Pak. J. Bot., 48(4): 1439-1445, 2016.

IMPROVEMENT OF SEED GERMINATION AND *IN VITRO* PROPAGATION OF A MULTIPURPOSE PLATEAU SHRUB SPECIES *SOPHORA MOORCROFTIANA*

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Abstract

Sophora moorcroftiana (Benth.) Baker (Fabaceae) with blue flowers is a shrub species in Qinghai-Tibet Plateau. This species is an important ornamental, ecological and economical species mainly distributed in the wide valleys of Yarlung Tsangpo and Lhasa River. It contains strong drought, solar radiation and sand burial resistance. However, it is on the verge of extinction because of over-exploitation in recent years, seedling production is a bottleneck in this plant's development, and information is lacking on an *In vitro* propagation system for *S. moorcroftiana*. Therefore, an effective propagation technique is urgently needed. In the present study, *S. moorcroftiana* seed germinations both *In vitro* and on an open moistened filter paper were significantly improved. The best germination percentage (88.67±0.67) and percentage of cotyledons presented (69.67±0.33) were obtained when the seeds were placed in moistened filter paper for 30 d after treatment with 98% concentration sulphuric acid for 70 min, and then treated with either Gibberellic acid (GA_3) 100 mg L^{-1} or 500 mg L^{-1}. Besides, an *In vitro* propagation system was developed for this species, the impacts of various media combined with different plant growth regulators (PGR) on multiple shoots proliferation were evaluated. The result showed Woody Plant Medium (WPM) supplemented with 1.0 mg L^{-1} N^6-Benzyladenine (BA) and 0.2 mg L^{-1} α-Naphthalene acetic acid (NAA) was found to be optimum for production of multiple shoots (89.33% shoot regeneration frequency and 18.67 shoots per explant) using cotyledonary node. The best rooted medium for regenerated shoots (89.72%, 4.68 roots per shoot) was half-strength Murashige and Skoog (1/2 MS) supplemented with 0.5 mg L^{-1} indole-3-butyric acid (IBA) and 0.02 mg L^{-1} NAA. The rooted plantlets were successfully acclimated, and survival of plantlets was 75% after transfer to soil. These results could be applied to rapid generation and conservation of this species in the future.

Key words: *Sophora moorcroftiana*; Seed germination; Cotyledonary node; Nodal explants; Plant regeneration; Plant growth regulators.

Introduction

Sophora moorcroftiana (Benth.) Baker (Fabaceae) with blue flowers is a shrub species in Qinghai-Tibet Plateau, China. It is mainly distributed in the wide valleys and the middle reaches of several main tributaries of Yarlung Tsangpo River (elevation in the range 2,800 m to 4,400 m) (Chen *et al*., 1978). As one of the few ornamental plant species in the Plateau, its flowering could last for a month. This specific *Sophora* species has strong resistance to drought and solar radiation (Zhao *et al*., 2007; Guo *et al*., 2014), and it also provides accessible resources for forage of local animal, fuel woods and traditional medicine of local people (Ma *et al*., 2006). In recent years, this species is on the verge of extinction because of over-exploitation, and no artificial culturing method for this species was reported so far.

Mature seeds of many woody plant species are dormant. The mechanisms underlying the restriction of germination vary among species. Generally, leguminosae species have low germination percentage in the wild because of their impervious, thick, and rigid seed coat, which prevents water entry, gaseous exchange, and radical emission (Nongrum *et al*., 2013; Baskin *et al*., 2004). However, germination of many leguminous species increases in response to pretreatments, such as oven-dry-heat (Ajiboye *et al*., 2011), warm water, physical scarification (Green *et al*., 2002), hot or boiling water, cold water, acid scarification (Nasr *et al*., 2013; Ruter *et al*., 1991; Fernandez *et al*., 2015), gibberellic acid (Jacobsen *et al*., 2002; Bewley *et al*., 1997), indole-3-butyric acid, potassium nitrate, salinity (Nasr *et al*., 2013), sand burial (Zhao *et al*., 2007) and others.

