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Shoot proliferation, callus induction and plant regeneration in *Tripsacum laxum* Nash

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Abstract

The peduncles of *Tripsacum laxum* Nash were used as explants to induce axillary shoots. Multiple shoots were proliferated on Murashige and Skoog (MS) medium to establish, for the first time, efficient shoot proliferation and plant *in vitro* regeneration systems. Optimal shoot proliferation medium was MS with 3.0 mg/L 6-benzyladenine (BA) and 0.2 mg/L α-naphthaleneacetic acid (NAA), resulting in a shoot proliferation coefficient of 11.0 within 45 d. Optimal rooting medium was MS with 0.1 mg/L NAA and/or 0.1 mg/L indole-3-butyric acid (IBA), inducing 100% root formation from shoots within 30 d. When young roots, leaf sheaths and shoot bases were used as explants, MS medium with 1.0 mg/L thidiazuron (TDZ) and 0.2 mg/L BA induced most shoots, with the least callus. Shoot bases induced beige-white callus and shoots directly on MS medium with 1.0 mg/L TDZ and 0.2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), while leaf sheaths induced beige-white callus and shoots directly on MS medium with 1.0 mg/L TDZ and 0.2 mg/L BA. Rooted plantlets showed 99.3% survival when transplanted into a substrate of vermiculite: peat soil (1:3, *v/v*).

Keywords: *Tripsacum laxum*; Axillary shoots; Callus; Adventitious shoots; Rooting; Regeneration

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BA; 6-benzyladenine; IBA, indole-3-butyric acid; KIN, kinetin; MS medium, Murashige and Skoog (1962) medium; NAA, α-naphthaleneacetic acid; PGR, plant growth regulator; SPC, shoot proliferation coefficient; TDZ, thidiazuron

Introduction

The genus *Tripsacum* (Maydeae tribe, Panicoideae, Gramineae) includes 16 species that grow in many ecologically distinct niches and habitats that are typically distributed in tropical and subtropical regions (Gray 1974; Wet et al. 1985). Since *Tripsacum* has a common ancestor with maize and teosinte, it may be important to better understand the origin and evolution of maize. *Tripsacum* is a perennial warm-season C_4 type of grass that is often used to produce high-quality forage and biomass energy, and control soil erosion (Zhao *et al*. 2020). *Tripsacum laxum* Nash (Guatemala grass) is widely used globally as a forage crop (Guyadeen 1951). Its strong roots and long period of growth and utilization allow it to be harvested for many years, and given its high yield, high nutritional value, and good taste, it is suitable for cutting green feed or process into silage material, and can thus be used as feed for cattle, ducks, geese and pigs (Guyadeen 1951; Boonman 1993). The roots of *T. laxum* develop well, and when tilled into soil and used as organic matter, this improves the physical and chemical structure of the soil, so it is often used as a multi-year cover crop (Shem et al. 1995). After *T. laxum* was introduced to China, it is now a major source of forage feed (Jiang *et al*. 2002; Zhong *et al*. 2011).

The diploid chromosome number of *T. laxum* is 2n = 72 (Dodds & Simmonds 1946; Zhong *et al*. 2011). Although most chromosomes are bivalents, there multiple chromosomal irregularities, ultimately resulting in male sterility (Dodds & Simmonds 1946). *T. laxum* is rarely propagated by stem cuttings because stems tend to shrink (Guyadeen 1951) and are prone to bacterial infections (Tuley 1961; Schieber 1975). To resolve limitations associated with proliferation and to overcome disease-related problems, the establishment of an *in vitro* regeneration system would allow this plant to be mass propagated and to create a platform that would allow for its genetic improvement through transgenic strategies. To our knowledge, there are no studies on the tissue culture or related biotechnologies of *T. laxum*. In this study, for the first time, we employed the peduncles of *T. laxum* as explants to induce axillary shoots that were then proliferated to establish an efficient *in vitro* regeneration system.

