Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Establishment of tissue culture regeneration system of Phalaris arundinacea L. mature embryo

Siriguleng Bai

sirinmg@163.com

Inner Mongolia Minzu University

Article

Keywords: Phalaris arundinacea L., mature embryo, tissue culture, callus, dormancy

Posted Date: April 18th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4147065/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations:
No competing interests reported.

Plate is available in the Supplementary Files section.

Establishment of tissue culture regeneration system of *Phalaris arundinacea* L. mature embryo

Siriguleng

College of Agriculture, Inner Mongolia Minzu University, Tongliao, China

Abstract: The present study used the mature embryos of two varieties of *Phalaris arundinacea* L. (Tongxuan No.1 and Chuancao No.3) as explants. These explants were treated with 70% ethanol for 12 minutes under sterile conditions, sterilized with 0.1% $HgCl_2$ for 5 minutes, washed with distilled water, and inoculated on media containing different ratios of hormones to overcome the dormancy caused by seed pupae and seed coats and obtain the regenerated plants. The results showed that the total recovery rate of Tongxuan No.1 and Chuancao No.3 on the induction medium N6+3.0–4.0 mg/L 2,4-D reached 70%; Tongxuan No.1got the best differentiation rate of 95.38% on N6+0.5 mg/L 2,4-D+2.0 mg/L 6-BA medium. Chuancao No. 3 on N6+0.5 mg/L 2,4-D+4.0 mg/L 6-BA medium had the highest differentiation rate of 89.33%. Both varieties produced new regenerated plants.

Keywords: *Phalaris arundinacea* L.; mature embryo; tissue culture; callus; dormancy

Phalaris arundinacea L. is a perennial C_3 highlight plant of the Poaceae family with high utilization value. *Phalaris arundinacea,* with high protein, nitrogen-free extractives, ash content, and yield, is used as a forage grass. In Tongliao (Inner Mongolia), the aboveground biomass of four-year-old grass is 561.22 g/m^2. It is used as a functional plant for soil and water conservation and sewage treatment and as an indicator for assessing the eutrophication degree of water bodies（Vymazal and Kropfelova, 2004; Chundu Wu *et al*., 2006; Schilling and Kiniry, 2007）. As an energy plant, it has huge development and utilization prospects. It is considered a potential biofuel due to its higher yield and higher content of cellulose and lignin in late growth stage （Sanderson and Adler, 2008; Wrobel *et al*., 2009）. In Europe and the United States, *Phalaris arundinacea* L. has attracted people's attention since the 1980s and has been used to produce ethanol and other fuels for people's daily life（Iris *et al*., 2003; Matthew

et al., 2010; Yuzui *et al*., 2009; Nancy *et al*., 2010）.

Phalaris arundinacea is naturally distributed in China and has strong environmental adaptability. Due to higher economic and ecological value, more people are showing interest in *P. arundinacea*; however, the monogenesis of varieties and dormancy of seeds limit its utilization to a certain extent. Seed dormancy is a biological feature that helps adapt to environmental changes and has been acquired through long-term evolution. It is a quantitative trait and is regulated by multiple gene loci. These genes affect different seed parts' physiological and biochemical activities （Foley and Fennimorse, 1998; Meyer and Pendleton, 2000）. Therefore, exploring the causes of P. arundinacea seed dormancy is necessary. A previous study found that both internal and external factors affect seed germination. Poor seed coat permeability affects air and moisture exchange and results in exogenous dormancy（Matus-Cádiz and Hucl, 2005; Zhanghao *et al*., 2012）. Meanwhile, germination inhibitory substances in the seed result in endogenous dormancy（Vose, 1962; Asbjörn and Olavi , 1979）.

The present study focused on overcoming the exogenous dormancy of the *P. arundinacea* seeds. The study developed a tissue culture regeneration system using the mature embryos of *P. arundinacea* L. to eliminate dormancy caused by the seed coat and obtain highly efficient regenerated plants for future application in agriculture and energy.

