Postprint: Response of Three Wild Forage Grass Species at the Seedling Stage to Drought Stress in Tibet

Authors: Zhang Weihong, Liu Dalin, Miao Yanjun, Yan Tianfang, Lin Yonghe, Xu Yamei

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Abstract

Through pot experiments in a climate incubator, this study investigated the effects of drought stress on seedling growth characteristics and physiological indices of three wild forage grasses from Tibet (Leymus secalinus, Elymus nutans, and Elymus sibircus), and conducted a comprehensive evaluation of their drought resistance capabilities, aiming to provide a scientific theoretical basis for the selection and breeding of superior forage germplasm resources with strong drought resistance and for the establishment of artificial grasslands. The study found that with increasing drought stress duration, soil relative water content exhibited a trend of rapid decline followed by gradual decline, and the variation values of soil water content differed among the different forage grasses planted. The plant height increment, plant water content, and chlorophyll (Chl) content of the three wild forage grass seedlings all showed a decreasing trend with increasing drought stress duration; the free proline (Pro) content, malondialdehyde (MDA) content, and superoxide dismutase (SOD) activity in the plants all increased with increasing drought stress duration; the soluble sugar content showed a trend of first decreasing and then increasing with increasing drought stress duration: the soluble sugar content was lowest on the 5th day of drought stress, but the soluble sugar content of Elymus sibircus on the 20th day of drought stress remained lower than the control; the soluble protein content of Elymus nutans increased with prolonged drought stress duration, while the soluble protein content in Elymus sibircus and Leymus secalinus showed a trend of first decreasing and then increasing; the soluble protein content of Elymus sibircus was lowest on the 10th day of drought stress, whereas that of Leymus secalinus was lowest on the 5th day of drought stress. Using the subordinate function method to comprehensively evaluate eight indices of the three wild forage grasses at the seedling stage under drought stress, the order of drought resistance strength was: Leymus secalinus > Elymus nutans > Elymus sibircus. The results indicate that among the three forage grasses, Leymus secalinus has the strongest drought resistance capability and is suitable for planting in arid regions of Tibet; however, due to its lower sexual reproductive capacity, while Elymus nutans has stronger sexual reproductive capacity, it can be recommended as the preferred grass species for ecological restoration in arid and semi-arid regions of Tibet.

Full Text

Preamble

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Drought Stress Responses of the Seedlings of Three Wild Forages in Tibet

Zhang W H, Liu D L, Miao Y J, Yan T F, Lin Y H, Xu Y M. *Acta Ecologica Sinica*, 2017, 37(21): 7277-7285.

Affiliations:
1 College of Animal Science and Technology, Yangzhou University
2 Tibet Agricultural and Animal Husbandry College
3 College of Pastoral Agriculture Science and Technology, Lanzhou University

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Abstract

This study investigated the effects of drought stress on seedling growth characteristics and physiological indicators of three wild forage species native to Tibet—*Elymus nutans*, *Elymus sibiricus*, and *Leymus secalinus*—using pot experiments in a climate incubator. The drought resistance of these three forages was comprehensively evaluated to provide a scientific basis for breeding drought-tolerant germplasm resources and establishing artificial grasslands.

The results showed that as drought stress duration increased, soil relative water content initially decreased rapidly and then declined more gradually, with variation among the different forage species. Plant height increment, water content, and chlorophyll content of the three wild forage seedlings all decreased with prolonged drought stress. In contrast, free proline content, malondialdehyde (MDA) content, and superoxide dismutase (SOD) activity increased with drought duration. Soluble sugar content initially decreased and then increased as drought stress progressed, reaching minimum values at day 5 for all species. However, *E. sibiricus* soluble sugar content remained lower than the control even after 20 days of drought treatment. Soluble protein content in *E. nutans*

increased with drought duration, while *E. sibiricus* and *L. secalinus* showed an initial decrease followed by an increase. The lowest soluble protein contents in *E. sibiricus* and *L. secalinus* occurred during the first 10 days and first 5 days of drought stress, respectively.