Materials and methods

Establishment of *in vitro* tissue culture

T. laxum plants growing on a farm in Guigang city, Guangxi province with taxonomy ID: 47471, were brought back to Guangzhou in 2010. All the studies comply with relevant institutional, national, and international guidelines and legislation. It has been specified under the appropriate permissions and licenses for the collection of plant specimens. Plants were propagated by cutting and grown in a test field of South China

Botanical Garden, Guangzhou, Guangdong Province. The plants flowered every year but no seed were produced (Fig. 1a). Stems were cut into 30 cm long cuttings, planted in a field and allowed to grow naturally. Plants were identified by Dr. Liu Qing, a botanist in South China Botanical Garden. When the plants began to flower, between March and April of 2016, young inflorescences of *T. laxum* were removed with a surgical knife (Fig. 1b). Segments (5 cm long) were first surface disinfected with 75% ethanol using cotton balls, dipped into 0.1% (w/v) mercuric chloride solution ($HgCl_2$) for 10 min, then washed three times with sterile distilled water. Surface-disinfected explants (2-3 cm long peduncles) were inoculated into Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) containing 1.0 mg/L 6-benzyladenine (BA) and 30 g/L sucrose. Medium pH was adjusted to 6.0 before being solidified with 0.7% (w/v) agar (Sigma-Aldrich, St. Louis, MO, USA), then autoclaved at 121°C for 20 min. Culture jars (height = 10 cm; diameter = 8 cm) were placed in an air-conditioned culture room at 25 ± 2°C with a 12-h photoperiod and 100 μM m^{-2} s^{-1} fluorescent light (Philips, Tianjin, China). Tissue culture conditions were identical to those used for another grass *Lepturus repens* (Xiong *et al*. 2021). After 15 d in culture, some axillary shoots buds (Fig. 1c) were induced from peduncle internodes. Axillary shoots were subcultured on the same medium every 45 d. When sufficient stock was proliferated, experiments were initiated.

Effects of plant growth regulators on axillary shoot proliferation

Using a similar technique as was employed for *Scaevola sericea* (Liang et al., 2020), axillary shoot clusters were cut into smaller clusters, each with three shoots. These were inoculated onto MS medium containing different combinations and concentrations of plant growth regulators (PGRs) for axillary shoot proliferation (Table 1). For each treatment, 10 jars were used. Each jar contained three shoot clusters. After culture for 45 d, axillary shoot proliferation coefficient (SPC) was assessed as: number of axillary shoots after proliferation for 45 d / number of axillary shoots before proliferation.

Adventitious root formation

Axillary shoots were separated and cultured on rooting medium (½MS) supplemented with different concentrations and combinations of IBA and NAA (Table 2). In each treatment, 10 jars were inoculated and each jar contained three shoots. PGR-free ½MS medium was used as the control. After 15 and 30 d of culture, rooting percentage was observed and assessed, as follows: (number of buds that rooted after 30 d / number of inoculated buds) × 100%.

Effects of plant growth regulators on callus induction from three explant types

Young roots, young leaf sheaths and shoot bases were used as explants. Roots were derived from 15 d-old plantlets that had been rooted in ½MS medium with 0.1 mg/L NAA. Roots were cut into 1.0 cm long explants. The young leaf sheaths and shoot bases were derived from shoots that had been proliferated on MS medium with 1.0 mg/L BA for 45 d. These tissues were cut into explants 0.5 cm^2 in size and inoculated onto MS-based media with different PGRs to induce callus and observe differentiation after 30 d (Tables 3-5).

Acclimatization and transplantation

Culture jars with shoots that were rooted in ½MS medium with 1.0 mg/L IBA for 30 d were transferred to natural light for 7 d. Using tap water, agar was gently rinsed off roots. Rooted plantlets were transplanted into plastic pots (height and diameter = 10 cm) containing yellow mud and peat soil (1:1, *v/v*), or peat and vermiculite (3:1, *v/v*). A single plantlet was planted in each plastic pot, and each treatment had 30 plantlets. Plants were watered every morning with tap water. After 30 d, plantlet height was determined. Survival percentage of transplanted plantlets was assessed as: (number of living plantlets before transplanting / number of living plantlets after transplanting for 30 d) × 100%.

