



Establishment of Tissue Culture Regeneration System for *Medicago ruthenica* L. cv. 'Zhilixing'

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ABSTRACT

Background: *Medicago ruthenica* L. 'Zhilixing' is a new variety with superior forage and seed yield compared to the wild type. The cold, drought and salt tolerance of Zhilixing are better than those of alfalfa, suggesting that this variety can serve as a high-quality genetic resource for improving the stress resistance of alfalfa. However, because of the lack of tissue culture regeneration system, it is difficult to perform genetic transformation studies on stress resistance genes. This study aimed to establish an efficient tissue culture regeneration system for Zhilixing variety.

Methods: Three types of explants were selected and tested on four types of basal media supplemented with different combinations of auxin and cytokinin for callus induction and differentiation, based on orthogonal tests to select the combinations of auxin and cytokinin suitable for callus induction and differentiation. Two-factor combination method was used to formulate a suitable rooting medium.

Result: The hypocotyledonary axis was found to be an excellent explant for callus induction on MS medium. The optimum callus induction medium contained thidiazuron (TDZ, 0.5 mg/L), 2,4-dichlorophenoxyacetic acid (2,4-D, 1.0 mg/L) and naphthaleneacetic acid (NAA, 0.5 mg/L) where the callus induction rate was 93.33%. The differentiation medium was supplemented with TDZ (0.75 mg/L), 2,4-D (0.25 mg/L) and 6-benzyladenine (6-BA, 1.5 mg/L) where the differentiation rate was 63.33 %. Thidiazuron played the key role in both processes of callus induction and differentiation. Half-strength MS containing 0.1 mg/L of NAA was the most efficient rooting medium.

Key words: Callus, Differentiation, *Medicago ruthenica*, TDZ, Tissue culture.

INTRODUCTION

Medicago ruthenica is a perennial forage plant of the Leguminosae that is widely distributed in high mountains (Balabaev, 1934) and typical and desertified grasslands in northern China (Shu *et al.*, 2018). It has a great potential for use in many fields such as grassland improvement, ecological management and grass industry development. The nutrient value utilization efficiency for *Medicago ruthenica* has been reported to be higher compared with alfalfa (Wu *et al.*, 2020). The cold and drought resistance as well as salt and alkali tolerance were reported to be better than those of alfalfa, but the yield was lower (Yang *et al.*, 2011). Because of its high tolerance to various extreme environmental conditions (Campbell *et al.*, 1999; Guan *et al.*, 2009), *Medicago ruthenica* can be used as a high-quality genetic resource for improving the stress resistance of alfalfa and other pastures.

In recent years, research on *Medicago ruthenica* mainly focused on the responses to abiotic stress (Shu *et al.*, 2018), evaluation and analysis of germplasm genetic diversity (Wang *et al.*, 2020) and screening, cloning and functional verification of resistance genes (Yin *et al.*, 2021; Liu, 2020). The functional verification of *Medicago ruthenica* resistance genes was mainly carried out on model plants. Reports on gene over-expression or knockout in *Medicago ruthenica* are scarce if at all, mainly because a genetic transformation and regeneration system for *Medicago ruthenica* is yet to be established.

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Successful genetic transformation requires an efficient tissue culture protocol (Mehmet *et al.*, 2021). The regeneration process from tissue culture is very complex and is affected by many factors, such as basal media, types and genotypes of explants, growth conditions and types and combinations of phytohormones (Xu *et al.*, 2021). Currently, the tissue culture of *Medicago ruthenica* traditionally utilizes mature embryos, stems, leaves and hypocotyledonary axis as explants to produce calli capable of regenerating plantlets (Bian *et al.*, 2009; Zhang *et al.*, 2009; Jin *et al.*, 1997; Liu *et al.*, 1990). However, it is costly and time consuming to cultivate donor plants to obtain mature embryos, stem and leaf explants (Mehmet *et al.*, 2021) and the results of the reported studies have shown that callus induction rates were less than 50% (Tao *et al.*, 2011). In a previous experiment,

we used hypocotyledonary axis as an explant to produce callus and the callus induction rate was 91%, but most of the calli were non-embryogenic. The differentiation rate was only 15% and few shoots generated roots (Bian *et al.*, 2009). In view of the above information, it is necessary to establish an efficient tissue culture regeneration system for *Medicago ruthenica*.

