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In vitro propagation of *Lepironia articulata* in Kuan Kreng Peatlands, Nakhon Si Thammrat

Premrudee Domyos[1] and Sompong Te-chato[1,2*]

[1]Marine and Coastal Resources Institiue (MACORIN), Prince of Songkla University, Hatyai, Songkhla 90112, Thailand, [2]Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University,Hatyai, Songkhla 90112, Thailand

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Induction of multiple shoots using young shoot and young spike explants of *Lepironia articulata* was achieved. Multiple shoots from cultured young shoot were enhanced by increasing the concentration of BA from 1 to 3 mg/L and the highest average number of shoots was observed in the medium containing 3 mg/L BA. In the case of multiple shoot induction from young spike, shoots were only obtained from the young spike inside 1 mm involcral bract and the highest average number of shoots was equal observed in 2 and 3 mg/L BA containing medium. A maximum of average number of shoots were observed on proliferation medium with the addition of 5 mg/L BA. The regenerated shoots were successfully rooted on MS medium without plant growth regulators together with 0.1% AC, and hardened at 100% after transfer to the greenhouse conditions.

Keywords: In vitro propagation, Lepironiaarticulata, Peatlands

Introduction

Lepironia articulata (Retz.)Domin, tube sedge or grey sedge, belongs to the family Cyperaceae and is widely distributed in Madagascar, India, Sri Lanka, Indo-China, South-East China, Malaysia, Micronesia, Australia, New Caledonia, and Fiji. Naturally, the plant occurs in swamps, ephemeral swamps, the margins of fresh water swamps, coastal and inland swamp, and swamp forest. It often occurs in acid sulfate soils (David and Tetsuo, 1998; Cowie *et al.*, 2000; Stevens and Dowling, 2002; Sim Cheng Hua, 2007; Shi Long Chu, 2010). In Australia, the plant has been used extensively for planting in wetlands constructed for urban run-off management and for decorative ponds and lakes. It is also useful as a filter plant in a natural swimming pool, providing stable habitat and food for water birds) Cowie *et al.*, 2000; Stevens and Dowling,

*Corresponding author: SompongTe-chato; e-mail: stechato@yahoo.com

2002).The woody underground stems can be eaten raw or cooked (Anonymous, 2011). In Thailand as well as other parts of the tropics, such as China, the culms are used for making mat and basket (David and Tetsuo, 1998; Shi Long Chu, 2010).

The plant is said to be a commercial plants of the communities around peat swamp areas in south Thailand, especially, KuanKrengPeatlands covered 3 provinces: Songkhla, Phatthalung, and Nakhon Si Thammarat. In the past, this natural plant in the peat swamp was harvested for weaving into different kinds of products, for instance sacks, mats, bags, hats, and glass support, etc. However, due to drought, wild fire, increase in exploitation of it as well as site expansion for other activities, there is now shortage of natural Lepironia articulate. Thus, farmers must utilize some areas in the peat swamp to grow additional Lepironia articulate. Nowadays, the area of this plant in KuanKrengPeatlands had drastically decreased due to the problem of slash and burn (Somboon *et al*., 2002). Moreover, over exploitation and destructive method for harvesting are also the major causes of the depletion of this species in KhuanKrengPeatlands (Khairul,).

