to the fact that root tip is more sensitive to colchicine toxicity than the other two explants (Katarzyna *et al.*, 2010).

Improved culture of non-embryogenic callus

Callus could be induced after both solid and liquid doubling treatment, but most of them were non-embryonic callus. This may be due to the colchicine toxicity which had a negative effect on embryogenic callus formation and multiple shoots regenerated (Karen *et al.*, 2003; Ganga and Chezhiyan, 2002).

In our study, hormone combination improved the non-embryogenic callus (Table 2). After solid culture with colchicine treatment, the browning callus became yellow-green obviously (Fig 2 E and F) when MS medium was supplemented with 2,4-D 0.5 mg/L and 6-BA 0.5 mg/L. After colchicine liquid treatment, adding only 2,4-D at 0.5 mg/L was more advantageous to the formation of embryogenic callus. The improvement rate after colchicine solid treatment was 17.46 to 36.76% and that after liquid culture was 17.65 to 47.27%. Even if the non-embryogenic callus was improved, the number of regenerated plants was not necessarily large (Karen *et al.*, 2003; Ganga and Chezhiyan, 2002). The efficiency was found to be genotype-dependent, primarily due to differences in the ability to regenerate plants from embryogenic callus (Karen *et al.*, 2003).

Effects of solid and liquid culture on the doubling rate of alfalfa haploid

The doubling rate of the two culture methods were different (Fig 3, Table 3). Under the condition of colchicine solid doubling treatment, double haploid plants only accounted

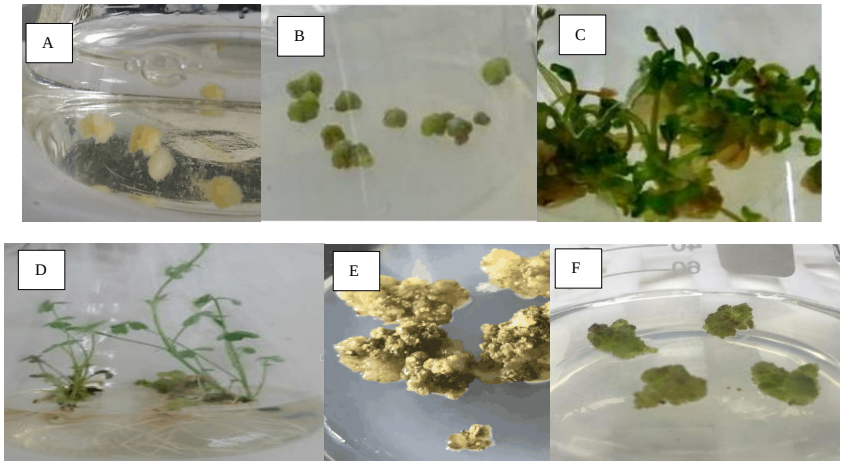

Fig 1: Anther tissue culture regeneration process and non-embryonic callus improvement of *Medicago sativa* L. 'XinJiangDaYe'. A- Callus induced by anther liquid culture; B- Callus after subculture; C- Regenerated shoots; D-Regenerated plants; E- Non-embryogenic callus induced by colchicine-treated explants; F- Embryogenic callus after improved.