1. Materials and methods

1.1 Overview of material collection sites

The materials used in this study were collected from two different sites, collection site 1 and collection site 2. Collection site 1 is the forage test base of Sichuan Provincial Academy of Grass Science, Hongyuan County, Sichuan Province (102° 55′ E, 31° 79′ N; 3600 m altitude). The base has an annual average temperature of 1.1 ℃, an extremely high temperature of 32.5 ℃, an extremely low temperature of -33.8 ℃, annual rainfall of 740 mm, and meadow soil; the region has been under irrigation. Collection site 2 is a Chajintai pasture in Tongliao City, Inner Mongolia (43°16'–43°24' N, 121°5'–121°45' E; 231.2 m altitude). The pasture has an annual average

temperature of 6.1 ° C, a frost-free period of 146 days, annual average precipitation of 385.5 mm, and meadow soil; the region has been under irrigation.

1.2 Materials

The seeds of *Phalaris arundinacea* L. Chuancao No. 3 were obtained from the Sichuan Academy of Grass Science after years of cultivation and domestication, and those of *Phalaris arundinacea* L. Tongxuan No. 1 from the wild selected varieties with stable biological and ecological traits after years of cultivation and domestication. The seeds were collected in 2020, cleaned, naturally dried at room temperature, placed in seed bags, and stored at room temperature for future use.

1.3 Preparation and disinfection of the seeds

The seeds were treated with 70% ethanol for 12 minutes, washed thrice with distilled water, sterilized with 0.1% $HgCl_2$ for 5 minutes, and finally washed 3 to 5 times with distilled water. The sterilized seeds were soaked in ddH_2O for 30 minutes on a clean bench to soften the seed coat and then placed on sterile filter paper. After excess water was removed from the seeds have been blotted with water, the embryo (with minimal endosperm) was separated using a dissecting needle under a microscope and inoculated on an N6 induction medium with different concentrations of auxin（Tongbao Qu *et al*., 2004; Jihong Xie *et al*., 2006）. Ten embryos were inoculated in each Erlenmeyer flask, and six replicates were maintained per hormone concentration for each species.

1.4 Callus induction

The N6 medium supplemented with different concentrations of 2,4-D (0 mg/L, 1.0 mg/L, 1.5 mg/L, 2.0 mg/L, 3.0 mg/ L, 4.0 mg/L) was used as the induction medium and marked from low concentration to high concentration as D1, D2, D3, D4, D5, and D6; 2.0% sucrose and 0.75% agar were added to the induction medium. The stripped embryos were inoculated on the induction medium, placed in a constant temperature incubator for seven days, and then transferred to a constant temperature light incubator with a light intensity of 2300–3000 Lx, a light time of 14 h (10 h in the dark), and a temperature of 23 °C. The callus growth was observed every two to three days, and the callus growth rate was calculated after 23 days as follows:

Callus rate (%) = (number of callus pieces/total number of seed embryos inoculated) × 100%

1.5 Callus generation and differentiation

The callus obtained after 30 days of induction was subcultured once every 30 days, and the differentiation was induced after 2 to 3 such subcultures. The N_6 medium containing 0.5 mg/L 2,4-D was used for subculturing. After the subculture, well-grown callus pieces were inoculated on the differentiation medium containing different ratios of auxin. The N_6 medium containing 0.5 mg/L 2,4-D with 6-BA (1.0 mg/L, 2.0 mg/L, 3.0 mg/L, 4.0 mg/L) was used for differentiation and numbered from low to high concentration as B1, B2, B3, and B4; four to five callus pieces were inoculated per bottle of differentiation medium containing 2.0% sucrose and 0.7% agar. The calli were examined for differentiation every five days, and the differentiation rate was calculated as follows:

Differentiation rate (%) = (number of differentiated plants/number of inoculated callus pieces) × 100%