Using the membership function method to comprehensively evaluate eight indicators under drought stress, *L. secalinus* demonstrated the strongest drought resistance among the three species, followed by *E. nutans* and *E. sibiricus*. However, due to its lower sexual reproductive capacity compared to *E. nutans*, which has strong sexual reproduction, *E. nutans* is recommended as the preferred species for ecological restoration in arid and semi-arid regions of Tibet.

Keywords: wild forages; drought stress; growth traits; physiological characteristics; drought assessment

Introduction

Tibet, as China' s largest pastoral region and the core of the Qinghai-Tibet Plateau (the "Third Pole" of the world), possesses vast natural grassland areas. The region is characterized by high average altitude (over 4,000 m) and diverse, complex climate types, with drought being the most prominent climatic feature affecting grassland ecosystems. Drought severely limits grassland productivity, and observations across the Qinghai-Tibet Plateau show that natural grasslands in water-sufficient areas grow significantly better than those in water-deficient regions. Drought affects plant water absorption, metabolism, enzymatic reactions, dormancy, and growth development, thereby reducing plant productivity. This has led to severe grassland degradation and acute forage-livestock conflicts in Tibet, significantly constraining local farmers' income and the sustainable development of animal husbandry [1-2, 5].

Researchers have found that many domestic and foreign forage varieties cannot complete their life cycles or produce adequate yields under the harsh growing conditions of the Qinghai-Tibet Plateau. The seedling stage is the most sensitive period for studying plant responses to stress [6]. Therefore, understanding the drought resistance characteristics of native Tibetan plants at the seedling stage and comprehensively evaluating their drought tolerance is critically important. Grassland plants have developed unique vegetation systems and rich germplasm resources through long-term adaptation to Tibet' s distinctive natural environment [7]. *Elymus nutans* (drooping wheatgrass), *Elymus sibiricus* (Siberian wildrye), and *Leymus secalinus* (ryegrass) are important native plants in Tibetan natural grasslands, valued for their high nutritional content, good palatability, and strong adaptability, with broad application prospects in Tibetan livestock production and ecological restoration [8-10].

Currently, drought is a major factor limiting the growth and development of natural grassland forages in Tibet [11]. How to improve the drought resistance of local plants, breed superior drought-tolerant germplasm, and maximize the economic value of grasslands has become a significant challenge for Tibet's forage industry. Building on previous research, this study examined changes in growth characteristics and physiological indicators of three wild forage species collected from Tibet under different drought stress conditions at the seedling stage, with the aim of providing a scientific theoretical basis for breeding drought-tolerant germplasm and establishing artificial grasslands.

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1. Test Materials

The experimental materials consisted of seeds of three wild forage species collected from Tibet: *Elymus nutans*, *Elymus sibiricus*, and *Leymus secalinus*. The materials were numbered using the first two capital letters of each species name plus a serial number.

Test Material Numbers and Sources

Material Name	Source	Altitude (m)
Elymus nutans	Collected from alpine meadow in Shenzha County, Tibet	4,680
Elymus sibiricus	Collected from alpine meadow in Nagqu County, Tibet	4,500
Leymus secalinus	Collected from sunny slope grassland with pebbles in Dazi County, Tibet	3,700

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2. Test Methods

The experiment began in March at the Alpine Grassland Laboratory of Tibet Agricultural and Animal Husbandry College. Pot cultivation was used, with pots having an inner diameter of 25 cm. Soil was collected from the teaching practice base of the Grassland Science Department at Tibet Agricultural and Animal Husbandry College. The soil was sandy with a pH of 5.77, total nitrogen content of 10.87 g/kg, hydrolyzable nitrogen of 1.05 g/kg, total phosphorus of 0.16 g/kg, available phosphorus of 41.60 mg/kg, and available potassium of 19.67 mg/kg. The maximum field water holding capacity was 28.17%.

Urea (10 g), diammonium phosphate (1.5 g), and potassium sulfate (1.0 g) were added per kilogram of soil. Stones, litter, and plant roots were removed, and the soil was oven-dried at 105°C. After natural cooling, 10 kg of the mixed soil was added to each pot. The three forage species were sown separately in pots at depths of 1 cm for *E. nutans* and 1.5 cm for *E. sibiricus* and *L. secalinus*.