In vitro propagation allows the cost-effective production of a high volume of elite planting materials throughout the year without any seasonal constraints. This technology can lead to rapid multiplication, germplasm conservation, and genetic transformation (Tomar *et al*., 1998; Schwarz and Beaty, 2000). *In vitro* regeneration of some plantlet has been reported previously, such as *Cassia alata* (Ahmed *et al*., 2013), *Clitoria ternatea* (Ismail *et al*., 2011), *Withania somnifera* (Nayak *et al*., 2013), *Acacia mangium* (Shahinozzaman *et al*., 2012), *Eucalyptus saligna* (Da Silva *et al*., 2015).

The germination percentage of *S. moorcroftiana* is low under natural conditions, information on its germination percentage and propagation *In vitro* is lacking. Therefore, the goal of this project was to break seed dormancy and to develop a reliable protocol for *In vitro* propagation of *S. moorcroftiana*.

Materials and Methods

Plant materials and treatments: In February 2013, seeds of *S. moorcroftiana* were collected from the Shigatse, Tibet (3,948 m, 29°18′N, and 89°33′E) and

stored at room temperature. Seeds were deep soaked and agitated in concentrated sulfuric acid for 0, 10, 30, 50, 70, 90, 120, 150, 180, and 210 min and then washed in running tap water for 30 min. Subsequently, the treated seeds were transferred to Petri dishes containing moistened filter paper in a chamber at 24°C. A seed was considered germinated when the radicle protruded through the seed coat. Germination percentages and percentage of cotyledons were evaluated 10, 20, and 30 d after the treatment. For plant growth regulator (PGR) treatment, seeds were deep-soaked in one of the PGR treatment concentrations [0, 50, 100, and 150 mg L^{-1} indole-3-butyric acid (IBA); 0, 50, 100, and 500 mg L^{-1} gibberellic acid (GA_3)] for 2 d at 30°C after sulfuric acid treatment for 70 min. Some seeds were sterilized in a chamber for *In vitro* culture, and other seeds were incubated on moistened filter paper in a chamber at 24°C under cool-white fluorescent light (16 hours photoperiod). Germination and percentage of cotyledons were evaluated after 10, 20, and 30 d of treatment.

***In vitro* germination and culture conditions:** *In vitro* seed germination was performed to ensure sterile conditions for the growth of seedlings and seed-derived explants. Seeds were sterilized as follows: (1) surface sterilization for 1 min in 75% (*V/V*) ethanol and rinsing thrice with sterile distilled water; and (2) immersion in 0.1% (*W/V*) aqueous solution of mercuric chloride ($HgCl_2$) for 8 min, followed by washing eight times in sterile distilled water. After sterilization, seeds were placed on full strength Murashige & Skoog (1962) (MS) medium or woody plant medium (WPM) (Lloyd & MoCown, 1980) supplemented with 3% sucrose and 0.65% agar. Before autoclaving, the medium's pH was adjusted to 5.8-6.0. After inoculation, the cultures were maintained at 24°C and incubated under cool-white fluorescent light (16 h photoperiod). Strong seedlings were selected for the following experiments when true leaves were approximately 1 cm long.

Multiple shoots induction: For multiple shoot induction, different types and concentrations of PGR were used. Primary shoots, apical points, and most of the parts of the radicles were carefully removed from 8 d to 10 d old axenic seedlings. The cotyledonary nodes contained two cotyledons and 3 mm radicles. The radicles were artificially damaged and transferred to the MS and WPM media supplemented with different combinations of N^6-benzyladenine (BA) (1, 2, and 3 mg L^{-1}) and α-naphthalene acetic acid (NAA) (0 and 0.2 mg L^{-1}). Eight PGR combinations were tested. After 20 d, the cotyledonary nodes were transferred to the same MS or WPM media for further shoot elongation. The percentage of explants forming shoots, mean number of shoots per explant, and mean shoot length (cm) were calculated after 60 d.

Rooting of *In vitro*-regenerated multiple shoots: After one sub-culture for shoot multiplications, small shoots (3-4 cm length) were excised and transferred to 1/2 MS supplemented with the PGRs for *In vitro* rooting, as follows: 3% sucrose, 0.65% agar, and NAA (0.3 mg L^{-1}); IBA (0.05 mg L^{-1}); NAA (0.3 mg L^{-1}) in combination with IBA (0.05 mg L^{-1}); and NAA (0.02 mg L^{-1}) in combination with IBA (0.5 mg L^{-1}). The rooting percentage, mean root length, number of roots per shoot, and root status were recorded and calculated at 45 d of culture.