1.6 Root induction and plant regeneration

The adventitious buds of 2–3 cm in length were divided into individual clusters with a scalpel and inoculated on a rooting medium, with two clusters per bottle. Here, 1/2 MS medium containing 1.5% sucrose and 0.75% agar was used as the rooting medium. The pH of the medium was adjusted to 6.0. Once the regenerating plant was about 6 cm long with a sufficiently developed root system, the sealing film was removed, and the seedlings were allowed to condense for three days. Once the medium was basically off the wall, the seedling was taken out, washed with water, and planted in the substrate (vertical: sand =1: 1). These plants were maintained under the following conditions: daily watering with 300 mL tap water, room temperature, and no direct sunlight; the survival was evaluated after one week.

2. Results and analysis

2.1 Effect of callus induction

The seed embryos of the two varieties induced callus formation on different

induction media; however, the callus situation was quite different. Four days after inoculation, the embryos began to swell (Plate I-A), producing white, transparent, and soft callus. After culturing under dark (1 week) and light conditions, the color of the callus gradually changed from white to yellow-green, and the texture appeared compact (Plate I-B,C). All the explants produced callus after about 20 days of culture. The effects of different hormone concentrations on the callus induction of the two varieties were significantly different. Tongxuan No.1 had the highest induction rate of 91.67% in the D6 (N6 medium + 2,4-D 4.0 mg/L) medium, and the induction rate increased with the increase in 2,4-D concentration (Table 1). Meanwhile, Chuancao No. 3 obtained the highest induction rate of 70.0% in the D5 (N6 medium + 2,4-D 3.0 mg/L) medium, and the induction rate increased with an increase in the concentration of 2, 4-D (Table 2). A high concentration of 2,4-D induced more callus in Tongxuan No. 1 than in Chuancao No.3; however, an excessively high concentration of 2,4-D decreased the induction rate, probably due to the endogenous hormone content of the variety. The induced tender green and dense texture callus was transferred to the subculture medium. The callus showed different degrees of browning during the subculture; these observations indicated the importance of reducing the number of subgenerations and the interval times between subgenerations.

Table 1 The embryo growth condition of *Phalaris arundinacea* L. Tongxuan No.1 on different content of hormone medium and variance analysis of induction rate

Medium No.	Concentration of 2,4-D (mg/L)	Callus condition	Induction rate (%)
D1	0	Milky white, watery	33.33^{c}
D2	1.0	milky white，harder	48.33^{bc}
D3	1.5	white，harder	45.0^{bc}
D4	2	tender green，soft	76.67^{ab}
D5	3	yellow green，soft	70.0^{ab}

	D6	4	yellow green，soft	91.67[a]

Note: The same letters after column of induction rate indicated no significant difference at 0.05 level

Table 2 The embryo growth condition of *Phalaris arundinacea* L. Chuancao No.3 on different content of hormone medium and variance analysis of induction rate

Medium No.	Concentration of 2,4-D (mg/L)	Callus condition	Induction rate (%)
D1	0	White, sticky	25．0[b]
D2	1.0	white，sticky	28.33[b]
D3	1.5	gray green，harder	33.33[b]
D4	2	gray green，harder	45.0[b]
D5	3	yellow green，soft	70.0[a]
D6	4	yellow green，close	30.0[b]

Note: The same letters after column of induction rate indicated no significant difference at 0.05 level

2.2 Adventitious bud differentiation

The calli that grew well after induction were cut into 0.5 cm × 0.5 cm pieces and inserted into the differentiation medium numbered B1 to B4, and the differentiation rate was counted. Tissue blocks transferred into the differentiation medium produced green shoot spots visible to the naked eye after seven days (Plate I-D,E). These green shoot spots gradually grew for 30 days to form new stems and leaves; a few shoots turned brown and died. Different hormones displayed different effects on the differentiation of adventitious buds. Tongxuan No. 1 exhibited the best differentiation (95.38%) on the B2 medium, while Chuancao No. 3 (89.33%) displayed on the B4 medium (Table 3). In the differentiation culture, the reduction of 2,4-D and the addition of 6-BA were beneficial for the regeneration of new plants, and 6-BA

160 demonstrated a greater impact on the differentiation of seed embryos.