In China, multiple mixed selection methods were used to breed the new variety hereafter named as *Medicago ruthenica* L. cv. 'Zhilixing'. Compared with the wild type, the new variety has an upright plant type, which is easier to harvest and has higher forage and seed yield. We plan for future in-depth research on the screening of resistance genes and genetic transformation of this variety. The whole genome of *Medicago ruthenica* has been published and this information together with the availability of a regeneration system could be of great significance for research on genetic transformation (Yin *et al.*, 2021). Therefore, in this study, three types of explants were selected and tested on four types of basal media for callus induction and plantlet regeneration. We adopted orthogonal experiments to select different combinations of auxins and cytokinins/mitogens, aiming to establish a complete and efficient tissue culture regeneration system of *Medicago ruthenica* L. cv. 'Zhilixing' and lay basis for research on its genetic transformation.

MATERIALS AND METHODS

The experiment was conducted *rabi* session of 2019-11 and 2020-7 at the research farm of Inner Mongolia Agricultural University, Hohhot in Inner Mongolia, China. The tissue culture and regeneration system was established using *Medicago ruthenica* L. cv. Zhilixing. The plants were grown to maturity and seeds were collected.

Preparation and culturing of explants

The seeds were soaked in H_2SO_4 for 7 min to break the hard coats, rinsed with running water for 30 min. The seeds were then sterilized with 75% ethanol for 1 min and rinsed with sterile water three times and cultured into bottles (ten seeds per bottle) containing MS, B5, N6, or Nitsch basal media. After 5-7 days, the cotyledons, hypocotyledonary axes and roots were separated where the length of each explant was 3-5 mm. The explants were inoculated onto the same basal media used for germination.

Callus induction

Based on 3-factor and 4-level orthogonal design, different concentrations (0 mg/L, 0.25 mg/L, 0.5 mg/L, 0.75 mg/L) of TDZ, 2,4-D and NAA as well as sucrose (20.0 g/L) and agar (6.0 g/L) were used to formulate the callus induction media. The pH of all media was set at 5.8. The gradient was set according to $L_{16}(4^3)$. Each culture bottle was inoculated with five explants and each treatment was repeated six times. The callus induction rate was determined after 20 days.

Callus differentiation

The callus were transferred to differentiation media after a sub-generation culture (25 days). According to 3-factor and 4-level orthogonal design, the regeneration media contained different concentrations of TDZ (0 mg/L, 0.25 mg/L, 0.5 mg/L, 0.75 mg/L), 2,4-D (0 mg/L, 0.25 mg/L, 0.5 mg/L, 0.75 mg/L) and 6-BA (0.5 mg/L, 1 mg/L, 1.5 mg/L, 2 mg/L), hydrolyzed casein (0.5 g/L), sucrose (20.0 g/L) and agar (7.0 g/L). The pH of all media was set to 5.8. Each culture bottle was inoculated with five calli. Each treatment included five replicates and the differentiation rates were analyzed after 15-20 days.

Rooting of shoots

The generated shoots were cultured onto the rooting media. The basic medium was either MS or half-strength MS supplemented with NAA (0 mg/L, 0.1 mg/L or 0.2 mg/L), sucrose (20.0 g/L) and agar (7.0 g/L). The pH of all media was adjusted to 5.8. Each culture bottle was inoculated with three differentiated shoots. Each treatment included five replicates and the rooting rate was determined after 15 days. The regenerated complete plantlets were transferred to the field.

Statistical analyses

The callus induction and differentiation rates as well as shoot rooting rates were analyzed *via* analysis of variance (ANOVA, $p < 0.05$).

