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Construction of Tissue Culture System of *Onobrychis viciaefolia* Scop 'Mengnong' Anthers

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ABSTRACT

Background: To accelerate the establishment of a genetic improvement system for *Onobrychis viciaefolia* Scop. by cultivating haploid plants and producing pure parental material.

Methods: We endeavored to set up the *Onobrychis viciaefolia* Scop. anther culture system, examine the suitable anther pre-treatment method and the factors influencing medium type and hormone ratio during anther regeneration.

Result: The regenerative capacity of intact anthers was preserved by 48 h low temperature (4°C) medium treatment. Or the broken anthers were obtained when the regenerated grass was budding and preserved in the medium after 48 hours of low temperature treatment; callus induction was based on MS and the callus rate was 18.33% when 1.0 mg/L 2,4-D + 0.75 mg/L 6-BA + 0.8 mg/L NAA + 2.0 mg/L KT was added. With N6 as the basic medium, the addition of 2.0 mg/L 2,4-D + 0.75 mg/L 6-BA + 0.4 mg/L NAA + 0.5 mg/L KT resulted in a healing rate of 16.19%; the addition of 0.5 mg/L 6-BA + 1.0 mg/L KT to the MS medium resulted in a differentiation rate of 68%; during the construction of the anther histoponic system, browning and waterlogging of the healing tissues and vitrification of the seedlings hindered the formation of regenerated plants.

Key words: Anther, Callus rate, Differentiation rate, *Onobrychis viciaefolia* Scop 'Mengnong'.

INTRODUCTION

Onobrychis viciaefolia Scop. is an excellent perennial leguminous fodder grass (Qiao *et al*., 2021), mainly found in temperate climates and widely cultivated in Europe and other parts of the world. In China, wild species of *Onobrychis viciaefolia* Scop. are mainly found in the northern foothills of the Tianshan and Altay mountains in Xinjiang and most of the current cultivars have been introduced from abroad (Tao 2021). The forage is suitable for livestock and, because it contains condensed tannins, it prevents bloating in grazing animals by precipitating proteins to reduce greenhouse gas emissions, thereby increasing animal growth rates and milk production (Girard *et al*., 2016). As a result, it is known as the 'Queen of Forages' and is considered to be the ideal forage to solve the problem of alfalfa-induced dropsy in livestock (Aufrère *et al*., 2013). *Onobrychis viciaefolia* Scop 'Mengnong' (Wu *et al*., 1995) was bred by Inner Mongolia Agricultural University and is cold hardy, high yielding and nutritious. It flowers twice a year, in summer and autumn, in long racemes with small flowers that bloom in an orderly fashion from the bottom up. *Onobrychis viciaefolia* Scop. has recently been the subject of a study focusing on yield quality, resistance, formation of nutrient regeneration systems and condensed tannins. Bhattarai *et al*. (2018) studied 38 *Onobrychis viciaefolia* Scop. materials from 21 different countries and concluded that the dry matter yield of these materials ranged from 74 to 239 g/plant. This suggests that plant genotype and environment have an effect on yield and breeding efforts to improve this plant will be helpful for the future growth of the forage industry. Therefore, to improve the efficiency of genetic improvement of *Onobrychis viciaefolia* Scop. and to increase the yield and quality of forage to reduce the pressure on food supply, new breeding techniques and rapid breeding of new varieties suitable for industrial needs are urgently needed.

The use of doubled haploid breeding technology allows the production of completely pure parental lines while saving a significant amount of time and money (Abdollahi *et al*. 2021). As haploid plants have only one pair of chromosomes, there is no recessive gene link and many desirable recessive traits can be expressed, improving the choice of qualities for breeding material. Therefore, the selection of qualities for breeding material can be improved. The main techniques for obtaining haploid material are: (1) solitary propagation; (2) interspecific hybridisation; (3) anther culture, *etc.* (Gu *et al*., 2022; Tripathy, 2021). Initially, anther and pollen culture techniques were used to produce haploid mandarins. As a result of further research, this approach has been utilised to produce haploids of alfalfa, tobacco, rice and other plants

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(Sood *et al*., 2021; Xu *et al*., 2020). At present, *Onobrychis viciaefolia* Scop. haploid plants are rarely studied in the United States and abroad because the cultivation of haploid plants is affected by a variety of factors, such as genotype, growth conditions, collection time, pre-treatment of donor material, composition and culture conditions of the induction regeneration medium and so on. To overcome the limitations of multiple anther tissue culture of *Onobrychis viciaefolia* Scop. in this study, *Onobrychis viciaefolia* Scop. 'Mengnong' as the material, determined the appropriate time of anther sampling to obtain regenerated plants, investigated the pretreatment method and regeneration system of anther tissue culture and analysed the results. The main variables influencing the establishment of the regeneration system were investigated. The tissue culture system of *Onobrychis viciaefolia* Scop 'Mengnong' haploid plants was established and the leaves of the regenerated plants were used to establish an effective group culture expansion system and provide new materials for the genetic improvement of *Onobrychis viciaefolia* Scop.