161

162 Table 3 The differentiation rate of two varieties on different ratio of hormone medium

Medium No.	Concentration of 2,4-D (mg/L)	Concentrationof6-BA (mg/L)	Differentiation rate (%)	
			Tongxuan No.1	Chuancao No.3
B1	0.5	1.0	52.0[b]	47.93[b]
B2	0.5	2.0	95.38[a]	63.22[ab]
B3	0.5	3.0	78.57[ab]	59.48[ab]
B4	0.5	4.0	69.36[ab]	89.33[a]

163

164 2.3 Rooting and transplanting of regenerated plants

165 The differentiated cluster plantlets were divided into individual plants and inserted
166 into the rooting medium（Plate I-F）. In 25 days, the plant gradually grew tall and
167 produced small roots. The roots filled the bottom of the entire triangular flask at about
168 40 days to form a developed root system (Plate I-G), and they developed into
169 complete plantlets (Plate I-H). The regenerated plants were cultured under relatively
170 sterile conditions with constant temperature and humidity（Plate I-I）. These plants
171 displayed physiological characteristics and adaptability better from ordinary plants.
172 Therefore, during refining, the regenerated plants were gradually adapted to the new
173 environmental conditions for transplanting. Then, to prevent excessive water loss in
174 the culture medium, a few small holes were created in the plastic sealing film, and the
175 system was placed under weak light to allow the plants to adapt to a dry and
176 germ-free environment. After about three to four days, the sealing film was removed
177 (Plate I-F), and the plant was moved to a place with sufficient light. After two to three
178 days, when the medium was not dry, the plant and the medium were transferred into
179 clean water to wash off the root-adhered medium; then, the plant was moved into the
180 substrate. These plants formed callus, regenerated new plants, and displayed a high
181 survival rate.