Callus induction rate (%) =

$$\frac{\text{Number of calli induced}}{\text{Number of explants inoculated}} \times 100\%$$

Differentiation rate (%) =

$$\frac{\text{Number of differentiated calli}}{\text{Number of inoculated calli}} \times 100\%$$

Rooting rate (%) =

$$\frac{\text{Number of rooted shoots}}{\text{Number of inoculated shoots}} \times 100\%$$

RESULTS AND DISCUSSION

Selection of suitable basal medium and explant

The growth and differentiation of calli were significantly affected by genotypes and explants (Alatar *et al.*, 2017). In four kinds of basal media without hormones, the three kinds of explants induced calli. Among them, the hypocotyledonary axes showed the highest callus induction rate, followed by the cotyledons and roots (Fig 1). When the root was used as an explant, it was almost difficult to produce calli on the four basal media, in agreement with the previously reported results in wheat (Mehmet *et al.*, 2021; Sarker and Biswas, 2002). The hypocotyledonary axis had the highest callus induction rate on MS medium (over 30%), followed by B5 (20%), N6 (16.7%) and then Nitsch (10%). Thus, MS basal medium was recommended for follow-up research (Alatar *et al.*, 2017; Bian *et al.*, 2009).

The effect of different hormone concentrations on callus induction rate

Cytokinin and auxin play vital roles in the induction and differentiation of calli (Verma *et al.*, 2016). However, attention should be paid to calli induction rate and also to the state of calli, so as to increase the probability of formation of embryogenic calli (Liu *et al.*, 2016). As shown in Table 1, A10, A11 and A12 culture media induced compact embryogenic calli with light yellow or green color (Fig 2-A) where the callus induction rate was higher than on other media. Although A7 showed a high callus induction rate, the calli were almost non-embryogenic. In general, the optimum medium for callus induction contained TDZ (0.5 mg/L), 2,4-D (1.0 mg/L) and NAA (0.5 mg/L) (A7), where the rate of callus induction was 93.33%.

TDZ plays the key role in callus induction

When TDZ was omitted from the media (A1 through A4), the callus induction rates were relatively low (33.33%-40%) and most of the calli were non-embryogenic. In our previous research, TDZ was not used for inducing calli, which may have been one of the reasons for the low differentiation rates reported (Bian *et al.*, 2009). The present study shows that calli derived from hypocotyledonary axes were stimulated by TDZ to regenerate shoots (Miroshnichenko *et al.*, 2017). Compared with the previous study, TDZ obviously improved the development of embryogenic calli (Bian *et al.*, 2009; Ruduś *et al.*, 2001). The results of the range analysis (Table 3) also proved that TDZ played the most crucial role in callus induction from the hypocotyledonary axes (Aslam *et al.*, 2020).

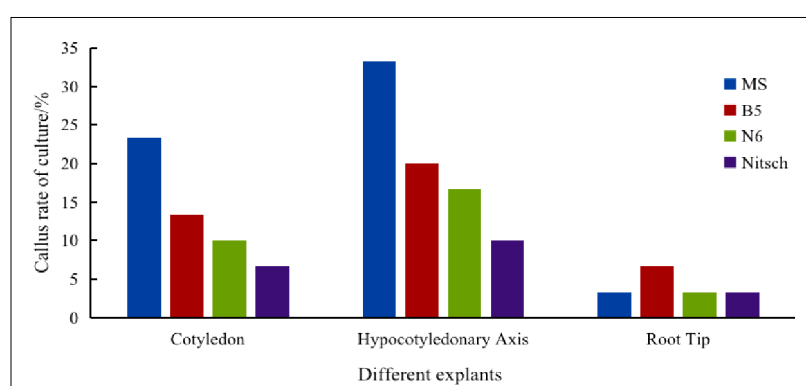


Fig 1: The callus rate of different explants in 4 kinds of basic medium.