Vegetative propagation of Lepironia articulate from rhizome has generally been successful, whereas propagation from seed was limited (Panaia *et al.*, 2009). Thus, development of in vitro propagation protocols could further facilitate the production of difficult wetland species including rare or endangered species. Moreover, this procedure could also help the selection of elite clonal lines exhibiting superior growth, form, stress tolerance and other commercially valuable characteristics (Kane, 1989). There have been some reports on in vitro micropropagation of dry-land Cyperaceae species, for example CarexluridaWahl., C. stipataMuhl., C. scoparisSchk., Chinese Puzzle (Caustisdioica R.Br.) (Addison, 1952; Meney and Dixon, 1988; Rossetto, 1990, Rossetto *et al.*, 1992; Kathy *et al*., 1995; Johnston *et al.*, 1994; Webber *et al*., 2003;) and wetland Cyperaceae species, e.g.Tetraia capillaries, T. octandra, LepidospermadrummondiandL. tenue(Panaia et al., 2009; Panaia et al., 2011; Andria *et al*., 2010). However, no information is available concerning in cultural requirements for the efficient in vitro propagation of Lepironiaarticulata. Therefore, in the present study, we report a method of adventitious shoot induction from young shoot from underground rhizome and young inflorescence of Lepironiaarticulata. To our knowledge, this is the first report of successful for high frequency of shoot induction in Lepironiaarticulata.

Materials and methods

Plant material

Underground rhizomes of Lepironiaarticulata were collected from KuanKrengPeatlands in Krengsubdistrict, Nakhon Si Thammrat. The rhizomes with young shoots were washed with running tap water for 20 minutes, followed by soaking in Teepol soap solution for 5 minutes and then kept in running tap water for 1 hour. Two young shoots were excised from those rhizomes, the first young shoot of about 0.5-1 cm and the second of about 2-3 cm in height. The explants were then surface sterilized, initially in 70% ethanol for 10 minutes, followed by 5% (v/v) sodium hypochlorite (NaOCl) and 0.2% Tween 80 for 30 minutes, rinsed in sterile distilled water for three times, trimmed the roots and peeled greyish brown scales. Trimmed shoots were again surface sterilized in 2% (v/v) NaOCl in the presence of 0.2% Tween 80 for 20 minutes, rinsed in sterile distilled water for three times and the outer leaf sheathes removed. Finally surface sterilization was conducted in 1% (v/v) NaOCl in the presece of 0.2% Tween 80 for 20 minutes, rinsed three times with sterile distilled water and a culm of the second young shoot excised. The inner leaf sheathes were removed and the culm of young shoots used as initial explants for culture.

Culture conditions

MS (Murashige and Skoog, 1962) medium with 3% sucrose (w/v) adjusted to pH 5.7 with 1N KOH or 1N HCl and solidified with 0.8% agar (w/v) was employed for culturing. The medium was sterilized using autoclave under 1.5 kg/cm^2 at 121^{o}C for 15 minutes. All cultures were grown at 25±2^{o}C under 16/8 (light/dark) hours photoperiod supplied by two bulbs of cool white Phillips TL 40W fluorescent.

Adventitious shoot induction

The shoot apices were cultured on MS medium supplemented with BA (1, 2 and 3 mg/L) alone or in combination with NAA (0.2 mg/L) for multiple shoot induction. MS medium without plant growth regulators was used as control. After 8 weeks of culturing, the frequency of explants producing multiple shoots, the average number of shoots per explants and average shoot height were recorded.

Young spikes located near the tip of vitro-grown culms enclosed by 1 and 2 mm subulateinvolucral bract were excised and immediately placed on MS

medium supplemented with BA (1, 2 and 3 mg/L). After 4 weeks of culture, the frequency of explants producing multiple shoots, the average number of shoots per explants and average shoot height were recorded.

Shoot proliferation

To facilitate multiple shoot proliferation and growth, small clump of shoots derived from culturing shoot apices were transferred to MS medium supplemented with KN (1, 3 and 5 mg/L) or BA (3, 5 and 7 mg/L) alone or in combination with 15% (v/v) coconut water (CW), whereas small clump of shoots from young inflorescences were transferred to MS medium containing BA (3, 5 and 7 mg/L). After 8 weeks of culture, the average number of shoots per explants and average shoot height were recorded.

Rooting of shoot

Well developed of regenerated shoots at 2 cm in height were excised from mother rhizome and transferred to MS medium without plant growth regulators in the absence or presence of 0.1% (w/v) activated charcoal (AC). After 6 weeks of culture, the frequency of shoots producing roots, the average number of roots per shoot and root length were recorded.