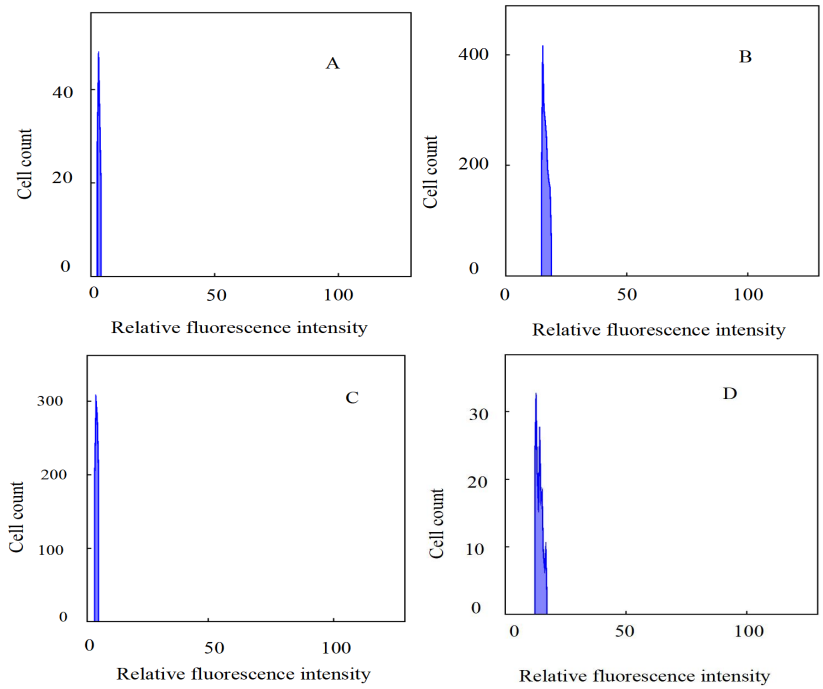

Fig 2: Flow cytometry identification map of another culture regenerated plants of *M. sativa* L. 'Xinjiangdaye'. A- *Medicago ruthenica* (2x=16); B- *Medicago varia* Martin. 'Caoyuan No.1' (2x=32); C- Haploid of *Medicago sativa* L.'Xinjiangdaye'(2x=16); D- Tetraploid of *Medicago sativa* L. 'Xinjiangdaye'(2x=32). When the position of the peak is consistent with the external standard control (A, B), the two materials have the same ploidy.

Table 1: Callus rate of stem tip, leaf and root tip after colchicine treatment.

Number	Cc/%	DMSO/%	Tt/h	Callus rate of solid culture/%			Callus rate of liquid culture/%		
				Shoot tip	Leaf	Root tip	Shoot tip	Leaf	Root tip
A1	0.05	0.5	12	76.67a	86.67a	23.33b	53.33b	56.67ab	6.67b
A2	0.05	1	24	36.67cd	33.33de	10.00cd	36.67c	33.33d	16.67a
A3	0.05	1.5	36	16.67de	43.33cd	0de	36.67c	46.67bc	6.67b
A4	0.05	2	48	26.67d	33.33de	6.67cd	30.00cd	33.33d	10.00ab
A5	0.1	0.5	24	46.67bc	46.67cd	3.33d	46.67bc	53.33b	3.33bc
A6	0.1	1	36	33.33cd	33.33de	3.33d	33.33cd	33.33d	3.33bc
A7	0.1	1.5	48	43.33c	50.00c	33.33a	26.67cd	33.33d	10.00ab
A8	0.1	2	12	53.33b	56.67bc	13.33c	46.67bc	43.33c	6.67b
A9	0.15	0.5	36	20.00de	20.00e	10.00cd	23.33d	16.67e	10.00ab
A10	0.15	1	48	23.33de	23.33e	23.33b	6.67e	3.33f	6.67b
A11	0.15	1.5	12	53.33bc	56.67bc	16.67bc	46.67bc	53.33b	6.67b
A12	0.15	2	24	53.33bc	53.33bc	3.33d	46.67bc	46.67bc	3.33bc
A13	0.2	0.5	48	13.33e	10.00f	13.33c	6.67e	6.67f	0c
A14	0.2	1	12	56.67b	63.33b	3.33d	66.67a	63.33a	3.33bc
A15	0.2	1.5	24	36.67cd	36.67d	3.33d	33.33cd	33.33d	3.33bc
A16	0.2	2	36	16.67de	16.67ef	3.33d	3.33e	6.67f	3.33bc
Mean value	-	-	-	37.92	41.46	10.63	33.96	35.21	6.25
CK	-	-		76.67	73.33	56.67	76.33	73.33	56.33

Cc-Colchicine; DMSO-Dimethyl sulfoxide; Tt-Treatment time; CK-Control check. Control check was the callus induction rate on the medium without add the colchicine and DMSO. Different letters in the same line indicate significant differences at 0.05 level based on Duncan's multiple range test.

Table 2: Effects of different subculture media on the improvement rate of non-embryonic callus.

Hormones		Solid culture			Liquid suspension culture		
2,4-D mg/L	6-BA mg/L	Number of callus inoculated	Number of embryonic callus	Improvement rate %	Number of callus inoculated	Number of embryonic callus	Improvement rate %
0.5	0	64	13	20.31	55	26	47.27
0.5	0.5	68	25	36.76	58	15	25.86
1	0	63	11	17.46	51	9	17.65
1	0.5	65	17	26.15	54	11	20.37

2,4-D - 2,4-Dichlorophenoxyacetic acid; 6-BA - 6-Benzylaminopurine..