MATERIALS AND METHODS

The material under study is *Onobrychis viciaefolia* Scop 'Mengnong', which is planted in the grassland (40°36′N, 111°45′E) on the south campus of the Inner Mongolia Agricultural University. This region has a temperate continental climate, with an arid environment and a wide temperature range. The average annual temperature is 1-15°C, while the average annual rainfall is 337-418 mm and the frost-free period is approximately 130 days. The first flowering season is from May to June and the second flowering season of regenerated grass after seed collection is from July to August.

Anther collection and low temperature processing method

At the early stage of bud emergence, branches with 10 cm long inflorescences were collected between 7:00 and 9:00 am and placed in plastic bags and rooted solid media. These were then returned to the room for dark incubation in a 4°C incubator for 24 h, 36 h, 48 h and 72 h.

Anther sterilisation and culture method

The inflorescences with branches were placed in gauze mesh bags, washed under running water for 30 minutes, cleaned with 70% alcohol for 30 seconds, rinsed twice with sterile water, cleaned with 0.1% $HgCl_2$ for 3 minutes, washed three times with sterile water and then placed on sterile Petri plates. After disinfection, the anthers were removed by peeling the buds with a dissecting needle and the intact anthers and those pierced through the anther wall were inoculated onto different media, with 15 anthers in each medium and 8 replicates per treatment.

Healing tissue culture

40-50 days at 25°C in a dark environment with medium changes every 20 days. The medium was changed to the proliferation medium after the growth of mung bean-sized transparent white healing tissues, which were reconstituted according to the healing rate (N6 medium + KT 1 mg/L + 6-BA 0.5 mg/L + agar 7.8 g/L + sucrose 30 g/L + acid hydrolysed casein 0.2 g/L pH=5.8) and placed in light culture at 25°C for 15-20 d. After one pass, the green, soft, non-vitrifying and non-water damaged healing tissues with good growth were selected and inoculated into differentiation medium with different hormone ratios after 15 days. Five healing tissue samples and five replicates were injected into each petri dish. After the healing tissues developed green buds, they were placed in medium containing 1/2 MS (plant gel 3.5 g/L + sucrose 15 g/L + acid hydrolysed casein 0.2 g/L pH=5.8), four pieces were inoculated in each flask and the number of seedlings was counted (Fig 1).

The media used were MS and N6 and different hormone concentrations were selected in comparison to existing legume anther tissue culture methods. The experimental design was based on the L_{16} (4^4) orthogonal table (Table 1).

Healing tissue differentiation culture

The basic medium used was MS, supplemented with varying concentrations of 6-BA and KT hormones (Table 2), as well as additional nutrients (agar 7.8 g/L + sucrose 30 g/L + acid hydrolysed casein 0.2 g/L pH=5.8).

Result statistics method

After transferring the healing tissue proliferation culture twice (30 days apart) to differentiation medium, the healing rate was calculated. The differentiation rate was then calculated after differentiation of green seedlings.

$$\text{Healing rate} = \frac{\text{Number of produced guaiac tissue blocks}}{\text{Number of inoculated anthers}} \times 100\%$$

$$\text{Differentiation rate} = \frac{\text{Number of mature buds}}{\text{Total number of healing tissues inoculated}} \times 100\%$$

Excel was utilized to process the basic data, whereas Origin was utilized for ANOVA.