Hardening of plantlets to soil

In vitro rooted plantlets were washed carefully in running tap water to remove the traces of agar. They were transferred individually to culture in bottles containing sterilized sand: coconut dust: rice husk charcoal mixture (1:1:1 v/v/v) and maintained under culture room conditions. After a week of hardening, the plantlets were transferred to plastic cups (6 cm in diameter) containing the same mixture of planting material and maintained for further one week and finally transferred to pots (12 cm in diameter) containing sand: lateritic soil: cow dung mixture (2:1:1 v/v/v) and maintained under shade area in the green house conditions. After 2 weeks of transferring, the survival rate was recorded.

Statistical analysis

The experiments of multiple shoot induction and shoot proliferation were set up in a completely randomized design (CRD). Each treatment consisted of four replications and each replication contained three explants. At the end of culturing period parameters on the percentage of response, number of shoots

per explants and shoot height were statistically analyzed and compared. Means and standard deviations were calculated for each treatment. Mean values were analyzed using a one-way analysis of variance (ANOVA). Significant differences among treatments were detected using least significant difference (LSD) at the 0.05 level of probability. In case of rooting experiment, parameters on the percentage of response, number of roots per shoot, root length were recorded and statistically analyzed. Means and standard deviations were calculated for each treatment. Mean values were compared using a t-test.

Results and discussions

Multiple shoot induction

BA alone gave significantly induction of multiple shoots at all levels in comparison with the control treatment (without PGR) or in combination with NAA. Multiple shoots were not observed in MS medium without PGR and the medium supplemented with 1 and 2 mg/L BA in combination with 0.2 mg/L NAA. On the other hands, MS medium supplemented with various concentrations of BA (1, 2 and 3 mg/L) alone could induce multiple shoots with same percentage of explants response (100). The induction of multiple shoots was enhanced by increasing the concentration of BA from 1 to 3 mg/L. The highest average number of shoots per explants at 3.33 ± 0.69 was observed in culture medium containing 3 mg/L BA and the highest average shoot height at 5.83 ± 0.33 was obtained on the media containing 2 mg/L BA (Table 1; Fig. 1). Similar results were also observed in Caustisdioica (Chinese Puzzle) by Sieler *et al*. (1997) who reported that BA significantly increased the production of multiple shoots at all concentrations (0.5, 1, 2, 5 µM). Addition of NAA play inhibitory effect on multiple shoot formation. Moreover, this auxin has also been reported in a declining in shoot quality and size of Chinese Puzzle (Rossetto *et al*., 1992).

Table 1. Effect of different concentrations of BA alone or in combination with NAA on multiple shoot induction from young shoots of Lepironiaarticulata

Type and concentration (mg/L)of plant growth regulators	Percentage of explants response	Average no. of shoots/explants	Average shootheight (cm)
PGR-free medium	0.00	0.00 ± 0.00 ^{d}	0.00 ± 0.00 ^{c}
BA (1)	100.00	1.33 ± 0.29 ^{c}	7.50 ± 0.89 ^{a}
BA (2)	100.00	2.33 ± 0.47 ^{b}	5.83 ± 0.33 ^{b}
BA (3)	100.00	3.33 ± 0.69 ^{a}	5.50 ± 0.55 ^{b}
NAA (0.2) + BA (1)	0.00	0.00 ± 0.00 ^{d}	0.00 ± 0.00 ^{c}
NAA (0.2) + BA (2)	0.00	0.00 ± 0.00 ^{d}	0.00 ± 0.00 ^{c}
NAA (0.2) + BA (3)	8.33	0.08 ± 0.00 ^{d}	0.25 ± 0.50 ^{c}
F-test	-	**	**
$LSD_{.05}$	-	0.50	0.67
C.V. (%)	-	33.78	16.79

** significant difference at $p \leq 0.01$

Each value represents the mean ± SD of 4 replicates. Values with the same superscript are not significantly different at the 0.05% probability level according to LSD.