Plantlet acclimatization: The plantlets with three or more stout roots were washed gently under running tap water to remove the agar. The plantlets were transferred to autoclaved soil and covered with transparent polythene bags to ensure maximum humidity. For primary acclimatization of transferred plantlets, the potted plantlets were maintained in a chamber for 15 d. The polyethylene bags were gradually removed after 6 d. The plantlets were watered once with 1/2 liquid MS at 2 or 3 d. The plantlets were transferred to large clay pots containing garden soil in a chamber for 15 d before transferring to a greenhouse.

Statistical analysis: The experimental unit was established using 100 seeds for each germination treatment, 100 explants for each combination for multiple shoot inductions, and 100 shoots for each combination for root induction. Each experiment was performed thrice. SPSS 17.0 was used to analyze the data. The data were subjected to one-way ANOVA. A significance level of 5% was used for all the statistical analysis. All data homogeneity was tested by using Levene's test. If the tests were not significant, data are not transformed. Tukey test (Tukey, 1949; Song *et al*., 2013) was utilized to detect the effect of 98% concentrated sulfuric acid to germination percentage and percentages of cotyledons for multiple comparisons among means. Duncan's new multiple range test (DMRT) (Gomez and Gomez, 1984) was used to separate the mean values of multiple shoot induction, nodal explants proliferation, and rooting for significant effect.

Results

Seed germination: Treatment with 98% concentrated sulfuric acid showed significant effect on seed germination ($F_{9, 20}$ = 708.64, p = 0.000; $F_{9, 20}$ = 1312.88, p = 0.000; $F_{9, 20}$ = 1891.38, p = 0.000) and the percentages of cotyledons ($F_{9, 20}$ = 434.72, p = 0.000; $F_{9, 20}$ = 502.91, p = 0.000; $F_{9, 20}$ = 501.55, p = 0.000) after 10, 20, 30 d. When the seeds were incubated in concentrated sulfuric acid for different durations, the germination percentage ranged from 16.00% to 72.33% after 30 d. Germination was not observed in the control (Table 1). Seeds treated with concentrated sulfuric acid for 70 min showed the highest germination percentage (72.33%), and the highest percentage of cotyledons were 35.33%, 42.67%, and 43.67%, respectively, after 10, 20, and 30 d of culturing, pretreated with concentrated sulfuric acid for 70 min in a chamber (Table 1).

To evaluate germination on moistened filter paper in open Petri dishes, we treated the seeds with concentrated sulfuric acid for 70 min and with different PGRs (GA_3 and IBA) for 2 d and then incubated for 10, 20, and 30 d. Those different treatments showed significant effect on seed germination ($F_{6, 14}$ = 92.49, p = 0.000; $F_{6, 14}$ = 103.50, p = 0.000; $F_{6, 14}$ = 179.73, p = 0.000) and the percentages of cotyledons ($F_{6, 14}$ = 125.00, p = 0.000; $F_{6,}$

$_{14}$ = 215.80, p = 0.000; $F_{6, 14}$ = 334.94, p = 0.000) after 10, 20, 30 d. The highest germination was showed by GA_3 (100 mg L^{-1}) treatment, in which the highest germination percentages were 82.33%, 88.33%, and 88.67% after 10, 20, and 30 d, respectively. The best result of cotyledon germination from seeds was showed by IBA (100 mg L^{-1}) treatment at 10 d. However, the best result of cotyledon germination was showed by GA_3 (500 mg L^{-1}) treatment at 20 and 30 d, the highest percentage of cotyledons were 69.33% and 69.67% after 20 and 30 d of culture in a chamber, respectively (Table 2). *In vitro*, different treatments showed significant effect on seed germination ($F_{6, 14}$ = 92.95, p = 0.000; $F_{6,}$ $_{14}$ = 106.78, p = 0.000; $F_{6, 14}$ = 98.12, p = 0.000) and the percentages of cotyledons ($F_{6, 14}$ = 88.36, p = 0.000; $F_{6,}$ $_{14}$ = 126.97, p = 0.000; $F_{6, 14}$ = 215.21, p = 0.000). Germination and percentage of cotyledons from seeds subjected to the same treatments were significantly lower *In vitro* than those in opened moistened filter paper after 10, 20, 30 d. Germination percentages were 65.67%, 69.33%, and 70.67% under *In vitro* condition after 10, 20, and 30 d, respectively. Percentage of cotyledons were 47.33% and 47.67% under *In vitro* condition, whereas 69.33% and 69.67% on opened moistened filter paper after 20 and 30 d of culture, respectively (Tables 2 and 3).

