日本 暖地 畜産 学会 報

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Journal of Warm Regional Society of Animal Science, Japan

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第5回 日本暖地畜産学会 福岡大会の開催について

第5回日本暖地畜産学会福岡大会を下記により開催します。会員多数の御参加をお待ちしております。大会の詳細は、学会ホームページ（http://waras.ac. affrc.go.jp/）にも掲載していますのでご覧下さい。

1. 開催期日
平成24年11月10日（土）～11月11日（日）

2. 会場
九州大学箱崎キャンパス（福岡市東区箱崎6丁目10番1号）

3. 参加費
大会参加費：会員4,000円（当日）非会員4,000円（当日）

4. 日程

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5. 大会受付
日時：11月10日（土）08:00～
11月11日（日）08:30～
場所：農学部5号館入口

6. 役員会
日時：11月9日（金）15:00～
場所：役員会（大会議室、農学部1号館6階）

7. 研究発表
日時：11月10日（土）08:45～11:45
11月11日（日）09:30～11:42
場所：農学部4号館4-110教室（第1会場）、5号館5-117教室（第2会場）

8. シンポジウム
テーマ：「日本畜産の将来と東太平洋戦略的経済連携協定（TPP）を考える」
日時：11月10日（土）15:30～18:00
場所：附属図書館視聴覚ホール
テーマ内容：環太平洋戦略的経済連携協定（TPP）と日本の畜産業の将来をテーマとし、基調講演では、「TPP締結による日本農業、経済への影響について」および「TPPと日本畜産を考える～前門の虎、後門の狼と如何に戦うか」というタイトルでそれぞれ吉田健治先生（前九州大学大学院教授）と甲斐論先生（中村学園大学学長、九州大学名誉教授）にご講演頂き、総合討論でこれからの西南暖地の畜産業が進むべき道について考える。

9. 編集委員会
日時：11月10日（土）12:00～
場所：農学部7号館1階 セミナー室

10. 総会
日時：11月10日（土）13:30～14:10
場所：附属図書館視聴覚ホール

11. 学会賞・優秀発表賞授賞式及び学会賞受賞講演
日時：11月10日（土）総会終了後～15:20
場所：附属図書館視聴覚ホール

12. 慶親会
日時：11月10日（土）19:30～21:30
場所：西鉄グランドホテル（福岡市中央区大名2丁目6番60号）電話：092-781-0211・0311（直通）
会費：6,000 円（当日）

13. 昼食
九州大学箱崎キャンパス理農食堂（農学部6号館1階）をご利用ください
（11月10日（土）のみ営業、営業時間11:30～14:00）

14. 宿泊
以下のウェブサイトなどを参考に、宿泊予約は各自で手配してください。

よかなびWeb http://www.yokanavi.com/jp/stay/index
るるぶ.com http://www.rurubu.com/Pref/list.aspx?ChikuCD=4013016&MeshCD=40001#stay

15. お問い合わせ先
九州大学大学院農学研究院 動物・海洋生物資源学講座
畜産化学分野 池内義秀
E-mail:likeuchiyo@agr.kyushu-u.ac.jp、 電話 092-642-2949

16. 会場へのアクセス
利用される市内各バス停を出発地に、「九大北門」あるいは「鹿岡駅」を到着地にして以下のウェブサイトで検索してください。

にしてつ時刻表
http://jik.nnr.co.jp/cgi-bin/Tschedule/menu.exe?pwd=h/menu.pwd&mod=F&menu=F
17. 大会会場内の見取り図
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シンポジウム 服部英彰（九州大学）
会場 古瀬充宏（九州大学）・安尾しのぶ（九州大学）
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日本暖地畜産学会報

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Original Article (Full Paper)

Efficient nursery production and multiple-shoot clumps formation from shoot tiller-derived shoot apices of dwarf napiergrass (Pennisetum purpureum Schumach.)

Nafiulat UMAMI\textsuperscript{1,4}, Takahiro GONDO\textsuperscript{2}, Genki ISHIGAKI\textsuperscript{2}, Mohammad Mijanur RAHMAN\textsuperscript{3} and Ryo AKASHI\textsuperscript{2}

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\textsuperscript{4} Faculty of Animal Science, Gadjah Mada University, Yogyakarta 55281, Indonesia.

