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Establishment of a Regeneration System for Wild Alfalfa in Alxa, China

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ABSTRACT

Background: The Alxa Region in Inner Mongolia, northwest China is a desert grassland area. Wild alfalfa growing in this area is a precious wild plant resource that can provide resources for improving the stress resistance of alfalfa varieties. Alfalfa is a cross-pollinated plant and its genes are commonly heterozygous. Therefore, establishing a complete tissue culture regeneration system for a single plant is an effective method of protecting wild plant resources. This study used the bud and leaf of wild alfalfa from the Alxa Region as explants to establish an efficient and stable tissue culture regeneration system.

Methods: Two kinds of wild alfalfa tissues were selected as explants. Different concentrations and types of plant growth hormones and regulators were tested, to determine the optimal medium for callus induction, differentiation and rooting culture, including 6-Benzylaminopurine (6-BA), Kinetin (KT), 2, 4-Dichlorophenoxyacetic acid (2, 4-D), Naphylacetic acid (NAA), Indole-3-butyric acid (IBA) and activated carbon (AC).

Result: The callus induction rate was 93.3% when the bud of wild alfalfa was used as an explant for propagation. With a basic medium (Murashige and Skoog medium with Ca^{2+}, Mg^{2+} and Mn^{2+} halved) and hormone regulator (6-BA, KT, NAA, AC), the differentiation rate and rooting rate reached as high as 66.67% and 93.33%, respectively. When leaves were used as explants, the callus induction rate and differentiation rate were 100% and 73.33%, respectively. In the rooting culture process, adding IBA (0.2 mg/L) and AC (0.5 mg/L) to the medium improved the rooting rate of wild alfalfa. Both buds and leaves can be used as explants for plant tissue regeneration and culture, which can help expand and preserve wild alfalfa resources.

Key words: Callus, Differentiation, Explant, Root, Wild alfalfa.

INTRODUCTION

As a perennial legume forage with many excellent characteristics such as high yield and nutritional value, Alfalfa is known as the “king of forage” and is widely cultivated around the world (Markovi *et al*., 2019). However, in the context of global climate change, abiotic stresses such as land salinisation and frequent extreme weather events seriously restrict the planting and development of Alfalfa (Geng, 1995). In China, Alfalfa cultivation is mainly concentrated in the northern mountains and grassland areas. However, in recent years, the aggravation of drought and salinisation caused by climate change has restricted alfalfa cultivation (Shu *et al*., 2018). The Alxa Region in Inner Mongolia is a typical desert grassland area with a dry climate and little rain all year round. The wild Alfalfa growing here is adapted to the climate of this area (Baoler, 2021). Therefore, wild alfalfa varieties can be used as high-quality planting materials for breeding alfalfa varieties and can provide resources to improve the stress resistance of cultivated Alfalfa.

Alfalfa is a tetraploid plant and cross-pollinated, so its offspring are prone to heterozygosity, resulting in the separation of its excellent phenotypic traits. The use of plant tissue culture technology can maintain the purity of varieties and maximise their potential breeding value. Establishing a stable and efficient tissue culture regeneration system is critical to enable utilisation of stress-resistant genes in wild plants and cultivation of new stress-resistant alfalfa varieties (Zhao *et al*., 2020). The tissue culture regeneration process is very complex and is affected by many factors, such as the basic medium, the type and genotype of explants, growth conditions and the type and combination of plant hormones used (Xu *et al*., 2021). Traditionally, tissue culture of Alfalfa uses mature embryos, stems, leaves and hypocotyls as explants to produce calli that can regenerate plants (Bian *et al*., 2009; Zhang *et al*., 2009; Liu *et al*., 1990). Tissue culture regeneration systems have been established for many varieties of alfalfa, including the salt- and alkali-resistant Zhongmu series, cold-resistant Aohan alfalfa, high-yield Xinjiangdaye alfalfa, the Longmu series, Baoding alfalfa and grassland series alfalfa which is suitable in Inner

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Mongolia (Zhang *et al*., 2008; Zhang *et al*., 2013; Yang *et al*., 2004; Ma *et al*., 2008).

In order to better preserve and propagate the wild Alxa alfalfa more efficiently, a variety of explants were used to establish a tissue culture regeneration system. Different concentrations of hormones were tested to establish a complete and efficient tissue culture regeneration system as a foundation for preserving varietal diversity and stabilising genetic material.