Table 1. Germination percentage and percentage of cotyledons for *S. moorcroftiana* treated with concentrated sulfuric acid for 10 min to 210 min

Duration of treatment (min)	Germination percentage (%)			Percentage of cotyledons (%)		
	10 d	20 d	30 d	10 d	20 d	30 d
0	0.00 (±0.00) i †	0.00 (±0.00) j	0.00 (±0.00) j	0.00 (±0.00) g	0.00 (±0.00) g	0.00 (±0.00) g
10	14.67 (±0.67) h	15.33 (±0.33) i	16.00 (±0.58) i	12.33 (±0.33) e	12.67 (±0.33) e	13.00 (±0.58) e
30	22.67 (±0.67) g	26.00 (±0.58) h	26.33 (±0.88) h	16.33 (±0.33) d	19.67 (±0.88) d	20.33 (±0.33) d
50	48.00 (±0.58) c	59.67 (±0.33) b	60.00 (±0.58) b	33.33 (±0.88) b	35.67 (±0.33) b	37.00 (±0.58) b
70	67.67 (±0.88) a	72.00 (±0.58) a	72.33 (±0.33) a	35.33 (±0.33) a	42.67 (±0.33) a	43.67 (±0.33) a
90	50.67 (±0.67) b	56.67 (±0.88) c	57.00 (±0.58) c	33.33 (±0.33) b	37.33 (±0.33) b	38.33 (±0.33) b
120	47.67 (±0.88) c	50.67 (±0.67) d	51.00 (±0.58) d	30.67 (±0.67) c	31.00 (±0.58) c	31.33 (±0.88) c
150	44.33 (±0.88) d	48.67 (±0.88) e	49.33 (±0.33) e	29.00 (±0.58) c	29.67 (±0.88) c	29.67 (±0.88) c
180	40.33 (±0.88) e	43.00 (±0.58) f	43.67 (±0.33) f	13.33 (±0.88) e	13.67 (±0.67) e	13.67 (±0.67) e
210	30.33 (±0.88) f	33.33 (±0.67) g	33.67 (±0.33) g	9.67 (±0.88) f	9.67 (±0.88) f	9.67 (±0.88) f

[1]Germination percentages and Percentage of cotyledons were evaluated 10, 20, and 30 d after culturing in a chamber (±SEM). Different letters in superscript indicate significant between treatments at p<0.05 (Tukey test)

Table 2. Germination percentage and percentage of cotyledons obtained from *S. moorcroftiana* seeds grown on opened moistened filter paper. The seeds were pretreated with concentrated sulfuric acid for 70 min, and then subsequently incubated with plant growth regulators (PGRs) at different concentrations.

Incubation PGRs (mg L^{-1})	Duration of treatment in H_2SO_4 (70 min)					
	Germination percentage (%)			Percentage of cotyledons (%)		
	10 d	20 d	30 d	10 d	20 d	30 d
0	67.67 (±0.88) d†	72.00(±0.58) cd	72.33(±0.33) d	35.33(±0.33) e	42.67(±0.33) f	43.67(±0.33) f
GA_3(50)	75.33 (±0.88) b	76.67(±0.33) b	77.00(±0.58) b	36.33(±0.33) e	56.67(±0.67) c	57.00(±0.58) c
GA_3(100)	82.33 (±0.33) a	88.33(±0.88) a	88.67(±0.67) a	47.33(±0.67) b	61.33(±0.88) b	61.67(±0.67) b
GA_3(500)	69.33 (±0.33) cd	70.67(±0.67) d	71.00(±0.58) d	40.67(±0.67) d	69.33(±0.67) a	69.67(±0.33) a
IBA(50)	70.33 (±0.33) c	71.00(±0.58) d	71.67(±0.33) d	43.33(±0.33) c	47.67(±0.88) e	48.00(±0.58) e
IBA(100)	73.67 (±0.67) b	74.00(±0.58) c	74.33(±0.33) c	51.33(±0.88) a	52.67(±0.67) d	53.00(±0.58) d
IBA(150)	65.33(±0.33) e	67.67(±0.88) e	68.00(±0.58) e	36.33(±0.33) e	40.67(±0.67) f	46.67(±0.33) e