Statistical analyses

All experiments were repeated three times within one week. Data are reported as mean ± SD (standard deviation). Means were statistically analyzed by one-way analysis of variance (ANOVA). Treatment means were considered to be significantly different from controls after applying Duncan's multiple range test ($P \leq 0.05$) using SPSS v. 19.0 (IBM, New York, NY, USA).

Results

Shoot proliferation on different media

BA induced shoots more effectively than KIN, as assessed by SPC, but not when its concentration exceeded 3.0 mg/L (Table 1). When BA was supplemented with 0.2 mg/L NAA, axillary shoot number increased significantly (Table 1; Fig 1d), with 3.0 mg/L BA and 0.2 mg/L NAA assessed as the optimal medium for shoot proliferation (Fig. 3a). When culture period was extended to 45 d, some shoots formed roots at their base (Fig. 3b), suggesting that rooting was easy.

Root formation

After 15 d, 67-75% of shoots induced roots when medium contained 0.1 mg/L NAA or IBA, or 100% if 0.1 mg/L of both these auxins were employed (Fig. 3c; Table 2). Control (no auxins) shoots did not induce roots within 15 d. However, after 30 d, 100% of shoots on any medium with an auxin formed roots (85% in the control) (Table 2).

Callus induction and adventitious shoot induced from root explants

When BA, 2,4-D and NAA were used alone, almost no callus was induced from root explants, and only TDZ induced some expansion of the root explant and the induction of some callus. In all cases, adventitious shoot buds developed (Fig. 2a). When TDZ and NAA were combined, the percentage of explants inducing callus increased to 13.3%, ultimately forming 2.2 adventitious shoot buds per explant after 30 d. Callus induction percentage (20% of explants) and number of adventitious shoot buds/explant (3.8) were largest on MS medium with 1.0 mg/L TDZ and 0.2 mg/L BA (Fig. 2b; Table 3).

Callus and adventitious shoot buds induced from young sheath explants

PGRs, when used alone, or a combination of BA/TDZ with NAA, could not induce callus from young sheath explants while TDZ with BA induced a low frequency (3.3-4.3%) of callus after 30 d. This callus was granular and beige-white (Fig. 2c). Adventitious shoot buds were visible after 30 d. Optimal medium contained 1.0 mg/L TDZ and 0.2 mg/L BA, resulting in highest callus induction frequency (30.8%) most adventitious shoot buds/explant (4.6) (Fig. 2d; Table 4).

Callus inducing from shoot basal meristem explants

When TDZ or 2,4-D were used alone, some hyperhydric pink callus was induced, but it was unable to differentiate, and eventually turned brown and died. BA did not induced callus, instead inducing adventitious shoots from callus. When 2,4-D was combined with BA and TDZ, they induced a low frequency of callus in 1-2% of explants after 30 d (Table 5). Milky white or yellow granular callus possessed a strong ability to develop adventitious shoot buds directly, especially the combination of 1.0 mg/L TDZ and 0.2 mg/L 2,4-D (9.2 adventitious shoot buds/explant) (Fig. 2e, 2f), followed by 1.0 mg/L TDZ and 0.2 mg/L BA (5.3 adventitious shoot buds/explant) (Table 5).

151

152 **Acclimatization and transplanting**

153 Both treatments resulted in a high survival percentage, 99.3% in vermiculite: peat (1:3, *v/v*), and 96.7% in
154 yellow mud and peat (1:1, *v/v*) (Table 6; Fig. 3d).

155

156 **Discussion**

157 The tissue culture of several species of the Gramineae employed various explants. For example, callus was
158 induced from leaves in sugarcane on MS with 1.0 mg/L 2,4-D (Garcia *et al*. 2007), callus were induced from
159 meristem tips in MS with 4.0 µM BA and 40.0 µM NAA (Lakshmanan *et al.* 2006; Tang *et al*. 2011), and
160 callus were induced from sorghum immature embryos in MS with 2.0 mg/L 2,4-D (Assem, et al. 2014). In our
161 experiment, we selected peduncles as explants because seed are not produced in the wild (Dodds and
162 Simmonds 1946; Zhong *et al*. 2011). Since explants derived from field-grown plants are easy to become
163 contaminated *in vitro* after inoculation on medium, despite surface disinfection, peduncles were selected as
164 explants, reducing contamination-associated problems to about 3% in our initial trial.