RESULTS AND DISCUSSION

Exosome pre-treatment methods and tissue culture regeneration

Treating flower buds with low temperatures increases the induction rate of anther healing tissue. Alfalfa anthers were better induced by pretreatment at 4°C for 24 h and 48 h than for 72 h . In this study, the low temperature pretreatment for 48 hours increased the healing rate of the anther sample of *Onobrychis viciaefolia* Scop 'Mengnong' ($p<0.05$). Among the anthers collected in June (Fig 2), the intact anthers treated with low temperature for 48 hours showed a greater potential for regeneration as measured by a healing emergence rate of 15.37%. While the anthers collected in

August (Fig 3) treated with low temperature for 48 h had a higher healing rate (35.6%) after anther fragmentation in culture, the healing tissue cultured from intact anthers treated with low temperature for 24 h had a lower healing rate (27%). Hypothermic pretreatment involves cooling the entire anther to four degrees celsius. Guy (1990) hypothesised that low temperature alters plant metabolism and physiology. The combination of flowering spike and low temperature corresponds to the plant entering the vernalisation stage, which stimulates the growth of the floral apparatus and supports the expansion of the anther regeneration capacity (Zhang 2014). Breaking the anther wall makes it difficult to maintain the fleece layer, which reduces the ability of pollen to regenerate (Jiang 2017). Therefore, the anther culture with present buds collected in August and anthers treated at low temperature for more than 24 hours was more favourable for healing formation.

Table 1: The concentration of each hormone in the anther guarantor tissue culture medium of *Onobrychis viciaefolia* Scop 'Mengnong' (mg/L).

Level	2,4-D	6-BA	NAA	KT
1	0.5	0.25	0.2	0.5
2	1.0	0.5	0.4	1.0
3	1.5	0.75	0.6	1.5
4	2.0	1.0	0.8	2.0

Table 2: Hormone types and concentrations for differentiation culture of *Onobrychis viciaefolia* Scop 'Mengnong' anther healing tissue.

Level	Factors and hormone concentrations (mg/L)	
	6-BA	KT
1	0.5	0.25
2	0.5	0.5
3	0.5	0.75
4	0.5	1.0
5	1.0	0.25
6	1.0	0.5
7	1.0	0.75
8	1.0	1.0

Effect of exogenous hormones on the activation of healing tissue

The ratio of exogenous growth hormones to cytokinins controls plant regeneration in isolates and regulates the internal and external balance by influencing endogenously produced phytohormones (Xu *et al*., 2022). It has been shown that different phytohormones have different abilities to detect receptor protein target cells and that different combinations of hormones alter the induction and proliferation of repair tissues. Nan Cui used the L_{16} (4^5) orthogonal table to design four media, such as MS and N6 and four hormones, 2,4-D, 6-BA, NAA and KT, at different concentrations to determine the optimal healing medium for the induction of *Medicago varia Martin*. cv. Caoyuan No.1 Cui (2017). According to Table 3, the hormone combination with the highest efficiency of anther healing tissue induction was MS + 1.0 mg/L 2,4-D + 0.75 mg/L 6-BA + 0.8