[1]After 2 d post PGR treatment at 30°C. Germination percentages and percentages of cotyledons on moistened filter paper were evaluated 10, 20, and 30 d after culturing in a chamber (±SEM). Different letters in superscript indicate significant between treatments at p<0.05 (Tukey test)

Table 3. Germination percentage and percentage of cotyledons of *S. moorcroftiana* seeds *In vitro*. The seeds were pretreated for 70 min in concentrated sulfuric acid, and then incubated with plant growth regulators (PGRs) at different concentrations

Incubation PGRs (mg L^{-1})	Duration of treatment in H_2SO_4 (70 min)					
	Germination percentage (%)			Percentage of cotyledons (%)		
	10 d	20 d	30 d	10 d	20 d	30 d
0	45.33(±0.67) e†	51.67(±0.67) d	52.00(±0.58) e	25.33(±0.33) e	26.67(±0.33) d	27.33(±0.33) d
GA_3(50)	57.67(±0.88) b	58.67(±0.33) b	59.33(±0.67) c	26.67(±0.33) e	33.33(±0.88) c	33.67(±0.33) c
GA_3(100)	65.67(±0.88) a	69.33(±0.88) a	70.67(±0.33) a	37.33(±1.20) b	43.33(±0.88) b	43.67(±0.88) b
GA_3(500)	49.67(±0.67) d	51.00(±0.58) d	53.33(±0.88) e	29.67(±0.88) d	47.33(±0.88) a	47.67(±0.33) a
IBA(50)	52.33(±0.33) c	54.33(±0.88) c	56.33(±1.20) d	33.33(±0.88) c	33.67(±0.33) c	34.33(±0.33) c
IBA(100)	56.67(±0.88) b	58.33(±0.88) b	62.67(±0.88) b	42.67(±0.33) a	43.00(±0.58) b	43.67(±0.67) b
IBA(150)	44.67(±0.88) e	47.33(±0.33) e	48.33(±0.33) f	25.67(±0.33) e	27.67(±0.88) d	28.33(±0.67) d

[1]After 2 d post PGR treatment at 30°C. Germination percentages and Percentages of cotyledons *In vitro* were evaluated 10, 20, and 30 d after culturing in a chamber (±=SEM). Different letters in superscript indicate significant between treatments at p < 0.05 (Tukey test)

Multiple shoots induction: The number of shoot formation per explant should be considered to obtain large-scale propagation (Andreu, 2005). *In vitro* multiple shoot propagation was highly affected by PGR type, PGR concentration, and media type. Multiple shoot proliferation was performed using cotyledonary node tissue, which was cultured on MS or WPM media added with different PGRs (BA and NAA). The percentage of explants forming shoots ($F_{7, 16}$ = 1.67e7, p = 0.000), mean number of shoots per explant ($F_{7, 16}$ = 37096.55, p = 0.000), and mean shoot length ($F_{7, 16}$ = 25303.13, p = 0.000) varied among the different combinations after 60 d of induction (Table 4; Fig. 1a, 1b, and 1c). The best results for these parameters (89.33%, 18.67, and 2.69 cm, respectively) were obtained from seeds cultured in WPM and treated with 1.0 mg L^{-1} BA and 0.2 mg L^{-1} NAA.

Rooting: Root induction was performed using regenerated multiple shoots that were cultured on 1/2 MS supplemented with various concentrations of NAA or NAA and IBA. Combinations type showed significant effect on the rooting percentage ($F_{3, 8}$ = 159.16, p = 0.000), mean root length ($F_{3, 8}$ = 779.70, p = 0.000) and number of roots per shoot ($F_{3, 8}$ = 3842.65, p = 0.000). The best PGR combinations were 0.02 mg L^{-1} NAA and 0.5 mg L^{-1} IBA, in which the rooting percentage, mean root length, and number of roots per shoot were 89.72%, 3.73 cm, and 4.68, respectively (Table 5). The root status was light yellow, stout, and long on the best media combination (Fig. 1d, 1e, and 1f).