165 Axillary shoot proliferation (i.e., SPC) was enhanced in the presence of a cytokinin and NAA (Table 1),
166 similar to the tissue culture of *Lepturus repens*, another Gramineae plant (Xiong *et al*. 2021). In *T. dactyloides*,
167 mature zygotic embryos were used to induce embryogenic callus cultures on MS medium with dicamba (10 or
168 20 µM) and sucrose (3 or 6%), while plantlets were regenerated on PGR-free MS medium containing 2%
169 sucrose (Furini and Jewell 1991). In our study on *T. laxum*, only TDZ was able to induce callus from root
170 explants, while the further addition of BA also stimulated shoot formation (Table 3). In dicotyledonous plants,
171 the use of TDZ or BA are popular PGRs to induce shoot buds (Zhang *et al.* 2017; Liang *et al.* 2020), although
172 TDZ might also induce somaclonal variation (Dewir *et al*. 2018). In monocotyledonous plants, 2,4-D has been
173 used to induce callus and shoots from roots in rice (Guo *et al*. 2018) and maize (Wang *et al*. 2021).

174 The base of leaf sheaths were used as explants to induce callus, although only TDZ combined with BA
175 successfully induced callus, which differentiated into adventitious buds (Table 4). Transverse sections of
176 young leaf spindle rolls in sugarcane oriented distal end into medium were critical for shoot regeneration,
177 which was observed within 3 weeks on MS medium with 10-60 µM NAA and 4-8 µM BA (Lakshmanan *et al.*
178 2006). Sugarcane explants cultured in the dark, and exposed to 4.5 µM 2,4-D, induced callus from stem
179 parenchyma while pre-embryogenic masses formed from immature leaves (Garcia *et al*. 2007).

180 In *Sorghum bicolor*, callus was induced from thin seedling-derived root or epicotyl explants when KIN or

BA were used (Gendy *et al*. 1996). The use of 3.0 mg/L BA and 1.0 mg/L TDZ in MS most efficiently induced multiple shoots from immature seeds of *S. bicolor* (Liu *et al*. 2015). When *S. bicolor* leaf bases were used as explants, callus was induced and ultimately plantlets could be regenerated on MS medium with 2.0 mg/L 2,4-D (Mishra *et al*. 2003). Also in *S. bicolor*, most callus was induced on MS medium containing honey and sucrose (80.0% of explants), and when further supplemented with BA, shoots were induced (Dreger *et al*. 2019). The use MS medium with 4.0 mg/L 2,4-D and 0.2 mg/L BA could induce callus from maize zygotic embryos (Huang *et al*. 2004).

Conclusion

We developed a protocol for the regeneration of shoots from *T. laxum* peduncles via a direct route and an indirect (callus-induced) route. The development of a protocol that will allow for the mass propagation of this plant, will allow the resource allocation needs of this forage crop to be met, and allow for additional research such as genetic engineering to fortify abiotic stress tolerance.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

GHM designed the experiment and provided guidance for the study. YPX and JHP prepared samples for all analyses. KLW, XHZ, YL and SJZ participated in the statistical analyses. YPX and GHM were also involved in all statistical analyses and co-wrote the manuscript. JATdS provided interpretation of the experimental data, and co-wrote and edited the manuscript. All authors read and approved the manuscript for publication.

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270

Table 1 Effect of PGRs in MS medium on SPC of *Tripsacum laxum* after 45 d.

PGRs (mg/L)	SPC
BA 1.0	4.9 ± 0.4 d
BA 3.0	6.9 ± 0.4 b
BA 5.0	7.0 ± 0.4 b
BA 1.0 + NAA 0.1	6.1 ± 0.3 c
BA 3.0 + NAA 0.1	11.0 ± 0.5 a
BA 5.0 + NAA 0.1	10.8 ± 0.5 a
KIN 1.0	3.1 ± 0.4 f
KIN 3.0	5.1 ± 0.5 d
KIN 5.0	5.2 ± 0.3 d
KIN 1.0 + NAA 0.1	4.0 ± 0.3 e
KIN 3.0 + NAA 0.1	5.9 ± 0.4 c
KIN 5.0 + NAA 0.1	6.2 ± 0.3 c

Values represent means ± SD. Different letters within a column indicate significant differences according to the Duncan's multiple range test ($P \leq 0.05$). n = 30 per treatment. BA; 6-benzyladenine; KIN, kinetin; NAA, α-naphthaleneacetic acid; SPC, shoot proliferation coefficient.