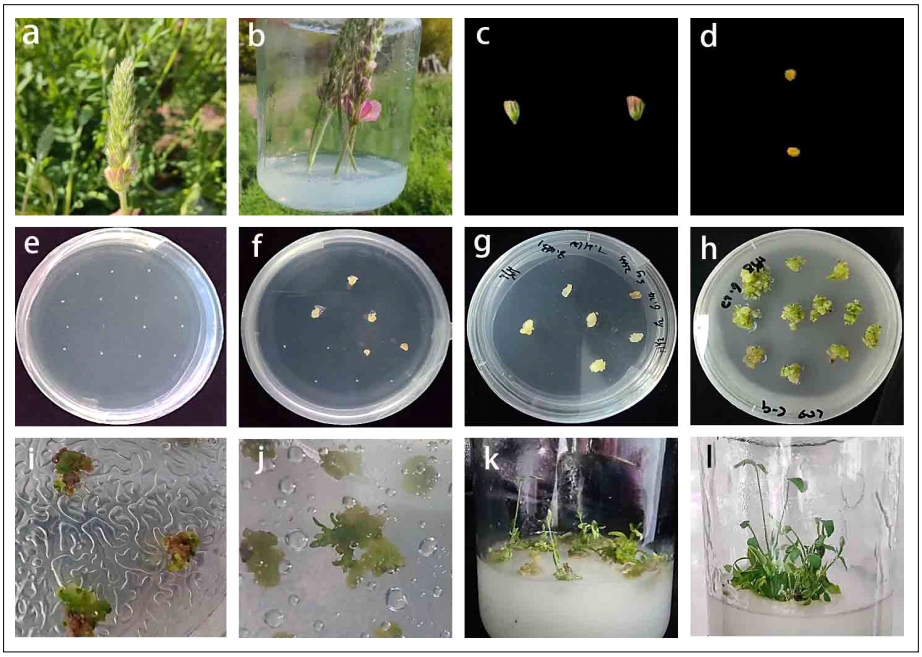


Fig 1: a. Inflorescence b. Collection of inflorescences stored in solid medium c. Bud d. Anther e. Inoculation of anthers in guaiac medium f. Healing tissue g. Healing tissue cultured in the light h. Healing tissue differentiation culture i. Differentiation of green bud sites j. Bud point differentiation into seedlings k. Transfer of differentiated seedlings into tissue culture flasks l. Seedlings multiply and expand Induction of healing tissue.

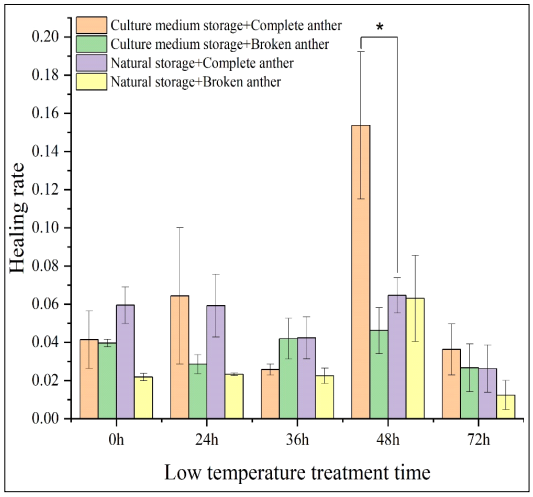


Fig 2: Anther pretreatment healing rate for June sample.

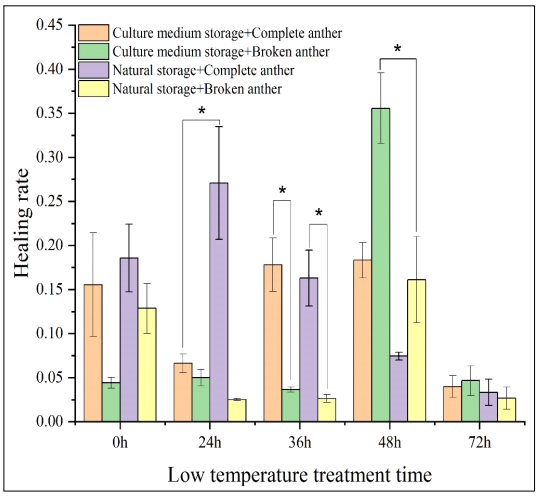


Fig 3: Anther pretreatment healing rate for August sample.

mg/L NAA + 2.0 mg/L KT and compared with A8, the hormone ratios A1, A3, A4, A5 and A7 had a lower induction ability for anther healing tissue, with flat healing rates ranging from 4.17% to 14.2%. The total healing rates for the remaining hormone ratios varied between 5.17% and 12.50%. The study on N6 medium, which was the same as the MS base culture (Table 4), showed that the hormone ratio of B15 resulted in an anther emergence rate of 16.19%. When B6 was added, the formation of healing tissue in the anthers was prevented. Overall healing rates ranged from 5.71-11.11% for hormone ratios other than B15, B6 and B7. Several studies have found that cells can be kept in a proliferative state by using high levels of growth hormone and low levels of cytokinin (Perianez-Rodriguez *et al*., 2014). This analysis is consistent with the results of the current study, where 2,4-D concentrations between 1.0 and 2.0 mg/L resulted in higher anther healing rates than other concentrations.

Effect of exogenous hormones on adventitious shoot development from healed tissues

During the period of adventitious shoot differentiation, a high concentration of cytokinin promoted shoot differentiation (Atta *et al*., 2009). As shown in Table 5, the addition of different ratios of 6-BA and KT to MS basic medium resulted in the differentiation of healing tissues, with the ratios causing significant differentiation differences, particularly in the formulation MS + 0.5 mg/L 6-BA + 1.0 mg/L KT, where the differentiation rate of healing tissues reached 68%; Compared to the differentiation of F4, the addition of 1.0 mg/L 6-BA significantly decreased the adventitious shoot induction rate. When growth hormone 0.5 mg/L and cytokinin KT 0.75 mg/L were used, the differentiation rate increased. Therefore, the selection of the optimal concentration ratios for the different growth phases of the anther media could accelerate the regeneration rate of the plants.