Plantlet acclimatization: The rooted shoots were transferred to plastic pots after hardening (Fig. 1g). When the leaf color turned dark green and the villi existed on the leaf surface at 15 d of culture, the plantlets were transplanted to larger clay pots. After 15 d of culturing in a chamber, the plantlets were transferred to a greenhouse, where their survival percentage was 75% after 15 weeks (Fig. 1h).

Table 4. Effects of different plant growth regulator (PGRs) and media on multiplication of shoots from the cotyledonary node of *S. moorcroftiana* after culturing for 60 d.

Medium	Concentration of PGRs (mg L^{-1})		% of explants forming shoots	Mean shoots/ explant	Mean shoot length (cm)
	BA	NAA			
MS	1.0	0.2	81.67 b[†]	15.81 b	2.46 b
	2.0	0.2	65.42 c	9.89 e	1.54 c
	3.0	0.2	55.42 g	7.27 h	1.03 g
	2.0	0.0	59.58 d	9.62 g	1.15 ef
WPM	1.0	0.2	89.33 a	18.67 a	2.69 a
	2.0	0.2	59.00 e	14.66 c	1.38 d
	3.0	0.2	49.67 h	9.79 f	1.28 de
	2.0	0.0	58.67 f	13.26 d	1.21 e

[1]Mean values within a column followed by different letters in superscript are significantly different ($p<0.05$; Duncan's new multiple range test). Date were recorded following transfer to a rooting medium