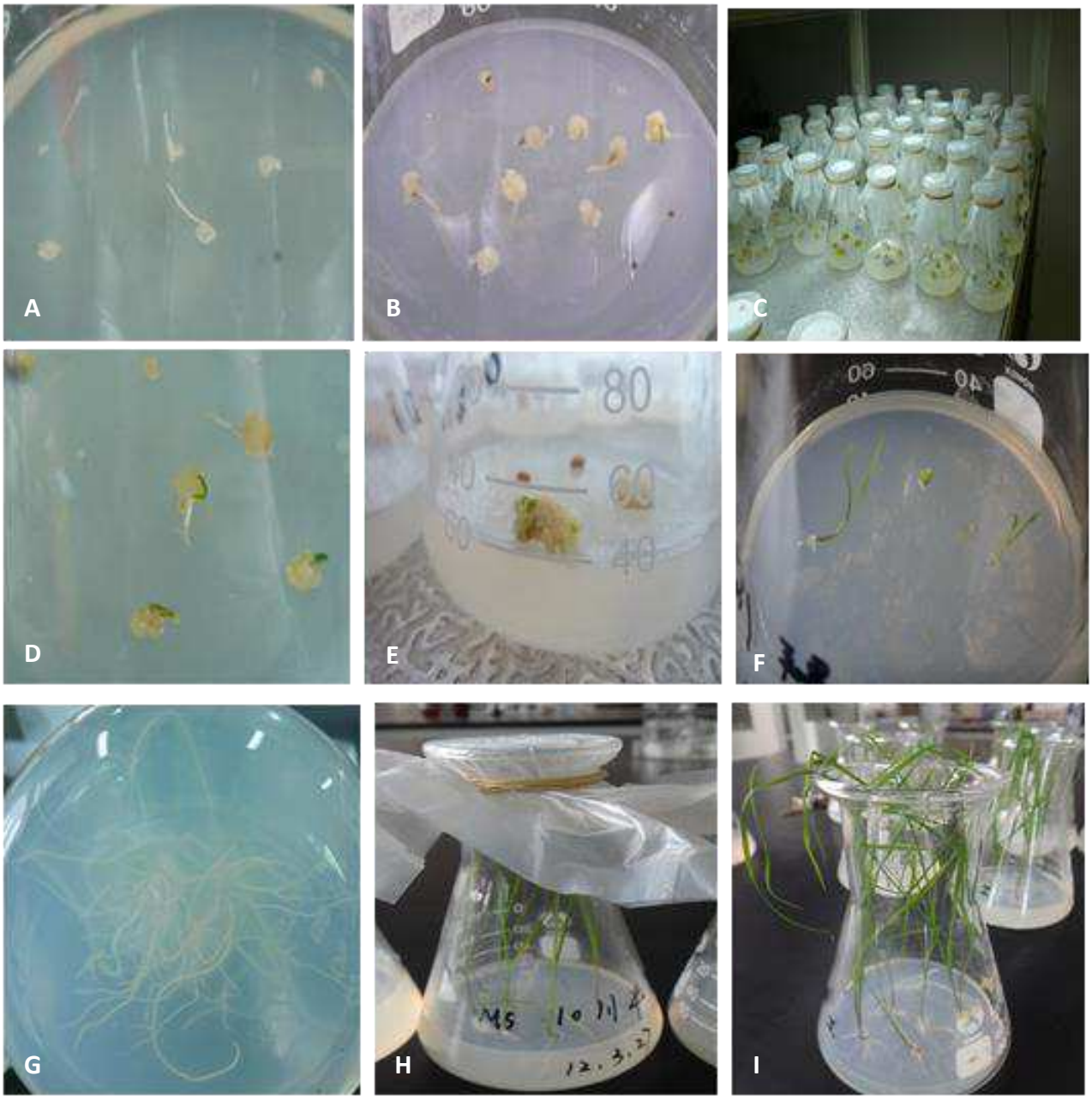


Plate Ⅰ The establishment of regeneration system of mature embryo of *Phalaris arundinacea by tissue culture*

Note: (A) Embryo culture of 4 days; (B) embryo culture of 7 days; (C)subculture; (D)the green bud from callus; (E)stems and leaves formed by callus; (F)individual plants formed by differentiation; (G)the root system of regenerated plant; (H)the regenerated plant

3. Discussion

3.1 Establishment of the regeneration system

The embryos of the two *P. arundinacea* varieties were stripped and cultured in vitro. These embryos formed callus on the induction medium with different concentrations of 2,4-D but at significantly different rates. Studies have shown that 2,4-D effectively induces callus formation in Poaceae plants. Generally, 1–6 mg/L of 2,4-D is added to the induction medium to induce callus（Fengxia *et al*., 2004; Tongbao Qu *et al*., 2004;Heyong *et al*., 2005; Jihong Xie *et al*., 2006）. The selected Tongcao No.1 embryo had the highest induction rate in the D6 (N6 + 2,4-D 4.0 mg/L) medium, and the induction rate was significantly different from that at lower concentrations (D1, D2, and D3). As the concentration increased, the induction rate increased. Meanwhile, Chuancao No. 3 got the highest induction rate on D5 (N6 + 2,4-D 3.0 mg/L) medium. The induction rates were lower at lower or higher concentrations of 2,4-D. These observations indicated differences between the two varieties in response to hormones, which may be due to the differences in the endogenous hormone content and sensitivity between the two varieties. The results of this study are consistent with Cui Kairong and others who reported that a higher concentration of 2,4-D is required to induce dedifferentiation of monocotyledons（Kairong Cui and Ruolan Dai, 2000）.