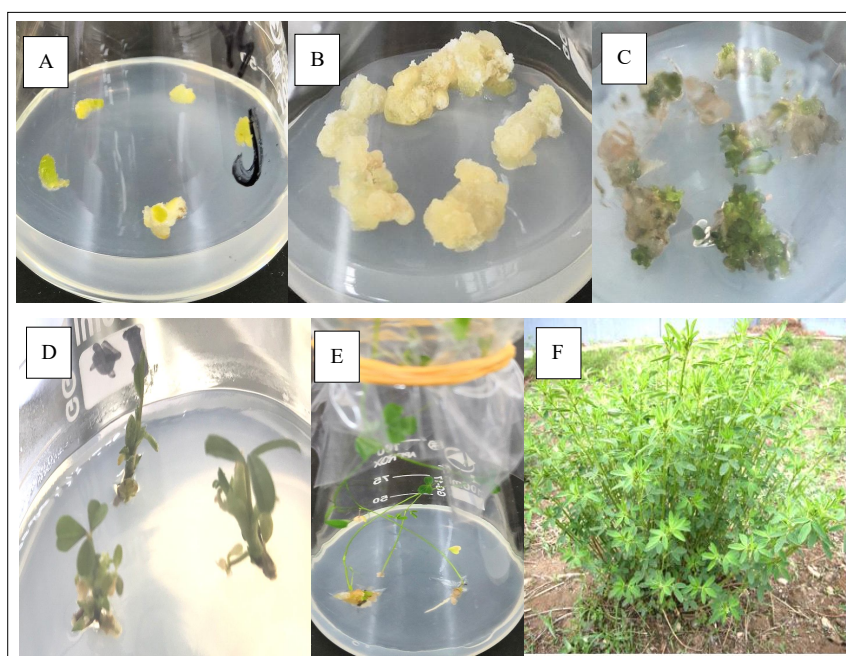


Fig 2: Anther tissue culture regeneration process of *Medicago ruthenica* L. 'Zhilixing'.

A- Callus induced by hypocotyledonary axis; B- Callus after subculture; C- Callus differentiation; D- Differentiated seedlings;

E- Regenerated plants; F- Live plants after transplanted.

Table 1: Callus induction rate of the hypocotyledonary axes cultured on different hormone concentrations.

Number	TDZ (mg.L ⁻¹)	2,4-D (mg.L ⁻¹)	NAA (mg.L ⁻¹)	Callus induction rate (%)	Callus appearance
A1	0	0	0	36.67ef	Loose, brownness
A2	0	0.25	0.25	33.33f	Loose, slight green
A3	0	0.5	0.5	36.67ef	Loose, light yellow
A4	0	0.75	0.75	40.00def	Loose, green with slight brownness
A5	0.25	0	0.25	46.67cdef	Compact, light brownness
A6	0.25	0.25	0.5	50.00cde	Compact, slight green
A7	0.25	0.5	0.75	60.00c	Loose, light brownness
A8	0.25	0.75	0	50.00cde	Loose, light yellow
A9	0.5	0	0.5	53.33cd	Loose, light brownness
A10	0.5	0.25	0.75	76.67b	Compact, light yellow
A11	0.5	0.5	0	60.00c	Compact, light green
A12	0.5	0.75	0.25	93.33a	Compact, light yellow
A13	0.75	0	0.75	36.67ef	Loose, light yellow
A14	0.75	0.25	0	50.00cde	Loose, green
A15	0.75	0.5	0.25	50.00cde	Loose, green
A16	0.75	0.75	0.5	50.00cde	Loose, light yellow

TDZ- Thidiazuron; 2,4-D - 2,4-Dichlorophenoxyacetic acid; NAA- Naphthylacetic acid.

Table 2: Callus differentiation rate of *Medicago ruthenica* L. cv. Zhilixing under different hormone ratio.