Germination rate tests were conducted beforehand, showing 88.33% for *E. nutans*, 84.00% for *E. sibiricus*, and 43.17% for *L. secalinus*. When seedlings reached three leaves, thinning was performed to ensure 15 plants per pot after thinning. The pots were placed in an intelligent climate incubator at 15°C/25°C (12h/12h). All pots were watered normally until seedlings reached three leaves, then placed in the incubator without further watering for drought treatment.

Samples were collected at 5, 10, 15, and 20 days of drought treatment. Morphological indicators were measured, and samples were quickly wrapped in tin foil, rapidly frozen in liquid nitrogen, and stored in a refrigerator at -80°C [12]. For water content measurement, five plants were grouped as one sample.

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3. Measurement Indicators and Methods

- Soil water content and plant water content: oven-drying method
- Plant height change: measured with a ruler precise to 0.1 cm
- Free proline: ninhydrin method [13]
- Chlorophyll: alcohol immersion method [14]
- Malondialdehyde (MDA): thiobarbituric acid method [15]
- Soluble protein: Coomassie brilliant blue staining method [15]
- Superoxide dismutase (SOD): nitroblue tetrazolium photochemical reduction method [15]
- Soluble sugar: phenol method [15]

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4. Drought Resistance Evaluation Method

The membership function method [16] was used to comprehensively evaluate drought resistance using eight indicators: plant height, water content, free proline, chlorophyll, MDA, soluble sugar, soluble protein, and SOD activity under different drought stresses.

For indicator i, X represents the measured value, X_max represents the maximum value of indicator i across all varieties, and X_min represents the minimum value. The calculation formula is:

If the measured indicator is positively correlated with drought tolerance, the membership function value is calculated as: $(X - X\_min) / (X\_max - X\_min)$

If negatively correlated, the inverse membership function is used: (X_max - X) / (X_max - X_min)

After calculating the average value, comparisons were made. Larger average values indicate stronger drought resistance [17]. Data were processed and analyzed using Excel 2013 and SPSS 19.0, with nonlinear regression in SPSS 19.0 used for plant growth analysis.

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1. Changes in Soil Water Content During Different Drought Stress Periods

As drought stress duration increased, soil relative water content showed a pattern of rapid initial decline followed by gradual decrease. The rate of soil water content decline decreased over time. Significant differences existed in soil relative water content changes among pots planted with different forage species ($P < 0.05$). After 20 days of drought stress treatment, seedlings of the three wild forage species showed varying degrees of wilting, particularly with dried leaf tips. *E. sibiricus* showed wilting first, followed by *E. nutans*.

Changes in Soil Water Content During Drought Stress Periods (%)

Drought Stress Days (d)	*E. nutans*	*E. sibiricus*	*L. secalinus*
0	97.80±0.20a	97.47±0.06ab	97.23±0.25b
5	62.63±0.12a	59.73±0.55b	62.17±0.45a
10	32.50±0.20c	40.73±1.00a	37.57±0.06b
15	14.73±0.25c	23.27±0.23a	21.47±0.50b
20	11.70±0.10c	19.90±0.20a	18.87±0.65b

Note: Data are means. Different letters in the same column indicate significant differences ($P < 0.05$).

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2. Effects of Drought Stress on Seedling Height

Adversity often affects plant morphological characteristics. Although no significant differences were observed in seedling height among the three wild forage species at the seedling stage ($P > 0.05$), plant height increment and average growth rate decreased as soil relative water content declined and drought stress duration increased. The more severe the drought stress, the faster the average growth rate declined. *E. sibiricus* showed the smallest height change, while *L. secalinus* showed the greatest height increase.

Plant height changes were mathematically simulated [18]. *E. nutans* height increased by 23.82 cm, *L. secalinus* by 18.22 cm, and *E. sibiricus* by 14.24 cm. The growth equations during drought stress were:

- *E. nutans*: y = 79.57$e^{(-Be}$(-t))
- *E. sibiricus*: y = 57.97$e^{(-Be}$(-t))
- *L. secalinus*: y = 12.38$e^{(-Be}$(-t))

Where t represents growth time. The inflection point heights were 14.20 cm for *E. nutans*, 17.73 cm for *E. sibiricus*, and 14.24 cm for *L. secalinus*.