(Received: 5 March 2012 / Accepted: 16 April 2012)

ABSTRACT The objective of this study was to improve an \textit{in vitro} tissue culture system for the dwarf napiergrass (\textit{Pennisetum purpureum} Schumach.). Additionally, it was needed special treatments for acclimatization, and carried the risks of somaclonal variation and physiological aberrations. Shoot apices as initial explants were isolated aseptically from shoot-tillers, and cultured on \textit{in vitro} MS (Murashige and Skoog, 1962) medium containing 3.0% sucrose and 0.3% phytagel. The most effective phytohormone treatment for multiple-shoot clumps (MSCs) induction was 0.1 mg L\(^{-1}\) 2,4-D plus 2.0 mg L\(^{-1}\) BAP. The addition of 50 \(\mu\) M CuSO\(_4\) could increase the percentage of MSCs proliferation. Plant regeneration frequency was achieved up to 84\% by culturing the MSCs on solid MS medium containing 0.1 mg L\(^{-1}\) NAA and 2.0 mg L\(^{-1}\) BAP. All regenerants were successfully grown up in the soil. Compared to control plants, \textit{in vitro} regenerated plants did not reveal any significant difference \((P > 0.05)\) on morphological characteristics and DNA content. The results of this study suggest that improved protocols for \textit{in vitro} propagation would provide high quality nursery plant production of dwarf napiergrass in grassland field.


Key words: dwarf napiergrass; flow cytometry; multiple-shoot clumps formation; plant regeneration.

Introduction

In tropical and sub-tropical areas of the world including Asian and south-Asian countries, napiergrass (\textit{Pennisetum purpureum} Schumach.) has been widely adopted as cut-and-carry grass by smallholder farmers for feeding dairy cattle (Valk 1990). It is a tall, predominantly clonally propagated, rapidly regenerates, high yielding perennial grass that is very palatable to cattle in the leafy stage (Lowe et al. 2003). Recently, Timbo et al. (2010) reported that it has also great energetic potential for bio-ethanol, bio-oil and biogas production as well as for electricity in thermoelectric power plants and rural properties.

Napiergrass has two different plant types in the genetic resources: one type is normal type including cultivars like "Merkuron", "Wruk wona" and "Rhodesia" (Davies 1963, Burton 1989, Mukhtar et al. 2003, Orodo 2006), and the another type is dwarf type (Hanna and Monson 1988). The both plant types are same chromosome number (2n=4x=28). Normal type "Rhodesia" was introduced to southern part of USA in 1913 (Burton 1944, 1989; Pongtongkam et al. 2006). On the other hand, dwarf type is considered a cross-pollinated plant, which was originally found in Florida, USA. There are several negative properties of dwarf type, which prevents this grass to be developed as planned (Pongtongkam et al. 2006).

The dwarf type is leafier and can be grown in a wide variety of soil types. The biomass of dwarf type is lower than normal type, but it contains higher nutrients than normal type to feed the livestock. Their flowers are very small, while the pollens are short-lived which results in low level of seed formation. The flowering period of each type of napiergrass is different, which makes it more difficult and time consuming to have them cross-pollinated. Therefore, tissue culture has been used as a tool for production of high quality nursery and improvement of napiergrass cultivars (Pongtongkam et al. 2006).
in vitro culture techniques enable the propagation of high plant numbers on a small area under controlled conditions with free from microorganisms. Rapid growth and propagation as well as the recovery and maintenance of disease-free plants are the major benefits of plant tissue culture. There were several reports on tissue culture of napiergrass to establish plant regeneration system (Bajaj and Dhanju 1981; Haydu and Vasil 1981; Wang and Vasil 1982; Chandler and Vasil 1984; Pontongkam et al. 2006; Xiao-Xian et al. 2007). An efficient in vitro tissue culture system, however, is in great need for nursery plant production. Therefore, improvement of an in vitro tissue culture method is a one of the most important factors for commercial propagation of dwarf napiergrass.

In this study, we described an in vitro propagation system of dwarf napiergrass via multiple-shoot clumps (MSCs) from shoot apices with especial emphasis on effectiveness of CuSO4. In addition, we evaluated the morphological characteristics and stability in DNA content of regenerated plants using flow cytometry (FCM), which was compared with native dwarf napiergrass. It is considered that this system will be a suitable method for nursery plant production on dwarf napiergrass.

**Materials and Methods**

**Explants sterilization**

Shoot-tillers of dwarf variety of late-heading type napiergrass used for explants were collected from Experimental Field of University of Miyazaki, Japan. The shoot-tillers were washed with running tap water to remove sand and dust particles, and then sterilized by 70% (v/v) ethanol for 2 minutes followed by 2% (v/v) sodium hypochlorite solution. The solution contained shoot tips were agitated for 15 minutes followed by washing of three times with sterilized water for 2 minutes.