MATERIALS AND METHODS

The experiment was conducted in the dry seasons from July 2020 to November 2021 at the research farm of the Inner Mongolia Agricultural University, Hohhot, Inner Mongolia, China. The tissue culture regeneration system was established using leaves and buds of wild alfalfa plants obtained from the Alxa Desert Grassland, Inner Mongolia.

Preparation and culturing of explants

Leaves and buds of wild Alfalfa were rinsed with running water for 1 hour. The explants were then sterilised with 75% ethanol for 30 s and rinsed with sterile water three times. They were then rinsed with 0.1% HgCl for 3 min, after which they were again rinsed with sterile water three times. Leaves and buds were separated and each explant was cut to a length of 3 mm. The explants were inoculated onto the same basal media used for germination.

Callus induction

The callus induction media were formulated as MS medium modified with different concentrations of 6-Benzylaminopurine (6-BA; 0.1 mg/L, 0.2 mg/L, 0.3 mg/L) and 2, 4-Dichlorophenoxyacetic acid (2, 4-D; 0.5 mg/L, 0.75 mg/L). The MS medium comprised 0.1-0.5 mg/L of Kinetin (KT), 0.1 mg/L of Naphylacetic acid (NAA), 0.5 mg/L or 0.75 mg/L of activated carbon (AC), sucrose (30.0 g/L) and agar (8.0 g/L). The pH of all media was set at 5.8. Each culture bottle was inoculated with five explants and each treatment was repeated six times. The callus induction rate was determined after 16 days.

Callus differentiation

After a sub-generation culture, the callus was transferred to differentiation media (25 days). The regeneration media contained different concentrations of MS (MS, MS with Ca^{2+}, Mn^{2+} and Mg^{2+} halved), KT (0.05 mg /L, 0.1 mg/L, 0.2 mg/L), 6-BA (0.2 mg/L, 0.25 mg/L), NAA (0.05 mg/L, 0.1 mg/L), sucrose (30.0 g/L) and agar (8.0 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 analysed after 15-20 days.

Rooting of shoots

The generated shoots were cultured onto rooting media. The basic medium was either half-strength MS or half-strength MS (Ca^{2+} and Mg^{2+} halved) supplemented with NAA (0.1 mg/L), indole-3-butyric acid (IBA) (0.2 mg/L), 6-BA (0.25 mg/L, 0.5 mg/L), AC (0.5 mg/L, 0.75 mg/L), sucrose (20.0 g/L) and agar (5.5 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 analysis

The callus induction and differentiation rates, as well as shoot rooting rates, were analysed using analysis of variance (ANOVA, $p< 0.05$). The following evaluation indicators were calculated (Xu *et al*., 2022):

$$\text{Callus induction rate (\%)} = \frac{\text{Number of calli induced}}{\text{Number of explants inoculated}} \times 100$$

$$\text{Differentiation rate (\%)} = \frac{\text{Number of differentiated calli}}{\text{Number of inoculated calli}} \times 100$$

$$\text{Rooting rate (\%)} = \frac{\text{Number of rooted shoots}}{\text{Number of inoculated shoots}} \times 100$$

RESULTS AND DISCUSSION

Selection of suitable explant

Different physiological states and regeneration functions of different plant parts affect their ability to form complete plants; thus, the selection of explants directly affects the process of plant dedifferentiation and redifferentiation (Zhao *et al*., 2013). In the study of Alfalfa, the main explants are roots, stems, leaves, cotyledons and hypocotyls (Weeks *et al*., 2008; Xu *et al*., 2015; Bai *et al*., 2010). This study found that the bud of Alfalfa can also be used as an explant. When it is necessary to propagate from limited materials, the bud is also an option and our study found that healing rate when using buds as explants can reach 93.33%, which was not significantly different from the induced healing rate when using leaves as explants ($P>0.05$).