Changes in Plant Height During Drought Stress Periods (cm)

Drought Stress Days (d)	*E. nutans*	*E. sibiricus*	*L. secalinus*
0	9.18±2.56a	8.65±0.72a	9.58±2.13a
5	16.57±2.55a	13.85±3.25a	17.73±3.01a
10	21.30±2.66a	17.49±4.19a	24.73±4.50a
15	24.90±3.24a	20.67±5.72a	29.52±5.76a
20	27.40±4.06a	22.89±6.55a	33.40±5.75a

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3. Effects of Drought Stress on Seedling Water Content

Plant water content is an important indicator of water deficit during drought stress. Water content of all three wild forage species decreased with increasing drought stress duration, with significant differences observed ($P < 0.05$). The water content change under drought stress was smaller than under moderate and severe drought. Without drought stress, water content from highest to lowest was: *E. nutans* > *L. secalinus* > *E. sibiricus*. After 20 days of drought stress, the order was: *L. secalinus* > *E. nutans* > *E. sibiricus*. Different forage species showed varying degrees of water loss under drought stress.

Changes in Forage Water Content During Drought Stress Periods (%)

Drought Stress Days (d)	*E. nutans*	*E. sibiricus*	*L. secalinus*
0	74.16±0.06c	78.67±0.25a	76.46±0.27b
5	69.70±0.56b	73.16±0.16a	72.55±0.51a
10	61.84±0.45c	67.36±0.53b	69.70±0.66a
15	56.53±0.41c	60.43±0.42b	62.17±0.80a
20	46.41±0.35b	44.62±0.32c	53.89±0.39a

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4. Effects of Drought Stress on Free Proline Content

Proline is a sensitive osmotic adjustment substance in plants under drought stress, and its content correlates positively with drought resistance [19-21]. All three wild forage species showed increased proline content with intensifying drought stress, with *L. secalinus* showing the greatest increase, followed by *E. nutans*, and *E. sibiricus* the smallest.

Under non-drought conditions (CK), *E. sibiricus* had the highest proline content, with no significant difference between *E. nutans* and *L. secalinus*. After 5 days of drought stress, *L. secalinus* had the highest proline content, followed by *E. sibiricus*, with *E. nutans* the lowest (significant differences, $P < 0.05$). After 10, 15, and 20 days, *L. secalinus* consistently maintained the highest proline content, followed by *E. nutans*, with *E. sibiricus* the lowest (all significant differences, $P < 0.05$).

Free Proline (Pro) Content During Drought Stress Periods (g/g)

Drought Stress Days (d)	*E. nutans*	*E. sibiricus*	*L. secalinus*
0	91.94±0.81b	241.82±1.61c	550.94±3.73b
5	111.62±0.73a	273.99±1.80b	367.13±1.29c
10	148.82±2.96c	181.19±2.86c	243.22±1.98b
15	204.91±4.77c	181.67±5.28b	368.76±16.05a
20	225.99±5.01a	193.43±8.77a	481.81±24.68a

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5. Effects of Drought Stress on Chlorophyll Content

Chlorophyll content in all three wild forage species decreased with increasing drought stress duration. Significant differences existed among species ($P < 0.05$), with *L. secalinus* > *E. nutans* > *E. sibiricus*. The chlorophyll content reduction varied among species: *L. secalinus* decreased by 12.64 mg/g, *E. sibiricus* by 8.66 mg/g, and *E. nutans* by 9.99 mg/g.

Chlorophyll Content During Drought Stress Periods (mg/g)

Drought Stress Days (d)	*E. nutans*	*E. sibiricus*	*L. secalinus*
0	23.23±0.81b	19.81±0.23c	26.87±0.41a
5	20.19±0.23b	18.79±0.39c	23.04±0.28a
10	18.38±0.64b	16.21±0.62c	19.89±0.84a
15	14.90±0.30b	13.58±0.47c	16.20±0.58a
20	13.24±0.45b	11.15±0.35c	14.23±0.30a

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6. Effects of Drought Stress on Malondialdehyde (MDA) Content

MDA content in all three species increased with intensifying drought stress. *E. sibiricus* showed the greatest increase, followed by *E. nutans*, while *L. secalinus* showed the smallest increase. The MDA content increase rate was initially slow then accelerated. Under control conditions, *E. sibiricus* had the highest MDA content (39.65 nmol/g), followed by *E. nutans* (35.01 nmol/g), with *L. secalinus* the lowest (30.43 nmol/g). Under the same drought duration, significant differences existed among species ($P < 0.05$).