277 Table 2 Rooting of *Tripsacum laxum* in ½MS medium supplemented with different auxins.

Auxins (mg/L)	Rooting percentage at different culture periods (d)	
	15 d	30 d
Control	0 c	78.7 ± 7.3 b
NAA 0.2	67.4 ± 5.3 b	100 a
IBA 0.2	74.3 ± 6.7 b	100 a
NAA 0.2 + IBA 0.2	100 a	100 a

278 Values represent means ± SD. Different letters within a column indicate significant differences according to
279 Duncan's multiple range test ($P \leq 0.05$). n = 30 per treatment. IBA, indole-3-butyric acid; NAA,
280 α-naphthaleneacetic acid.
281
282

283 Table 3 Effect of PGRs in MS medium on callus induction and adventitious bud differentiation from young
284 root explants of *Tripsacum laxum* after culture for 30 d

PGRs (mg/L)	Roots forming callus (%)	Callus differentiation into shoots (%)
2,4-D 1.0	2.8 ± 1.2 d	0 c
NAA 1.0	2.4 ± 1.3 d	0 c
BA 1.0	3.6 ± 1.2 d	0 c
BA 1.0 + NAA 0.2	4.1 ±1.4 d	0 c
TDZ 1.0	10.3 ± 1.1 c	1.6 ± 0.5 b
TDZ 1.0 + NAA 0.2	13.3 ± 0.9 b	2.2 ± 0.6 b
TDZ 1.0 + BA 0.2	20.0 ± 1.2 a	3.8 ± 0.8 a

285 Values represent means ± SD. Different letters within a column indicate significant differences according to
286 Duncan's multiple range test ($P \leq 0.05$). n = 30 per treatment. 2,4-D, 2,4-dichlorophenoxyacetic acid; BA;
287 6-benzyladenine; NAA, α-naphthaleneacetic acid; PGR, plant growth regulator; TDZ, thidiazuron
288

289 Table 4 Effect of PGRs in MS medium on callus induction and adventitious shoot formation from young leaf
290 sheath explants of *Tripsacum laxum* after culture for 30 d

PGRs (mg/L)	Callus induction (% of explants)	Number of shoots/explant (%)	Callus induction and differentiation
2,4-D 1.0	3.5 ± 1.2 d	0 c	Little callus, no shoots
NAA 1.0	3.3 ± 1.1 d	0 c	Little callus, no shoots
BA 1.0	4.3 ± 1.3 d	0 c	Little callus, no shoots
TDZ 1.0	3.5 ± 1.4 d	0 c	Little callus, no shoots
BA 1.0 + NAA 0.2	4.4 ± 1.5 d	0 c	Little callus, no shoots
BA 2.0 + NAA 0.2	5.6 ± 1.6 d	0 c	Little callus, pink, no shoots
TDZ 0.2 + BA 1.0	15.1 ± 1.4 c	2.3 ± 0.5 b	Beige-white, shoots
TDZ 0.2 + BA 2.0	16.7 ± 1.6 c	2.1 ± 0.6 b	Beige-white, shoots
TDZ 1.0 + NAA 0.2	4.6 ± 1.3 d	0 c	Little callus, no shoots
TDZ 2.0 + NAA 0.2	6.3 ± 1.5 d	0 c	Little callus, no shoots
TDZ 1.0 + BA 0.2	30.8 ± 3.5 a	4.6 ± 0.8 a	Beige white, shoots
TDZ 2.0 + BA 0.2	23.1 ± 2.4 b	3.5 ± 0.7 a	Beige white, shoots