Table 3: Ratios of hormone concentrations for healing tissue induction in MS medium.

Number	2,4-D ($mg.L^{-1}$)	6-BA ($mg.L^{-1}$)	NAA ($mg.L^{-1}$)	KT ($mg.L^{-1}$)	Callus induction rate (%)
A1	0.50	0.25	0.20	0.50	4.17±1.75b
A2	0.50	0.50	0.40	2.00	5.71±3.06ab
A3	0.50	0.75	0.60	1.00	4.17±2.80b
A4	0.50	1.00	0.80	1.50	5.00±2.75b
A5	1.00	0.25	0.40	1.50	5.00±2.75b
A6	1.00	0.50	0.20	1.00	6.67±2.82ab
A7	1.00	0.75	0.80	2.00	5.00±1.67b
A8	1.00	1.00	0.60	0.50	18.33±3.02a
A9	1.50	0.25	0.60	2.00	7.50±2.65ab
A10	1.50	0.50	0.80	0.50	10.00±1.78ab
A11	1.50	0.75	0.20	1.50	5.00±2.09b
A12	1.50	1.00	0.40	1.00	7.50±2.65ab
A13	2.00	0.25	0.80	1.00	11.67±2.09ab
A14	2.00	0.50	0.60	1.50	12.50±2.94ab
A15	2.00	0.75	0.40	0.50	12.50±3.20ab
A16	2.00	1.00	0.20	2.00	8.33±2.44ab

Anomalous development of *Onobrychis viciaefolia* Scop. anthers in regenerated plants

The entire propagation process is carried out in a controlled environment, resulting in phenomena such as browning, vitrification, waterlogging and staining, which limit plant growth during the propagation phase . Healing rate and differentiation rate of *Onobrychis viciaefolia* Scop. tissue cultures were shown to be affected by the following conditions Fig 4a. waterlogging; b. browning; c. vitrification; d. overall granulation with difficulty in differentiating green bud points; e. The mature healing site differentiates new white healing tissue with soft texture; f. After bud point differentiation, buds grow into aerial roots and cannot become seedlings; g. Formation of vitrifying seedlings.

Browning of the healing tissue is primarily caused by enzymatic browning , which is caused by the oxidation of phenolic compounds released from the cut end of the explants by the polyphenol oxidase, peroxidase, in the tissue. To reduce browning of the healing tissue, anti-browning agents such as activated carbon (AC), polyvinylpyrrolidone (PVP) and vitamin C are added to the culture medium (Chen *et al*., 2022). In this study, the phenomenon of waterlogging was found to initially form a ring of water around the bottom of the healing tissue and over time, the green healing tissue gradually turned white and the whole tissue was filled with water internally, losing its ability to differentiate. Zhu *et al*. (2008) investigated the enhancement of the rice transformation system and discovered that the addition of 15 g/L mannitol to the medium significantly reduced the waterlogging of healing tissues and increased the healing tissue induction rate.

Vitrification is a complex process influenced by both chemical and physical factors. These include high cytokinin levels, low concentrations of gelling agent in the liquid medium and an excess of mineral elements. Several measures to reduce vitrification have also been discovered,

Table 4: Ratios of hormone concentrations for healing tissue induction in N6 medium.

Number	2,4-D ($mg.L^{-1}$)	6-BA ($mg.L^{-1}$)	NAA ($mg.L^{-1}$)	KT ($mg.L^{-1}$)	Callus induction rate (%)
B1	0.50	0.25	0.20	0.50	5.71±2.27abc
B2	0.50	0.50	0.40	2.00	4.17±1.75bc
B3	0.50	0.75	0.60	1.00	4.76±2.80bc
B4	0.50	1.00	0.80	1.50	5.71±2.27abc
B5	1.00	0.25	0.40	1.50	13.33±1.78ab
B6	1.00	0.50	0.20	1.00	0.00±0.00c
B7	1.00	0.75	0.80	2.00	5.00±1.67bc
B8	1.00	1.00	0.60	0.50	12.38±2.69ab
B9	1.50	0.25	0.60	2.00	9.52±1.98abc
B10	1.50	0.50	0.80	0.50	5.71±2.27abc
B11	1.50	0.75	0.20	1.50	7.78±2.05abc
B12	1.50	1.00	0.40	1.00	6.67±2.52abc
B13	2.00	0.25	0.80	1.00	11.43±2.80ab
B14	2.00	0.50	0.60	1.50	6.67±1.78abc
B15	2.00	0.75	0.40	0.50	16.19±2.46a
B16	2.00	1.00	0.20	2.00	11.11±2.22abc