Both varieties formed clusters at different hormone ratios on differentiation medium but with significantly different differentiation rates among the culture media. Tongcao No. 1 had the best differentiation effect on B2 (N6 + 0.5 mg/L 2,4-D + 2.0 mg/L 6-BA). Meanwhile, Chuancao No. 3 got the highest differentiation rate on B4 (N6 + 0.5 mg/L 2,4-D + 4.0 mg/L 6-BA). They were both significantly different from the B1 medium, and their differentiation rates reached more than 80%. Moreover, lower concentrations of 2,4-D but higher concentrations of 6-BA were beneficial for regenerating new plants in the differentiation medium. Thus, 6-BA demonstrated a greater effect on differentiation. Previous studies showed that adding low concentrations of 6-BA to the Mongolian grass immature embryo induction and differentiation medium benefited the formation of explants with embryogenic callus

and resulted in a higher differentiation rate（Jihong Xie et al., 2006）. Increasing the concentration of 6-BA resulted in different degrees of browning; however, the present study believes that higher concentrations of 6-BA are beneficial to callus differentiation, which may be related to the composition and content of hormones in different plants. Moreover, immature embryos in the development stage have unstable physiological and ecological indicators that are easily disturbed by external factors, such as the application of hormones. Therefore, low concentrations of hormones also probably resulted in changes in a series of cellular events in immature embryos[21]. After the embryos mature, their nutrients and hormones are balanced and stable; therefore, low concentrations of exogenous factors may have a small effect on them or may not cause any change in the cellular events. In general, mature embryos of the two varieties of *P. arundinacea* formed callus and regenerated new plants. Thus, mature embryos of *P. arundinacea* can be used as explants to establish an efficient regeneration system.

3.2 Exogenous dormancy and tissue culture

The exogenous dormancy of seeds is mainly due to the poor permeability of the seed coat to water and air and also is known as the seed coat effect. The dormancy induced by the seed coat includes the following five mechanisms: 1) inhibit water absorption; 2) mechanical limitation; 3) interfere with gas exchange; 4) prevent the exudation of inhibitors; 5) supply inhibitors to the embryo. Water and oxygen are crucial factors for seed germination. Water is necessary for all life activities and a prerequisite for seed germination. Dry seeds have a low water level, and the protoplasts are in a gel state. The physiological activity of the seeds in this state is fragile. After the seeds absorb water, protoplasts transform, and the internal changes and respiration are strengthened. At this time, if the water cannot meet the developmental requirements, seeds will not germinate. When the seed germinates, all life activities require the supply of energy, which is attained via breathing. However, when oxygen is insufficient, ethanol and lactic acid are produced in the seeds, which affects seed vitality and germination rate. Therefore, the seed coat's water and air permeability directly affect the seed's dormancy and the beginning of germination. Scientists have done a lot of research on

seed coat water permeability and air permeability. The impermeability of the seed coat affects the supply of oxygen to the embryo and promotes the generation of germination inhibitors (Edwards, 1969）.

Moreover, the seed coat absorbs light at different wavelengths. *Phalaris arundinacea* seeds are light-sensitive, and their germination rate is higher under white and red light than under far-red light（Roberto and Joy, 2001）. The damage to the seed coat may further enhance the sensitivity of the seeds to light and change the composition of light waves, thereby increasing the germination rate. He Xueqing et al. found that the seed pupae and seed coat have an important inhibitory effect on seed germination in *Leymus chinensis* (He Xueqing *et al*., 2010). Piercing the seed coat and removing pupae increased the seed germination rate significantly. Sunjie et al.showed that the bud structure was the main reason for the dormancy of bison grass（Sun Jie *et al*.,2009）. Shi Dongli found that the seed coat lipid or seed keratin has a certain degree of permeability in *Zoysia macrostachya*（Dongli Shi, 2007）. Generally, the lemma of the graminaceous plants has a protective effect over seeds; however, it hinders oxygen and water absorption and prevents hypocotyl emergence. Removing the lemma eliminates or reduces the hindrance to seed germination. Moreover, treating the seed coat makes it thin and improves permeability. Therefore, tissue culture after stripping mature embryos of *P. arundinacea* can eliminate the adverse effects of seed coat on germination and obtain a high frequency of regenerated plants.