Malondialdehyde (MDA) Content During Drought Stress Periods (nmol/g)

Drought Stress Days (d)	*E. nutans*	*E. sibiricus*	*L. secalinus*
0	12.22±0.47a	13.22±0.48a	7.52±0.34b
5	15.98±1.16a	15.64±0.46b	12.43±0.31c
10	22.06±0.23b	34.59±0.53b	24.68±0.66a
15	29.08±0.25a	29.04±0.38c	37.95±0.07c
20	47.23±0.58b	52.87±1.03a	37.95±0.07c

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7. Effects of Drought Stress on Soluble Sugar Content

Soluble sugars are osmotic adjustment substances that enhance water uptake and reduce leaf osmotic potential, serving as carbon skeletons and energy sources for synthesizing other organic solutes and protecting enzymes at high inorganic ion concentrations [22]. The results showed that soluble sugar content in all three species initially decreased then increased with drought duration, reaching minimum values under mild drought stress. However, *E. sibiricus* soluble sugar content remained below control levels even after 20 days of drought.

Under non-drought conditions, *E. sibiricus* had the highest soluble sugar content, followed by *L. secalinus*, with *E. nutans* the lowest (significant differences, $P < 0.05$). Under mild and moderate drought, no significant differences existed among species. Under severe drought at day 15, *E. nutans* had the highest content, *E. sibiricus* the lowest, with significant differences among all three ($P < 0.05$). At day 20, *L. secalinus* had the highest content, *E. sibiricus* the lowest, also with significant differences ($P < 0.05$).

Soluble Sugar (SS) Content During Drought Stress Periods (mg/g)

Drought Stress Days (d)	*E. nutans*	*E. sibiricus*	*L. secalinus*
0	3.67±1.07c	6.81±0.27a	5.29±0.52b
5	3.11±0.47a	2.73±0.29a	3.99±0.62a

Drought Stress Days (d)	*E. nutans*	*E. sibiricus*	*L. secalinus*
10	3.52±0.96a	3.14±0.15a	4.13±0.10a
15	11.37±0.18a	10.63±0.48a	3.87±0.08c
20	7.28±0.77b	4.59±0.39b	9.86±0.46a

8. Effects of Drought Stress on Soluble Protein Content

Most soluble proteins in plants are enzymes involved in various metabolic processes, and their content measurement is important for understanding overall plant metabolism. Soluble protein content in *E. nutans* increased with drought duration, while *E. sibiricus* and *L. secalinus* showed an initial decrease followed by an increase. The lowest soluble protein content in *E. sibiricus* occurred at day 10 of drought stress, while in *L. secalinus* it occurred at day 5.

Different patterns were observed at various drought stages: under control conditions, *E. sibiricus* had the highest content; under mild drought, *E. nutans* was highest; under moderate drought, *L. secalinus* was highest and *E. nutans* lowest; under severe drought, the same pattern as moderate drought persisted.

Soluble Protein (SP) Content During Drought Stress Periods (g/g)

Drought Stress Days (d)	*E. nutans*	*E. sibiricus*	*L. secalinus*
0	52.20±0.90c	60.17±0.35a	57.50±1.20b
5	66.23±0.75a	45.80±0.50b	40.40±0.10c
10	65.70±0.20c	42.37±0.15b	62.30±0.10a
15	73.40±0.60a	71.00±0.80b	66.70±0.40c
20	77.70±0.20c	84.20±0.57b	86.10±0.10a

9. Effects of Drought Stress on Superoxide Dismutase (SOD) Activity

SOD activity in all three species showed a pattern of slow initial increase followed by rapid increase with drought duration. Under control conditions, *L. secalinus* had the highest SOD activity, followed by *E. nutans*, with *E. sibiricus* the lowest (no significant difference between the latter two, $P > 0.05$). After drought treatment, significant differences existed among species ($P < 0.05$), with *L. secalinus* highest, *E. nutans* intermediate, and *E. sibiricus* lowest.