Fig. 1. Multiple shoots formation on MS medium supplemented with various concentrations of BA. (a) 1mg/LBA, (b):2 mg/L BA, (c): 3 mg/L BA

In the case of multiple shoot induction from young spikes growing near the tip of vitro-grown culms enclosed by 1 and 2 mm subulateinvolucral bract, size of the spikes inside the involucral bract was significantly affected the formation of adventitious shoots. The multiple shoots were only obtained from the young spikes inside 1 mm involucral bract. The highest percentage of explants response at 100 % and the highest average number of shoots per explants at 1.00 ± 0.00 were equal observed in 2 and 3 mg/L BA containing medium. On the other hands, all of the young spikes inside 2 mm involucral developed to large spikes and all of them flowered after 6 weeks of culturing on the same culture medium (Table 2; Fig. 2).

Table 2. Effect of different sizes of young inflorescences covered by involucral bract (mm) and different concentrations of BA (mg/L) on multiple shoot induction

Size / BA	Average no. of shoots/explants			Average shoot height/explants		
	1	2	Mean	1	2	Mean
1	0.58 ± 0.34 [b]	0.00 ± 0.00 [c]	0.29 ± 0.38 [b]	0.83 ± 0.31 [c]	0.00 ± 0.00 [d]	0.41 ± 0.49 [c]
2	1.00 ± 0.00 [a]	0.00 ± 0.00 [c]	0.50 ± 0.53 [a]	4.68 ± 0.76 [a]	0.00 ± 0.00 [d]	2.33 ± 2.55 [a]
3	1.00 ± 0.00 [a]	0.00 ± 0.00 [c]	0.50 ± 0.53 [a]	1.95 ± 0.21 [b]	0.00 ± 0.00 [d]	0.98 ± 1.05 [b]
Mean	0.86 ± 0.27 [a]	0.00 ± 0.00 [b]	0.43 ± 0.48	2.48 ± 0.24 [a]	0.00 ± 0.00 [b]	1.24 ± 1.75

Each value represents the mean ± SD of 4 replicates. Values with the same superscript are not significantly different at the 0.05% probability level according to LSD.

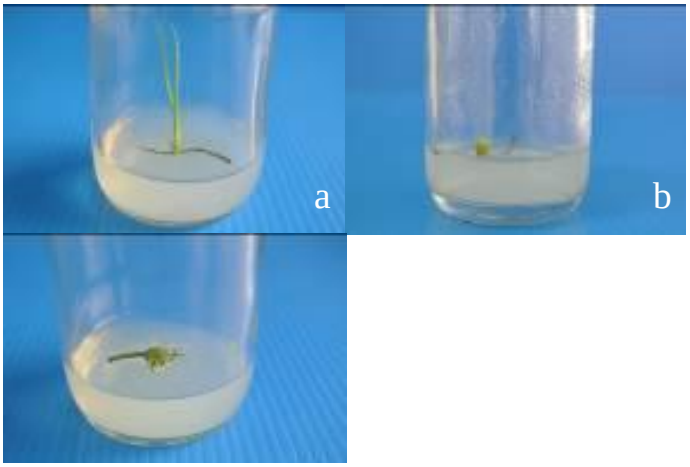

Fig. 2. Multiple shoot formation from inflorescences on MS medium supplemented with various concentrations of BA.
(a) Shoot formation, (b) Spike formation (c) Flowering

Shoot proliferation

The cytokinin types and concentrations have significantly increased the formation of multiple shoots. Each tested cytokinin promoted shoot regeneration with the concomitant of the concentrations used in proliferation medium. A maximum of average number of shoots at 4.08 ± 0.83 per explants were observed on proliferation medium supplemented with 5 mg/L BA alone. The highest average shoot height at 4.55 ± 0.64was recorded from the culture medium with 3 mg/L BA alone (Table 3; Fig. 3). Higher concentrations of BA reduced the number of shoots and shoot lengths. This finding is in agreement with an observed in other plant species such as Sorghum bicotor (Baskaran *et al.*, 2005). Combinations of BA at all concentration tested with 15% CW did not enhance shoot proliferation in comparison with BA alone. Five mg/L BA and 15% CW gave an average number of shoots at 2.73 ± 1.28 shoots/explant.