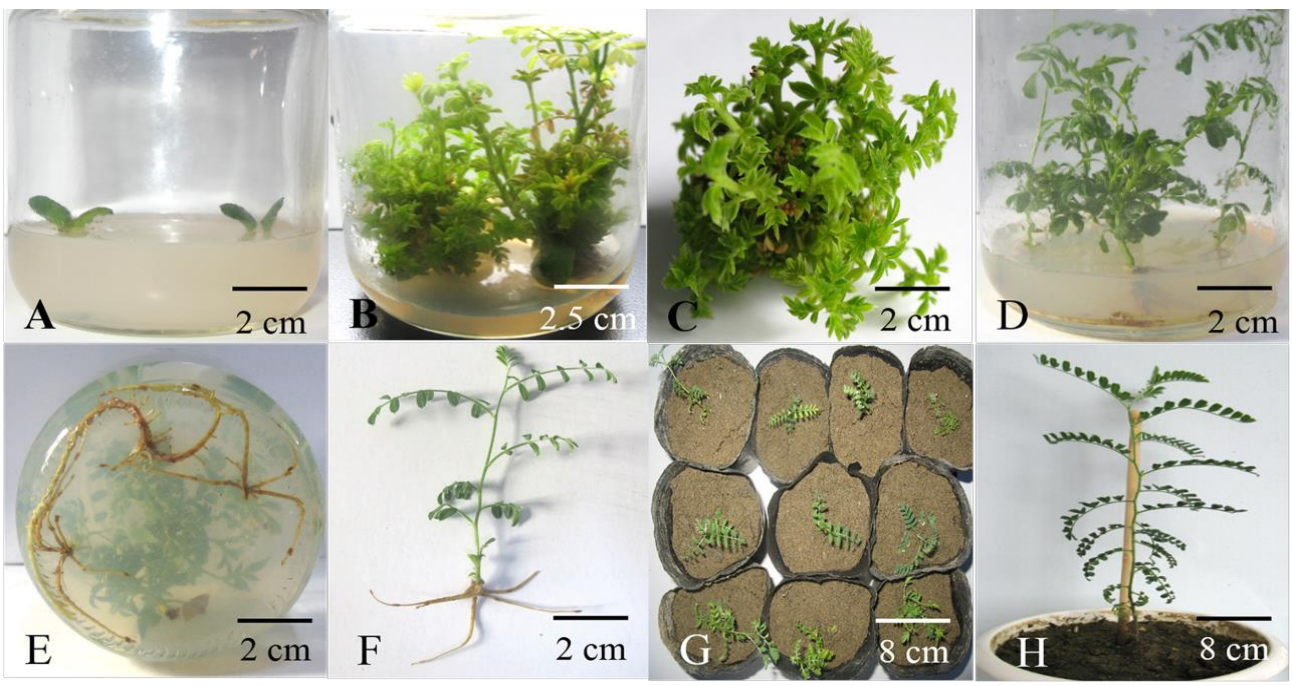

Fig. 1. Proliferation and rooting of *S. moorcroftiana*. Panel A, Cotyledonary node explants were inoculated on media. Panel B-C, Proliferation of multiple shoots from a cotyledonary node explant on WPM medium with 1.0 mg L-1 BA and 0.2 mg L-1 NAA for 60 d of culture. Panel D, Well-rooted shoots on 1/2 MS medium containing 0.5 mg L-1 of IBA and 0.02 mg L-1 of NAA after 45 d of culture. Panel E, Detailed roots obtained after 45 d of culture. Panel F, Small plantlet before transplanting. Panel G, Rooted plantlets in pots. Panel H, Regenerated plant after 15 weeks.

Table 5. Effects of different concentrations and types of plant growth regulator (PGRs) on root induction and growth of *S. moorcroftiana* after 45 d of culture.

Concentration of PGRs (mg L^{-1}) in 1/2 MS		Rooting percentage (%)	Mean root length (cm)	Roots/Shoot	Root status
IBA	NAA				
0.00	0.30	36.94 c[†]	1.40 c	2.34 b	Yellow, thin, short
0.05	0.00	69.17 b	2.12 b	1.10 c	Yellow, thin, long
0.05	0.30	26.67 d	0.34 d	0.81 d	Brown, thin,
0.50	0.02	89.72 a	3.73 a	4.68 a	light yellow, stout, long

[1]Mean values within a column followed by different letters in superscript are significantly different ($p < 0.05$; Duncan's new multiple range test)

Discussion

Semi-arid land plants have evolved complex mechanisms of dormancy to maximize their establishment in favorable environments (Tanaka-Oda *et al*., 2009). Mature seeds of numerous woody plant species frequently have dormancy issue thus avoiding the detrimental effects of unfavorable environmental conditions. Mechanical dormancy through the impermeability of the seed coat to water is the simplest but most effective means of preventing or delaying germination. Concentrated sulfuric acid is used to mechanically break seed dormancy of *Albizia chinensis* (Nongrum *et al*., 2013), *Spartium junceum* (Travlos *et al*., 2007), *Parkia biglobosa* (Aliero *et al*., 2004), *Acacia nilotica*, *Prosopis juliflora*, *Dodonaea viscosa* (Nasr *et al*., 2013), *Cassia fistula* (Soliman *et al*., 2013), *Mimosa flocculo* (Shibata *et al*., 2014), and *Urena lobata* (Awan *et al*., 2014). The seeds of these species were treated with concentrated sulfuric acid for less than 45 min, and satisfactory results were obtained. However, the seeds of other tree species require longer than 45 min of treatment to break dormancy; for example, 60 and 120 min of treatment were required for the seeds of *Tamarindus indica* (Muhammad *et al*., 2004) and *Sabina vulgaris*, respectively (Tanaka-Oda *et al*., 2009). In the present study, the seeds of *S. moorcroftiana* were treated with concentrated sulfuric acid for 70 min, and such treatment produced the highest germination percentage. Treatment with 98% concentrated sulfuric acid for a long duration can effectively break seed dormancy of species that are adapted to harsh environments.

PGR treatment is another effective way to break the seed dormancy of some species, such as *Withania somnifera*, *Abies pindrow*, *Cupressus torulosa*, *Picea smithiana*, *Melastoma dodecandrum*, and *Cupressus atlantica*. IBA pretreatment could lead to an efficient germination (Oguz *et al*., 2012; Khanna *et al*., 2013). Similar results were reported from *W. somnifera* (Shukla *et al*., 2010), *A. pindrow*, *C. torulosa*, *P. smithiana* (Rawat *et al*., 2006), *M dodecandrum* (Tang *et al*., 2012a), and *C. atlantica* (Youssef *et al*., 2012), in which GA_3 treatment significantly increased the germination percentage. In the present study, concentrated sulfuric acid broke the seed coat to a certain extent, but the obtained percentage of cotyledons was still low. GA_3 and IBA only slightly increased the germination percentage of seeds pretreated with concentrated sulfuric acid for 70 min. These two PGRs significantly increased the percentage of emerged cotyledons. Such effect is likely due to the induction of certain enzymes that are involved in the cotyledon and cotyledon node growth. Both germination percentage and percentage of cotyledons were higher on open moistened filter paper than *In vitro* when seeds subjected to the same treatments were used. The relative humidity in the flask may be much higher than on open moistened filter paper. *S. moorcroftiana* is an evolved drought-resistant species. Thus, high humidity may restrain its development.