The effects of different hormone ratios on the healing rate of different explants

Cytokinins and auxins play an important role in callus induction and differentiation. Different explants choose different cytokinins and auxins for callus induction (Xiao *et al*., 2007; Wang *et al*., 2010; Li *et al*., 2009). In previous studies, the combination of auxin 2, 4-D and cytokinin KT has been considered to be conducive to the formation of alfalfa callus, while the combination of cytokinin 6-BA and KT is conducive to the induction of callus differentiation, with concentration ranges of 2, 4-D at 1.0-3.0 mg/L, KT concentration at 0.1-1.0 mg/L and 6-BA concentration at 0.5-1.0 mg/L. Studies have also shown that different alfalfa varieties have different sensitivities to these three hormones during callus formation and differentiation (Bai *et al*., 2010; Xu *et al*., 2015; Week *et al*., 2008). In this study, cytokinin 6-BA and auxin (2, 4-D and NAA) were added when buds were used as explants for callus induction. We also found that an appropriate amount of AC added during callus

induction (0.5 mg/L) increased the induction rate of embryogenic callus. As shown in Table 1, the callus induced under the A3 hormone ratio had the highest healing rate and the callus was mainly yellow-green with many differentiation points. When the callus was induced with leaves as explants, the cytokinins and growth factors added were slightly different from those when buds were used as explants and only 2,4-D and KT were added. The callus induced using the A8 hormone ratio had the highest healing rate and most of the embryogenic calli were green (Fig 1A and B).

The effects of different basal media and different hormone ratios on callus differentiation

Plant parts vary in their regeneration ability and the accumulated growth stimulants and physiologically active substances also differ (Gallego *et al.*, 2001; Zare *et al.*, 2009; Barbulova *et al.*, 2002). Therefore, the basic medium used in inducing callus differentiation will differ, as will the contents of ions and trace elements. In this experiment, when the contents of Ca^{2+}, Mg^{2+} and Mn^{2+} were halved and appropriate cytokinins (6-BA and KT) and auxin (NAA) were added to the medium, the highest differentiation rate was 66.67%. When callus differentiation was induced from leaves, the best performing differentiation medium formula was the basic medium MS + 6-BA (0.5 mg/L) + KT (0.9 mg/L), with a differentiation rate of 73.33% (Table 2). This study showed that the flower organs of plants can also be used as explants for tissue culture to expand and propagate wild plants and protect their own preferred characteristics from being heterozygous. However, there may be differences in the types and contents of the basic medium and added hormones required to induce callus differentiation because the substances stored in buds are different from those in leaves (Fig 1C, D and E).

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

When rooting and culturing the plantlets induced by the two

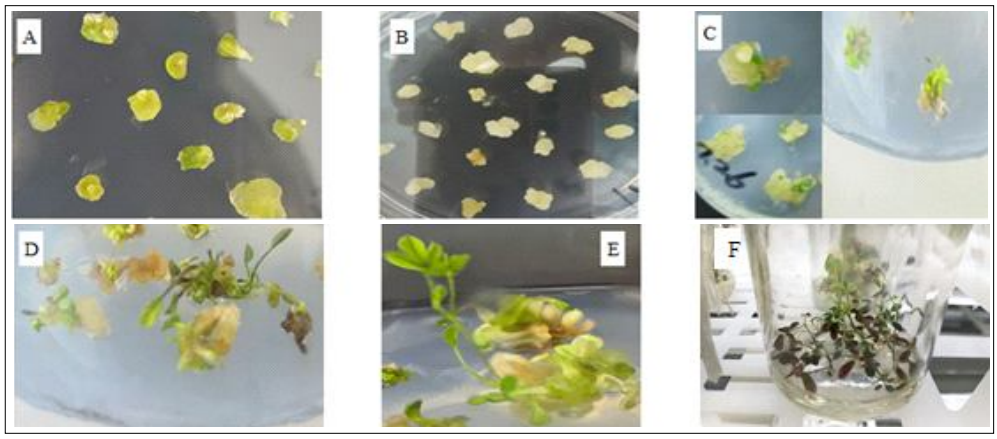

Fig 1: Wild alfalfa tissue culture regeneration process.
A: Callus induced by hypocotyledonary axis; B: Callus after subculture; C: Callus differentiation; D and E: Differentiated seedlings; F: Regenerated plants.

Table 1: Callus induction rate of different hormone concentrations and explants.