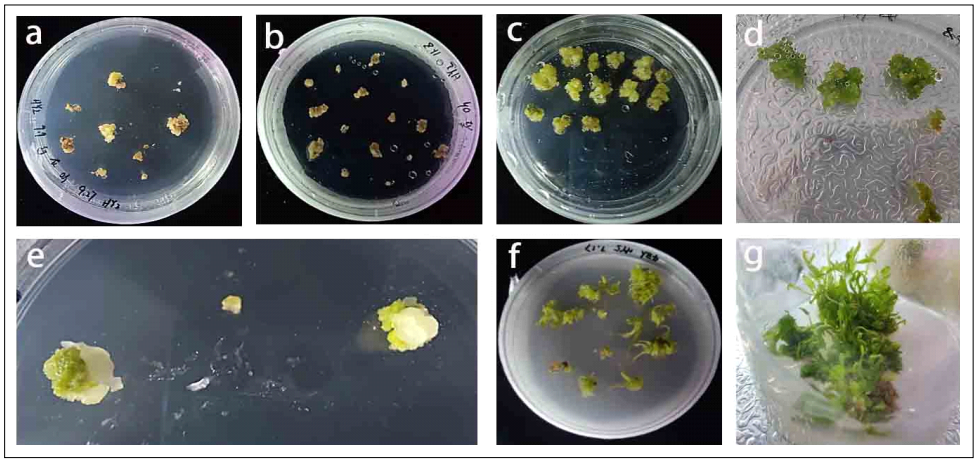


Fig 4: Abnormalities in the development of anther regeneration in plants.

Table 5: Differentiation rates of *Onobrychis viciaefolia* Scop 'Mengnong' guava healing under different hormone ratios.

Number	6-BA (mg·L^{-1})	KT (mg·L^{-1})	Induction rate (%)
F1	0.5	0.25	36.00±7.48bc
F2	0.5	0.5	20.00±8.94c
F3	0.5	0.75	48.00±10.20abc
F4	0.5	1.0	68.00±10.18ab
F5	1.0	0.25	40.00±8.94bc
F6	1.0	0.5	24.00±7.48c
F7	1.0	0.75	12.00±8.00c
F8	1.0	1.0	16.00±7.48c

including increasing the amount of carbohydrates in the medium, changing the light intensity, changing the agar concentration and reducing the humidity in the culture vessel (Xi, 2013). Shoots and roots are the most commonly differentiated organs in tissue culture. In the present study, it was observed that some of the materials first developed roots and then shoots. The main variables influencing the success of adventitious root induction are the presence of plant hormones and the choice of explants. In some studies it has been found that cytokinins at low concentrations can also induce adventitious roots (Arya *et al*., 2022), which is similar to the result observed in this work.

CONCLUSION

(1) The highest healing rate was obtained for culture by 48 h low temperature treatment of intact anthers in June or 48 h low temperature treatment of broken anthers in August.
(2) The healing rate formed by adding 1.0 mg/L 2,4-D + 0.75 mg/L 6-BA + 0.8 mg/L NAA + 2.0 mg/L KT to MS as the base medium was 18.33%, whereas the healing rate formed by adding 2.0 mg/L 2,4-D + 0.75 mg/L 6-BA + 0.4 mg/L NAA + 0.5 mg/L KT to N6 as the base medium was 16.19%. With the addition of 0.5 mg/L 6-BA + 1.0 mg/L KT on MS medium, the differentiation rate reached 68%.
(3) During the construction of anther group culture systems, the formation of regenerated plants may be affected by browning and waterlogging of the healing tissue, as well as the appearance of glassy seedlings.

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Conflict of interest: None.

REFERENCES

Abdollahi, M.R., Seguí-Simarro, J.M. (2021). Anther culture of chickpea (*Cicer arietinum* L.). Methods Mol Biol. 2289: 289-299.

Arya, A., Sharma, V., Tyagi, P.K., Gola, D., Husen, A. (2022). Environmental, Physiological and Chemical Controls of Adventitious Rooting in Cuttings: Academic Press. (pp. 239-249)

Atta, R., Laurens, L., Boucheron-Dubuisson, E., Guivarc'h, A., Carnero, E. *et al*. (2009). Pluripotency of arabidopsis xylem pericycle underlies shoot regeneration from root and hypocotyl explants grown *in vitro*. Plant J. 57(4): 626-644.