291 Values represent means ± SD. Different letters within a column indicate significant differences according to
292 Duncan's multiple range test ($P \leq 0.05$). n = 30 per treatment. 2,4-D, 2,4-dichlorophenoxyacetic acid; BA;
293 6-benzyladenine; NAA, α-naphthaleneacetic acid; PGR, plant growth regulator; TDZ, thidiazuron.
294
295

296 Table 5 Effect of PGRs in MS medium on callus induction and differentiation into shoot buds from shoot

297 bases of *Tripsacum laxum* after culture for 30 d

PGRs (mg/L)	Callus induction (% of explants)	Callus description	Number of adventitious shoots/explant
2,4-D 1.0	91.7 ± 5.2 a	Brown, hyperhydric	0 f
TDZ 1.0	41.7 ± 2.6 e	Yellow, compact	0 f
BA 1.0	11.3 ± 1.5 f	Compact	0 f
2,4-D 1.0 + BA 0.2	75.1 ± 3.3 c	Brown	1.1 ± 0.3 e
2,4-D 1.0 + NAA 0.2	92.7 ± 7.3 a	Friable, pink	1.3 ± 0.3 e
2,4-D 1.0 + TDZ 0.2	91.5 ± 6.2 a	Beige-white, yellow	2.2 ± 0.4 d
TDZ 1.0 + BA 0.2	50.0 ± 3.4 d	Beige, yellow	5.3 ± 0.4 b
TDZ 1.0 + NAA 0.2	75.0 ± 3.2 c	Beige-white	3.7 ± 0.5 c
TDZ 1.0 + 2,4-D 0.2	83.3 ± 3.5 b	Beige-white, yellow	9.2 ± 0.3 a

298 Values represent means ± SD. Different letters within a column indicate significant differences according to

299 Duncan's multiple range test ($P \leq 0.05$). n = 30 per treatment. 2,4-D, 2,4-dichlorophenoxyacetic acid; BA;

300 6-benzyladenine; NAA, α-naphthaleneacetic acid; PGR, plant growth regulator; TDZ, thidiazuron

301

302

303 Table 6: *Tripsacum laxum* plantlet survival and height in different substrates after 30 d

Substrates	Survival (%)	Plant height (cm)
Vermiculite: peat (1:3)	99.3 ± 0.7 a	25.3 ± 3.7 a
Yellow mud: peat (1:1)	96.7 ± 0.8 b	15.4 ± 2.5 b

304 Values represent means ± SD. Different letters within a column indicate significant differences according to
305 Duncan's multiple range test ($P \leq 0.05$). n = 30 per treatment.