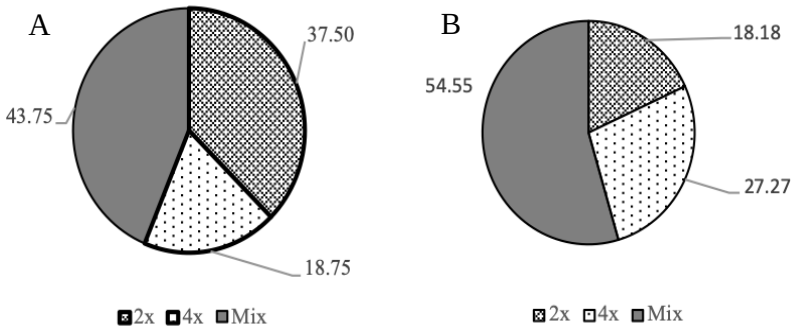

Fig 3: Effects of solid and liquid culture methods on doubling rate of alfalfa haploid. A indicates the ratio of ploidy on regenerated plants in a solid culture of colchicine; B indicates the ratio of ploidy of regenerated plants in liquid suspension culture of colchicine; 2x- Diploid; Mix- Mixoploid; 4x- Double haploid. The figures indicate the percentage of each ploidy plants.

Table 3: Ratio of ploidy of regenerated plants in two culture conditions.

Treatment	Explants	Regenerated plants/%	Diploid/%	Mixed ploidy/%	Double haploid/%
Solid culture	Shoot tip	43.75	12.50	25.00	6.25
	Leaf	50.00	18.75	18.75	12.50
	Root tip	6.25	6.25	-	-
Liquid suspension culture	Shoot tip	45.45	4.55	22.73	4.55
	Leaf	54.55	13.64	31.82	22.73
	Root tip	-	-	-	-

for 18.75% of the regenerated plants, and 37.50% of the regenerated plants did not have ploidy change, which were still diploid. Under the condition of colchicine suspension doubling treatment, the total ratio of ploidy changed percentage was 81.82%, the ratio of diploid was 27.28% lower than that of solid doubling treatment. In addition, the ratio of mixed ploidy increased by 24.69%. Double haploid plants accounted for 27.27% of regenerated plants, which is 45.44% higher than solid treatment. Colchicine and DMSO were added to the liquid medium, which was easier to penetrate the explants, and contributed to the improvement of the doubling rate (Currah and Ockendon, 1987). The doubling effect of colchicine liquid treatment was found to be better than that of solid medium treatment.

Effects of different explants on doubling rate

The ratio of each ploidy from three explants treated with colchicine was calculated. In both solid and liquid culture methods, at the explant level, the polyploidy efficiency of the leaves was the highest (Karen *et al.*, 2003). Although the number of regenerated plants was higher than the root tip, the proportion of double haploid was lower 6.25% to 18.18% than the leaf with the shoot tip as explants. When the root tip was used as explants, only 6.25% of the regenerated plants were obtained and all the plants were diploid without any change in ploidy. In general, the proportion of double haploid is low, most of which are either mixed ploidy or of original ploidy (James *et al.*, 1987). Similar results were reported from studies on cherry rootstocks (James *et al.*, 1987) and bananas (Ganga and Chezhiyan, 2002). Selection of suitable explants is a prerequisite for obtaining more double haploid plants, and in our study, the leaf as the explants had the best doubling effect.

The optimal doubling condition for alfalfa double haploid

In the medium treatment of double haploid plants obtained of this study, medium A7 and medium A6 were combined with low concentration for a long time, medium A15 was treated with high concentration for a short time. Considering the time and doubling rate of the test process, A15 was found to be the most appropriate treatment. Colchicine concentration and treatment time are the two most important and direct factors which have a certain correlation, and most of the results of doubling treatment study used were either low concentration for a long time or high concentration for a short time (Sharma *et al.*, 2019). Likewise, the addition of DMSO, a penetration aid, would also be beneficial to the doubling effect, which can reduce the toxic effect of colchicine and increase the rate of generation of double haploid (Currah and Ockendon, 1987).

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