Aufrère, J., Dudilieu, M. andueza, D., Poncet, C., Baumont, R. (2013). Mixing sainfoin and lucerne to improve the feed value of legumes fed to sheep by the effect of condensed tannins. Animal. 7(1): 82-92.

Bhattarai, S., Coulman, B., Beattie, A. D., Biligetu, B. (2018). Assessment of sainfoin (*Onobrychis viciifolia* Scop.) germplasm for agro-morphological traits and nutritive value. Grass and Forage Science. 73(4): 958-966.

Chen, X., Yu, C., Nie, J., Yang, H., Ji, W. *et al*. (2022). The effect of anti-browning agent activated carbon and polyvinyl pyrrolidone on the rooting of embryo seedlings of "fengdan" and its transcriptome analysis. Frontiers in Plant Science. 13: 832619.

Cui, N. (2017). Pollen cuture of the three Alfalfa varieties and its haploid identification. (Master), Inner Mongolia Agricultural University.

Girard, M., Dohme-Meier, F., Wechsler, D., Goy, D., Kreuzer, M. *et al*. (2016). Ability of 3 tanniferous forage legumes to modify quality of milk and Gruyère-type cheese. J. Dairy Sci. 99(1): 205-220.

Gu, R., Jia, Z., Ma, H., Liu, Y., Zhang, Y. *et al*. (2022). Establishment of a regeneration system for wild alfalfa in alxa, China. Legume Research-An International Journal. 45(7): 839-843. doi: 10.18805/LRF-679.

Guy, C. (1990). Cold acclimation and freezing stress tolerance: Role of protein metabolism. Annual Review of Plant Physiology. 41: 187-223.

Jiang, S.J. (2017). Studies on anther development and transcriptional regulation of cytoplasmic male sterile in alfalfa (*Medicago sativa* L.). (Master), Lanzhou University.

Perianez-Rodriguez, J., Manzano, C., Moreno-Risueno, M.A. (2014). Post-embryonic organogenesis and plant regeneration from tissues: two sides of the same coin? Front Plant Sci. 5: 219. https://doi.org/10.3389/fpls.2014.00219.

Qiao, Y., Cheng, Q., Zhang, Y., Yan, W., Yi, F. *et al*. (2021). Transcriptomic and chemical analyses to identify candidate genes involved in color variation of sainfoin flowers. BMC Plant Biol. 21(1): 61. https://doi.org/10.1186/s12870-021-02827-8.

Sood, S., Prasanna, P.S., Reddy, T.V., Gandra, S.V.S. (2021). Optimized protocol for development of androgenic haploids and doubled haploids in FCV tobacco (*Nicotiana tabacum*). Methods Mol. Biol. 2288: 293-305.

Tao, Y.T. (2021). Study on genetic diversity and cold resistance transcriptome of new strains of *Sainfoin*. (Master), Inner Mongolia Normal University.

Tripathy, S.K. (2021). High-throughput doubled haploid production for indica rice breeding. Methods Mol Biol. 2287: 343-360.

Wu, Y.F., Shi, F.L., Cui, Z.M. (1995). Breeding of *Onobrychis viciifolia* CV. 'Mengnong' and studies on its characteristics. Grassland of China. 4: 24-28.

Xi, H.J. (2013). Callus induction and bud proliferation of Arnebia euchroma (Royle) Johnst. (Master), Xinjiang Agricultural University.

Xu, B., Wu, R., Gao, C., Shi, F. (2022). Establishment of tissue culture regeneration system for *Medicago ruthenica* L. cv. 'Zhilixing'. Legume Research. 45(2): 162-167. doi: 10.18805/LRF-659.

Xu, B., Wu, R., Tang, F., Gao, C., Gao, X. *et al*. (2020). Haploid culture and double haploid induction in *Medicago sativa* L. cv. XinJiangDaYe. Legume Research-An International Journal. 44(3): 275-280. doi: 10.18805/LR-575.

Zhang, R. (2014). Effect of jarovization conditions on Scabiosa tschiliensis bud differentiation and study on the resistance. (Master), Shanxi Agricultural University.

Zhu, X.G., Dong, Z.Q., Sing, Z.S., Zhao, M. (2008). Modification of transformation system of NPT rice. Crops (5): 28-30.