Number	Explant	6-BA ($mg*L^{-1}$)	2,4-D ($mg*L^{-1}$)	KT ($mg*L^{-1}$)	AC ($mg*L^{-1}$)	NAA ($mg*L^{-1}$)	Callus induction rate (%)
A1	Bud	0.1	0.5	0	0.5	0.1	33.33±1.01c
A2		0.2	0.75	0	0.5	0.1	40±7.22b
A3		0.2	0.5	0	0.5	0.1	93.33±9.62a
A4		0.1	0.5	0	0.75	0.1	53.33±4.45b
A5		0.3	0.5	0	0.75	0.1	13.33±0.22d
A6	leaf	0	0	0.2	0	0	26.67±1.42d
A7		0	0.5	0.2	0	0	33.33±1.14c
A8		0	1.0	0.2	0	0	100±0.00a
A9		0	1.5	0.2	0	0	53.33±1.81b
A10		0	2.0	0.2	0	0	13.33±1.31e
A11		0	1.0	0.1	0	0	20±1.34d
A12		0	1.0	0.3	0	0	53.33±4.06b
A13		0	1.0	0.4	0	0	40±3.14c
A14		0	1.0	0.5	0	0	26.67±2.78d

2, 4-D: 2, 4-Dichlorophenoxyacetic acid; NAA: Naphthylacetic acid; 6-BA: 6-Benzylaminopurine; KT: Kinetin. AC: Activated carbon.

Table 2: Callus differentiation rate of wild Alfalfa under different hormone ratios and basic media.

Number	Explant	Basic medium	6-BA (mg*L-1)	KT (mg*L-1)	NAA (mg*L-1)	Differentiation rate (%)
B1	Bud	MS (reformed)	0.2	0.05	0.05	13.33±1.85e
B2		MS (reformed)	0.2	0.1	0.1	66.67±4.67a
B3		MS (reformed)	0.25	0.2	0.1	33.33±2.47c
B4		MS	0.2	0.05	0.05	20±1.54d
B5		MS	0.2	0.1	0.1	40±2.41b
B6		MS	0.25	0.2	0.1	26.67±1.06d
B7	leaf	MS	0.5	0.6	0	20±2.37d
B8		MS	0.5	0.7	0	33.33±2.87c
B9		MS	0.5	0.8	0	53.33±5.92b
B10		MS	0.5	0.9	0	73.33±6.63a
B11		MS	0.5	1	0	40±3.81b

NAA: Naphthylacetic acid; 6-BA: 6-Benzylaminopurine; KT: Kinetin.

Table 3: Comparison of callus rooting rate of wild alfalfa.

Number	Explant	Basic medium	6-BA (mg*L-1)	NAA (mg*L-1)	IBA (mg*L-1)	AC (mg*L-1)	Rooting rate (%)
C1	Bud	1/2MS (reformed)	0.25	0	0	0.5	13.33±0.54d
C2		1/2MS (reformed)	0.5	0	0	0.75	33.33±2.31b
C3		1/2MS (reformed)	0.5	0	0	0.5	93.33±8.21a
C4		1/2MS	0.25	0	0	0.5	20±1.13c
C5		1/2MS	0.5	0	0	0.75	13.33±1.02d
C6		1/2MS	0.5	0	0	0.5	33.33±2.54b
C7	leaf	1/2MS	0	0.1	0	0	20±3.63c
C8		1/2MS	0	0.1	0.2	0	53.33±4.85b
C9		MS	0	0.1	0.2	0	86.67±7.65a

NAA: Naphthylacetic acid; 6-BA: 6-Benzylaminopurine; IBA: Indole-3-butyric acid; AC: Activated carbon.

explants, different basic media were used for culture. The best basic medium for rooting culture of regenerated seedlings cultivated from flower-bud explants was 1/2MS, with the content of Ca^{2+} and Mg^{2+} halved. When cytokinin 6-BA (0.5 mg/L) and activated carbon (0.5 mg/L) were added to this medium, the rooting rate reached 93.33%. For the rooting of regenerated seedlings cultivated from leaf explants, the best-performing basic medium was MS. The hormones added were NAA (0.1 mg/L) and IBA (0.2 mg/L) and a rooting rate of 86.67% was achieved (Table 3, Fig 1F). After 25 days of rooting seedling culture, the plantlets could be transplanted into soil and the survival rate exceeded 95%.

CONCLUSION

In the tissue culture regeneration system established with the buds and leaves of wild Alxa alfalfa as explants, the best callus induction rate, differentiation rate and rooting rate were 100%, 73.33% and 93.33% respectively. Properly changing the content of Ca^{2+}, Mg^{2+} and Mn^{2+} in the basic medium and adding IBA and AC can improve the differentiation rate and rooting rate of callus. The results showed that both bud and leaf could be selected as explants for plant tissue regeneration andculture when protecting and expanding Wild Alfalfa varieties.

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Conflict of interest: None.

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