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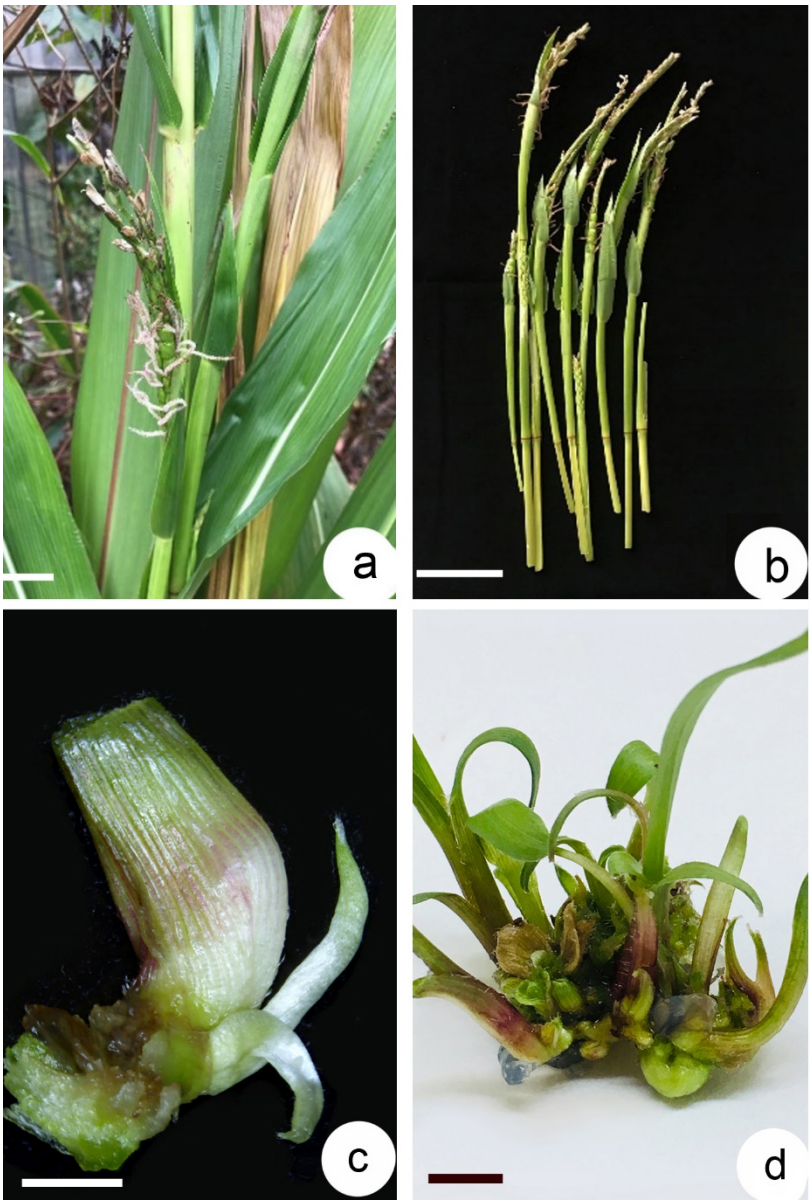

Fig. 1 Induction of axillary shoots from *Tripsacum laxum* peduncle explants derived from immature inflorescences. (a) Flowering plants growing outside a greenhouse (March, 2019); (b) peduncle explants collected from immature inflorescences of plants growing outdoors; (c) a few axillary shoot buds were induced from a peduncle explant on MS medium with 1.0 mg/L BA within 30 d; (d) multiple axillary shoots were induced from a peduncle explant on MS mdium with 3.0 mg/L BA and 0.1 mg/L NAA within 60 d. Bars: 2 mm (c, d); 1 cm (a, b).