Acknowledgements

We thank Mingjiu Wang, Liansheng Wang, Fan Huang and Weijun Xiao for assistance with seed collection and laboratory experiments. This project is supported by Educational science research "fourteen" planning subject of Inner Mongolia (NGJGH2021100) and Basic scientific research funds of colleges and universities for young teachers to enhance the scientific research and innovation ability project in Inner Mongolia (GXKY22145). Data is provided within the supplementary information files.The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

278

279

280 References

281 Cui K.R., and Dai R.L.（2000）. Molecular biology of plant somatic embryogenesis. Science Press,

282 Beijing, China, 13-14.

283 Feng X., Sun Z., Han L., and Peng Z.H.（2004）. Feature analysis of vegetation degradation on

284 Alpine Grassland in Yellow River source region. *Pratacultural Science*, **21**: 23-28.

285 <https://doi.org/10.3969/j.issn.1001-0629.2004.10.006>

286 He Y., Tian Z.H., and Zheng Y.L.（2005）.High frequency plant regeneration from mature embryos

287 in vitro culture of tall fescue. *Pratacultural Science*, **22**, 23-28. <https://doi.org

288 /10.3969/j.issn.1001-0629.2005.06.006 >

289 He X.Q., Hu X.W., and Wang Y.R.（2010）. Study on seed dormancy mechanism and breaking

290 technique of *Leymus chinensis. Acta Bot. Boreal*, **30**, 120-125.

291 <https://doi.org /CNKI:SUN:DNYX.0.2010-01-021>

292 Qu T.B., Wang P.W., Guan S.Y, and Liu L.Z.（2004）. Establishment of regeneration system of

293 *Leymus chinensis* culture. *ACTA Pratacultural Sinica*, **13**,91-94. <https://doi.org

294 /10.3321/j.issn:1004-5759.2004.05.016 >

295 Yu Z., Li J.L., and Li G.Y.（2009）. Prospects of producing bio-ethanol fuel from

296 perennial forage.*Pratacultural Science*, **26**,62-69.

297 < https://doi.org/CNKI:SUN:CYKX.0.2009-09-016>

298 Sun J., Xiong J.B., Liu Y.Z., Kang J.M., Lv H.G., Yun J.F., and Sun Y.（2009）. Analysis on factors

299 causing the seed dormancy of *Buchloe dactyloides* (Nutt .) Engelm. *Acta Agrestia Sinica*, **17**,

300 665-669. < https://doi.org/ 10.3969/j.issn.1672-5190.2010.06.122>

Shi D.L., Zhao L.P., and Yao Z.G. (2007). Study on the treatment techniques of breaking dormancy of *Zoysia macrostachya* Franchet seeds. Journal of Anhui Agri. Sci., **35**, 11384-11385. < https://doi.org/10.3321/j.issn:1000-4025.2009.07.020>

Wu C.D., Shen M.X., Chu J.Y., and Gao P. (2006). On the capacity of accumulation and transfer of nitrogen and phosphorus in *Phalaris arundinacea* Linn in Mount Beigu Wetland. *Acta Scientiae Circum stantiae*, **26**,674-678. < https://doi.org/CNKI:SUN:HJXX.0.2006-04-025>

Xie J.H., Yun J.F., Yang B., and Hou J.H.(2006). Influence of 2,4 -D and BAP on callus induction and growth of immature embryo of wheat grass.*Chinese Journal of Grassland*, **28**,44-47. < https://doi.org/10.3321/j.issn:1673-5021.2006.02.009>

Zhang H., Zhang Y.L., and Gao K. (2012). Preliminary study on the dormancy mechanism and doemancy breaking method in *Phalaris arundinacea* seeds.*Chinese Journal of Grassland*, **34**, 7-12. < https://doi.org/10.3969/j.issn.1673-5021.2012.06.002>