The cotyledonary node of Leguminosae demonstrated high potential for plant regeneration. In recent years, using the cotyledonary node for plant regeneration has become popular. The cotyledonary node is a juvenile meristematic part from axenic seedlings, as an alternative explant for high frequency *In vitro* propagation. This method allows bypassing of the callus culture process. Regeneration was achieved using cotyledonary nodes in some leguminous plant species, such as *Clitoria ternatea* (Ismail *et al*., 2011), *Ricinus communis* (Alam *et al*., 2010), *Abelmoschus moschatus* (Lithy *et al*., 2011), *Acacia mangium* (Shahinozzaman *et al*., 2012), *Cassia alata* (Ahmed *et al*., 2013), *Trichosanthes anguina* (Ambetkar *et al*., 2012), and *W. somnifera* (Nayak *et al*., 2013). BA and NAA are two PGRs that are used for shoot induction. However, high concentrations of PGRs reduce clustered shoot induction. High PGR concentrations may cause cytogenetic instability, thereby producing cells that are unsuitable for regeneration; thus, low BA concentrations were applied (Peddaboina *et al*., 2006). Consistent with these results, the best PGR combination in this study was used for shoot induction in cotyledonary nodes of *S. moorcroftiana*. The best PGR combinations were 1.0 mg L^{-1} BA and 0.2 mg L^{-1} NAA. WPM media were more effective than MS in some wood plant tissue cultures (Singh *et al*., 2013; Al-Khayri, 2011; Singh *et al*., 2014). Therefore, germination and shoot induction on WPM and MS media were compared. No difference was observed between MS and WPM for *In vitro* germination, but WPM media was more suitable for *S. moorcroftiana* shoot induction (Table 4).

IBA and NAA were the most commonly used PGRs for root induction in different plant species, such as *Operculina turpethum* (Sebastinraj *et al*., 2013), *Vigna unguiculata* (Tang *et al*., 2012b), *Cassia sopera* (Parveen *et al*., 2010), *Senna sopera* (Parveen *et al*., 2014), *T. anguina* (Ambetkar *et al*., 2012), *C. ternatea* (Ismail *et al*., 2011), *A. mangium* (Shahinozzaman *et al*., 2012), and *R. communis* (Alam *et al*., 2010). In some other species, a relatively high concentration of IBA seems effective

(Singh *et al*., 2013; Rajanna *et al*., 2011; Ahmad *et al*., 2013; Shahinozzaman *et al*., 2012). In the current study, the best PGR combinations was 0.5 mg L^{-1} IBA combined with 0.02 mg L^{-1} NAA for root induction.

Conclusions

Although desertification is a serious environmental problem in the QTP, *S. moorcroftiana* can adapt the harsh environment (Liu & Zhao, 2001), therefore *S. moorcroftiana* is an important species in the QTP ecosystem, but over-exploitation lead to its population declined sharply. In the present study, a method to easily break seeds dormancy of *S. moorcroftiana* was developed, which significantly improved germination of the species (Zang and Xin, 2012). Therefore, the seeds can be pretreated and then sowed in the field with expected successful germination, this method would be helpful in vegetation restoration and sustainable development of *S. moorcroftiana* as it is cost effective, reliable and easily available. Besides, breaking of the dormant seeds would be benefit to develop an effective *In vitro* propagation system for this species. Furthermore, *In vitro* propagation of the species is benefit of preserving the healthy plant material in a small space, and is to reproduce many plantlets within a short period easily. Hence, the *In vitro* propagation protocol can be applied to large-scale generation and conservation of this species in the future.

Acknowledgments

This work was sponsored by the National Natural Science Foundation of China (31260189).

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(Received for publication 28 June 2015)