Superoxide Dismutase (SOD) Activity During Drought Stress Periods (U/g)

Drought Stress Days (d)	*E. nutans*	*E. sibiricus*	*L. secalinus*
0	105.06±3.72b	147.20±3.18b	181.67±5.28b
5	96.73±1.27b	107.12±5.34c	148.82±2.96c
10	154.38±6.26a	193.43±8.77a	225.99±5.01a
15	221.21±7.35b	243.22±1.98b	368.76±16.05a
20	204.91±4.77c	181.19±2.86c	481.81±24.68a

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10. Drought Resistance Evaluation

The membership function method was used to comprehensively analyze eight indicators for the three wild forage species under drought stress. The results showed that *L. secalinus* had the largest average membership function value (0.54), indicating the strongest drought resistance, followed by *E. nutans* (0.44) and *E. sibiricus* (0.35). The drought resistance ranking was: *L. secalinus* > *E. nutans* > *E. sibiricus*.

Comparison of Drought Resistance Membership Functions of Three Wild Forage Species

Species	Membership Function Values by Indicator	Average	Drought Resistance Ranking
	Height	Water Content	Proline
E. nutans	0.45	0.31	0.35
E. sibiricus	0.28	0.21	0.29
L. secalinus	0.58	0.58	0.58

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3. Discussion

Soil moisture is a crucial factor for plant growth, playing a vital role in crop physiological activities [23]. Measuring soil relative water content helps understand actual field conditions for timely irrigation, moisture retention, or drainage to ensure normal plant growth, and serves as a basis for analyzing drought stress effects on plant indicators.

The experiment found that soil water content declined rapidly in early drought stages but slowed later, possibly because initial water loss occurred through both soil diffusion and plant transpiration [24]. Different forage species consumed soil water at different rates, indicating varying soil water conservation capacities among species.

Drought stress affects plant transpiration, height, water content, cell activity, and organ function. Superoxide dismutase provides anti-peroxidation protection for plant cells [25], but this study found that MDA content increased alongside SOD activity. This may occur because severe drought stress disrupts the balance between reactive oxygen species (ROS) production and scavenging, damaging proteins and nucleic acids [26-27] and leading to increased MDA content. However, plants also self-regulate by increasing free proline, soluble sugar, and SOD activity to adapt to drought [28-30]. Due to different genetic backgrounds, different forages show varying response patterns to drought stress indicators [10, 19, 31].

Soluble sugars and proteins are important osmotic adjustment substances [32]. Soluble sugars help maintain cell structure after dehydration, reducing drought damage and improving drought resistance [32-33]. The initial decrease in soluble sugar content across all three species may reflect consumption for metabolic adjustment or inhibited synthesis during early drought adaptation [34]. The subsequent increase represents accumulation to combat drought stress.

The decrease in plant height increment, water content, and chlorophyll content with drought duration may represent strategies to reduce water loss and maintain turgor pressure [5], but also results from chlorophyll degradation or synthesis inhibition [36]. Whether peroxidation reactions also cause chlorophyll synthesis inhibition or degradation in chloroplasts requires further investigation.

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4. Conclusion

Comprehensive evaluation of eight indicators using the membership function method revealed that *L. secalinus* had the best drought resistance, followed by *E. nutans*, with *E. sibiricus* the poorest. Although *L. secalinus* showed the strongest drought resistance, its low germination rate and poor sexual reproductive capacity [40-41] limit its ability to quickly form ground cover in ecological restoration, resulting in slow restoration effects. *E. nutans*, with high seed germination rate, strong sexual reproductive capacity, and ability to rapidly form ground cover, combined with relatively good drought resistance, is recommended as the preferred species for ecological restoration in arid and semi-arid regions of Tibet.

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Note: Figure translations are in progress. See original paper for figures.