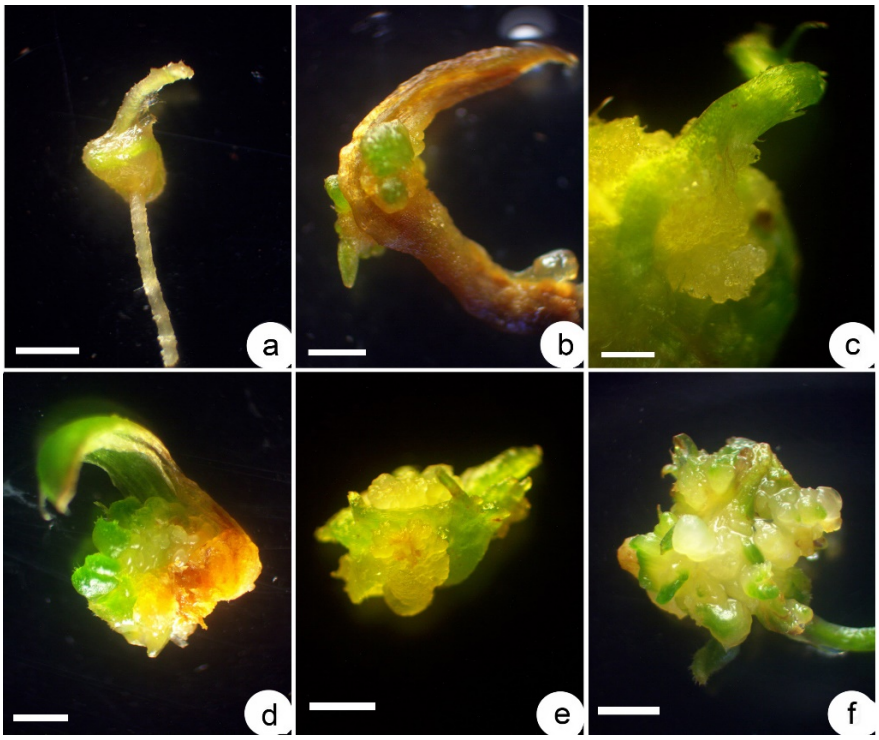

Fig. 2 Callus induction and differentiation of adventitious shoots from various explants (immature roots, young sheaths, base of shoots) of *Tripsacum laxum.* (a) expansion of immature root explants and induction of hard callus within 20 d on MS medium with 1.0 mg/L BA and 0.2 mg/L NAA. (b) expansion of immature root explants and induction of shoot buds within 30 d on MS medium with 1.0 mg/L TDZ and 0.2 mg/L NAA. (c, d) induction of friable callus and shoot buds within 30 d from young sheath explants on MS medium with 2.0 mg/L TDZ and 0.2 mg/L BA. (e, f) induction of friable callus and adventitious shoots buds from shoot base explants within 30 d on MS medium with 1.0 mg/L TDZ and 0.2 mg/L NAA. Bars = 3.0 mm.

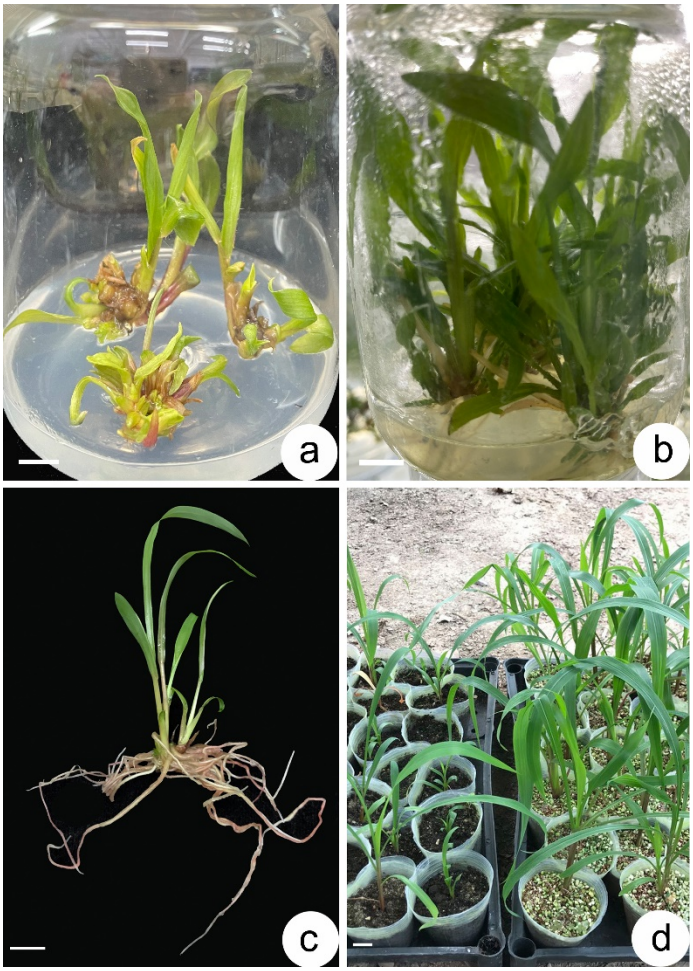

Fig. 3 Shoot proliferation, rooting, transplanting and acclimatization of *in vitro*-derived *Tripsacum laxum* plantlets. (a) shoot proliferation on MS medium with 2.0 mg/L BA and 0.1 mg/L NAA for 25 d; (b) shoot proliferation on MS medium with 2.0 mg/L BA and 0.1 mg/L NAA for 45 d, with the formation of some small roots at the base of multiple shoots; (c) rooting of shoots on ½MS medium with 0.2 mg/L IBA and 0.2 mg/L NAA for 30 d; (d) rooted plantlets were transferred to plastic pots containing peat and yellow mud (1:3, *v/v*) (left) and peat soil and vermiculite (3:1, *v/v*) (right) after 30 d, with more robust growth of plantlets on the right (also see Table 6). Bars = 1.0 cm.