Number	TDZ (mg.L ⁻¹)	2,4-D (mg.L ⁻¹)	6-BA (mg.L ⁻¹)	Differentiation rate (%)
B1	0.25	0.25	0.5	43.33b
B2	0.25	0.5	1	13.33def
B3	0.25	0.75	1.5	10.00def
B4	0.25	1	2	16.67cde
B5	0.5	0.25	0.5	20.00cd
B6	0.5	0.5	1	26.67c
B7	0.5	0.75	1.1	50.00b
B8	0.5	1	0.5	43.33b
B9	0.75	0.25	1.5	63.33a
B10	0.75	0.5	2	20.00cd
B11	0.75	0.75	0.5	46.67b
B12	0.75	1	1	40.00b
B13	1	0.25	2	3.33f
B14	1	0.5	0.5	6.67ef
B15	1	0.75	1	10.00def
B16	1	1	1.5	10.00def

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

The effect of different hormone concentrations on callus differentiation

Plant growth regulators play the key role in callus differentiation (Takatsuka and Umeda, 2014). Depending on the genotype, a regeneration system for a given species requires a specific combination of auxin and cytokinin (Mehtab *et al.*, 2020). In this study, the optimum concentration of hormones was TDZ (0.75 mg/L), 2,4-D (0.25 mg/L) and 6-BA (1.5 mg/L) (B9) where the differentiation rate was 63.33% (Table 2). Indeed, TDZ again determined the callus differentiation rate (Table 3). When the concentration of TDZ was 0.75 mg/L, a large number of differentiated shoots was obtained, but higher concentration (1 mg/L) caused the calli to lose viability, lose the ability to differentiate or even die. This suggests that TDZ was an effective plant growth regulator for *Medicago ruthenica* regeneration (Mehmet *et al.*, 2021). Furthermore, range analysis showed that 2,4-D and 6-BA also played roles in the differentiation process (Table 3). Addition of 2,4-D has been reported to promote the differentiation of calli and indirectly increase the differentiation rate (Mahmood and Razzaq, 2017). Appropriate levels of 6-BA have also been reported to reduce the browning rate and effectively improve the quality of calli (Xu *et al.*, 2021).

Table 3: Range analysis of callus induction and differentiation rates.

Induction rate of callus	TDZ	2,4-D	NAA	Differentiation rate	TDZ	2,4-D	6-BA
K1 mean value	0.37	0.43	0.48	K1 mean value	0.21	0.33	0.35
K2 mean value	0.51	0.53	0.55	K2 mean value	0.35	0.17	0.21
K3 mean value	0.71	0.51	0.48	K3 mean value	0.43	0.29	0.28
K4 mean value	0.46	0.58	0.53	K4 mean value	0.08	0.28	0.23
R	0.34	0.14	0.08	R	0.35	0.16	0.14

TDZ- Thidiazuron; 2,4-D- 2,4-Dichlorophenoxyacetic acid; NAA- Naphthylacetic acid; 6-BA- 6-Benzylaminopurine. K1, K2, K3 and K4 mean values were induction rate of callus and differentiation rate of three factors in every level. R was the range of the same factor in every level ($R = K_{\max} - K_{\min}$).

Table 4: Comparison of callus rooting rate of *Medicago ruthenica* L. cv. Zhilixing.

Number	Basal medium	NAA (mg.L ⁻¹)	Rooting rate (%)
C1	MS	0	46.6cd
C2	MS	0.1	53.4bcd
C3	MS	0.2	33.0d
C4	1/2MS	0	73.6b
C5	1/2MS	0.1	93.4a
C6	1/2MS	0.2	60.2bc

NAA- Naphthylacetic acid.

Effect of different types of media and hormone concentrations on the rooting of shoots

The rooting rate of differentiated shoots on half-strength MS basal medium supplemented with 0.1 mg/L NAA was significantly higher than these on C4 and C5 ($p < 0.05$, Table 4). Similar results have been obtained for alfalfa (Xu *et al.*, 2021). Adding a low concentration of NAA to the rooting medium was supportive to the rooting of differentiated shoots, but excessive NAA inhibited the rooting process (Liu *et al.*, 2016). After the rooted seedlings were cultured for 20 days, they were transplanted into soil where the survival rate was more than 90%.

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