Similar result was also reported by Kanchanapoom and Promsorn (2012) in Musa balbisiana. For KN, average number of shoots per explant was greater at low concentration. Increase in concentration of KN caused a progressively decreased in shoot number and shoot elongation. This present study indicated that BA enhanced shoot multiplication better than KN. Many authors reported that cytokinin was necessary for optimal proliferation of shoot in many plants (Tsay *et al.*, 1989; Shasany *et al.*, 1998; Sharma and Singh, 1997). The most effective cytokinin was reported to be BA. In the present study, we also found that BA containing medium was the best for shoot proliferation in L. articulate.

Table 3. Effect of different concentrations of KN, BA aloneor in combination with CW on shoots multiplication

Types and concentrations (mg/l)of plant growth regulators	**Average no. of shoots/explants**	**Average shootheight (cm)**
KN (1)	2.43 ± 0.51^{bc}	1.88 ± 0.29^{e}
KN (3)	1.75 ± 0.33^{cd}	2.25 ± 0.59^{de}
KN (5)	1.25 ± 0.33^{d}	2.60 ± 0.16^{cde}
BA (3)	3.33 ± 0.78^{ab}	4.55 ± 0.64^{a}
BA (5)	4.08 ± 0.83^{a}	3.03 ± 0.38^{bcde}
BA (7)	3.40 ± 0.71^{ab}	3.25 ± 0.53^{bcd}
BA (3) + CW 15%	3.58 ± 0.61^{ab}	3.68 ± 0.64^{abc}
BA (5) + CW 15%	2.73 ± 1.28^{bc}	4.05 ± 1.55^{ab}
BA (7) + CW 15%	2.48 ± 1.20^{bc}	4.48 ± 1.24^{a}
F-test	**	**
$LSD_{.05}$	1.16	1.15
C.V. (%)	28.75	23.98

** significant difference at $p \leq 0.01$

Each value represents the mean ± SD of 4 replicates. Values with the same superscript are not significantly different at the 0.05% probability level according to LSD.

Fig. 3. Shoot proliferation on MS medium supplemented with in combination with 15% CW (a) 1, 3 and 5 mg/L KN, (b) 3, 5 and 7 mg/L BA, (c) 3, 5 and 7 mg/L BA

Rooting of shoot

Well-developed shoots at 2 cm in height were transferred to MS medium without plant growth regulators in the absence or presence of AC at

concentration of 0.1% for in vitro rooting. The first period of root induction was observed at day 10 on MS medium without plant growth regulators in the presence of 0.1% AC. A maximum frequency of root formation at 100% and the average number of roots at 1.90 ± 0.74 roots with the average root length (1.75 ± 1.41 cm) were achieved on MS medium without plant growth regulators together with 0.1% AC. However, significant difference between culture medium with and without AC was not found (Table 4; Fig. 4). Johnstone*et al.* (1994) also reported that addition of AC in rooting medium could induce the greatest number of roots in Caustisblakei. However, the concentration added in culture medium was 0.12 g/L, slightly higher than this present study.