Zhang Y., Zhang C.D., Li D.X., Liu G., You H.M., and Bai S.Q. (2007). Study on regional experiment of "Chuan Cao No.3".*Prataculture and Animal Husbandry*, **144**,9-14. < https://doi.org/10.3969/j.issn.1673-8403.2007.11.003 >

Zhou B.H., and Wang L.S.(1998). The research on ecological and biological characteristics and forage value of *Phalaris arundinacea*.*Chinese Grassland*, **1**,33-34. < https://doi.org/CNKI:SUN:CDXU.0.2008-06-021 >

Asbjörn L., and Olavi J. (1979). Germination and dormancy of reed canary-grass seeds (*Phalaris arundinacea*).*PlantPhysiology*,**45**,96-102.<https://doi.org/10.1111/j.1399-3054.1979.tb01671 >

Edwards M. (1969). Oxegen and coat effects in *Sinapis arvensis*. *J. Exp. Bot*., **20**,876-894.< https://doi.org/10.1016/j.exppara.2016.06.011>

Foley M., and Fennimorse S. (1998).Genetic basis for seed dormancy. *Seed Sci. and Technol*, **8**,173-182.

Iris L., Jonathan M., and Scur L. (2003). The development and current status of perennial rhizomatous grasses as energy crops in the US and Europe. *Biomass and Bioenergy*, **25**,335–361. < https://doi.org/10.1016/S0961-9534(03)00030-8>

Matthew F., Kevin J., Richard E., and Bruce S. (2010). Full-scale on-farm pretreatment of perennial grasses with dilute acid for fuel ethanol production. *Bioenerg. Res,* **3,** 335–341.< https://doi.org/10.1007/s12155-010-9092-4>

Meyer S., and Pendleton R. (2000).Genetic regulation of seed dormancy in *Purshia tridentate* (Rosaceae). *Ann. Bot*, **85**,521-529.< https://doi.org/10.1006/anbo.1999.1099>

Matus-Cádiz M., and Hucl P.（2005）. Rapid and effective germination methods for overcoming seed dormancy in annual canarygrass.*Crop Science*, **45**, 1696-1703.< https://doi.org/10.2135/cropsci2004.0347>

Nancy N., Bruce S., and Michael A. (2010). Fermentation of bioenergy crops into ethanol using biological abatement for removal of inhibitors. *Bioresource Technology*, **101**, 7545–7550. < https://doi.org/10.1016/j.biortech.2010.04.097>

Roberto L., and Joy Z. (2001). Effect of light on seed germination in *Phalaris arundinacea* L. (reed canary grass).*Plant Ecology*, **155**,75–78. < https://doi.org/10.2307/20051109>

Schilling K., and Kiniry J. (2007). Estimation of evapotranspiration by reed canarygrass using field observations and model simulations. *Journal of hydrology*, **(3-4)**,356-363. <

https://doi.org/10.1016/j.jhydrol.2007.02.003>

Sanderson M., and Adler P. (2008). Perennial forages as second generation bioenergy crops. *International Journal of Molecular Sciences*, **9**,768-788. < https://doi.org/10.3390/ijms9050768>

Vymazal J., and Kropfelova L. (2004). Growth of *Phragmites australis* and *Phalaris arundinacea* in constructed wetlands for wastewater treatment in the Czech Republic. *Ecological Engineering*, **25**,606-621. < https://doi.org/10.1016/j.ecoleng.2005.07.005>

Vose P. (1962). Delayed Germination in Reed Canary-Grass *Phalaris arundinacea* L.. *Annals of Botany*, **26**,197-206. < https://doi.org/10.1046/j.1365-2494.2003.00372>

Wrobel C., Coulman B., and Smith D.(2009). The potential use of reed canarygrass (*Phalaris arundinacea* L.) as a biofuel crop . *ACTA Agriculturae Scandinavica Section B-Soil and Plant Science*, **59**, 1-18. < https://doi.org/10.1080/09064710801920230>

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Figures.zip
- rawdata.xls