Table 4. Effect of plant growth regulator-free medium (PGR-free medium) in the absence or presence of 0.1% AC on root induction from regenerated shoots

MS medium	Average no. of roots/explants		Average rootlength (cm)	
	$\bar{X}$	t	$\bar{X}$	t
PGR-free	1.70 ± 0.67	0.633^{ns}	1.13 ± 0.25	0.137^{ns}
PGR-free + AC (0.1%)	1.90 ± 0.74		1.75 ± 1.41	

$t_{.05}$= 2.101, $t_{.01}$=2.878

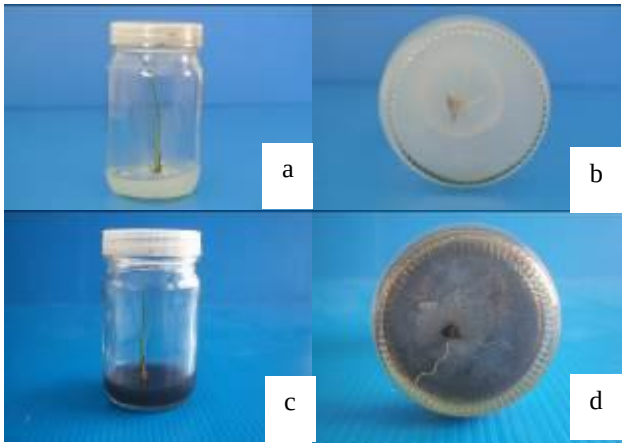

Fig. 4. Root induction on MS medium without plant growth regulators (a-b) in the absence of AC, (c-d) in the presence of 0.1% AC

Hardening of plantlets to soil

In vitro rooted plantlets or complete plantlets were transferred individually to culture in bottles containing sterilized sand: coconut dust: rice husk charcoal mixture (1:1:1 v/v/v) and kept in culture room conditions (Fig. 5a). After a week of hardening, the plantlets were transferred to plastic cups (6

cm in diameter) containing the same planting mixture and maintained under culture room conditions for further one week (Fig. 5b). Then the healthy plantlets were transferred to pots (12 cm in diameter) containing sand: laterite soil: cow dung mixture (2:1:1 v/v/v) and kept in a shaded area in the green house conditions. After 2 weeks of transferring, survival rate at 100% was obtained. Similar result was also reported in umbrella sedge (Cyperusalternifolius L.) which rooted shoots were easy transferring to soil (Mohamed-Yasseen *et al*., 1992). All of them were successfully acclimatized under these soil conditions. However, it was reported that short roots allowed the better acclimatization of plantlet due to avoiding of root damage during transplantation procedures (Anada *et al*., 2011). Morphological characteristics of plantlets after transfer to soil were normal (Fig.5c, d).

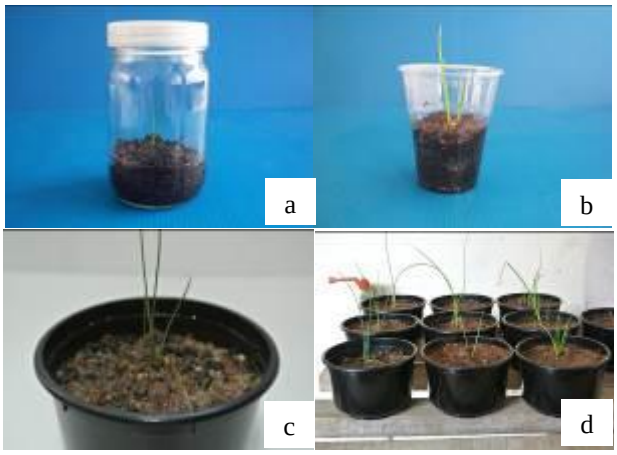

Fig. 5. Hardening plantlets to soil condition
(a-b) hardening of plantlet in Laboratory conditions, (c-d) hardening of plantlet under greenhouse conditions

Conclusion

The in vitro protocol for regenerating plantlets of *L. articulata* using young shoots and young spikes described in this study is simple and reproducible. Since the plantlets were developed directly without intervening of callus phases, thus, somaclonal variation among the regenerates was avoided. This protocol is useful for large-scale propagation and conservation of this important peatland plants as well. To our knowledge, this is the first report of successful in vitro shoot induction via micropropagation in *L. articulata*.

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