**Induction of multiple-shoot clumps (MSCs)**

Shoot apices were excised from shoot-tiller and cultured on MS medium (Murashige and Skoog 1962) containing 3.0% sucrose, 0.3% phytogel (Sigma-Aldrich, St. Louis, MO, USA) and 0.1% (v/v) PPM™ (plant preservative mixture, Plant Cell Technology, Inc., Washington DC, USA) supplemented with various concentrations of hormone. These induction medium contained 2,4-dichlorophenoxyacetic acid (2,4-D: 0.0, 0.01, 0.1 and 0.5 mg L⁻¹) and 6-benzylaminopurine (BAP: 0.0 and 2.0 mg L⁻¹). The induction frequency of MSCs was calculated. For proliferation, MSCs were transferred to MS induction medium (2.0 mg L⁻¹ BAP and 0.1 mg L⁻¹ 2,4-D) supplemented with several concentrations of CuSO4 (0.0, 5.0 and 50 µM). The original MS medium contained 0.1 µM CuSO4. The percentage of clump proliferation was observed after 14 days of culture.

**Plant regeneration conditions**

For plant regeneration, MSCs were transferred into MS basal medium containing 3.0% sucrose and 0.3% phytogel supplemented with 0.0 and 2.0 mg L⁻¹ BAP in combination with 0.0, 0.01, 0.1 and 0.5 mg L⁻¹ α-naphthalene acetic acid (NAA). After 14 days of culture, the plant regeneration percentage was observed. Elongated shoots were transferred to fresh half-strength MS medium to induce root development. All media were adjusted to pH 5.6-5.8 prior to being autoclaved at 121°C for 15 minutes. The cultures were incubated under fluorescent lights of 3500 lux for 16 hours at 27°C. The in vitro regenerants were taken out from the tubes carefully and washed in water to remove the nutrient and agar. Subsequently, they were transferred to soil directly in the greenhouse.

**Estimation of DNA content using flow cytometry analysis**

The regenerants dwarf napiergrass and control plants that grew well in aclimatization were used as material for FCM analysis. *Oryza sativa* cv Nipponbare was used as internal standard; the DNA content was 0.91 pg 2C⁻¹ (Uozu et al. 1997). The *Oryza sativa* seedlings were grown in a greenhouse. For estimation of DNA content, the FCM was performed using Beckman Cell Lab Quanta™ SC Flow cytometer machine (Beckman Coulter, Inc., Tokyo, Japan) following the method of Ishigaki et al. (2010). The analysis was replicated three times for each sample.

**Evaluation of morphological characteristics**

Twenty one regenerated plants from MSCs grew well in the greenhouse. These tillers of the regenerants were transferred into small pots (1/5000a size) and placed in a greenhouse for 1 month. After that, all plants were transplanted into larger pots (1/2000a size) and grown outside. The 10 control plants, which were obtained from tillers of dwarf napiergrass, were grown in the same conditions. After 60 days from plantation, the regenerant plants were compared with the control plants in relation to six morphological characteristics. These characteristics were as follows: (i) plant length, (ii) plant height, (iii) leaf blade length, (iv) leaf blade width, which was taken from trifoliate leaf of the longest stem, (v) number of tiller and (vi) yield. The mean of the ten measurements was used to define the six morphological characteristics. The data were analyzed by analysis of variance (ANOVA) and Tukey's test using SPSS 10.0 software.

**Results and discussion**

**Culture of multiple-shoots clumps and plant regeneration**

In general, in vitro pathways for propagation of plants include (i) axillary branching, (ii) adventitious shoot
formation and (iii) somatic embryogenesis. However, most of the commercially micro-propagated plants are derived from axillary shoot formation. In this study, we have established an *in vitro* propagation system from shoot apices of dwarf napiergrass. Shoot apices used as initial explants were excised from shoot-tiller of dwarf napiergrass in the field (Figure 1a). After 10 days, the size of the basal shoot apices was enlarged (Figure 1b), and the MSCs were produced from the basal of the shoot apices after 30 days (Figure 1c). Subsequently, the MSCs were grown for shoot development (Figures 1d).

The MSCs were induced on MS medium containing 20 mg L⁻¹ BAP and 0.1 or 0.5 mg L⁻¹ 2,4-D (Table 1).

![Figure 1 Plant regeneration via MSCs in dwarf napiergrass.](image)

**Figure 1** Plant regeneration via MSCs in dwarf napiergrass. (a) Excised shoot apex from shoot tiller as initial explant. (b) Induced shoot apex in MS medium containing 2 mg L⁻¹ BAP and 0.1 mg L⁻¹ 2,4-D after 10 days of culture. (c) Primary MSCs after 30 days. (d) MSCs after 40 days in the tube culture. (e) Proliferated clump in MS induction medium with 50 μM CuSO₄. (f) Germinated clump in MS medium containing 0.1 mg L⁻¹ NAA and 2.0 mg L⁻¹ BAP. (g) Numerous green regenerated plants in MS medium containing 0.1 mg L⁻¹ NAA and 2.0 mg L⁻¹ BAP. (h) Rooting regenerants in half strength hormone-free MS medium. (i) Regenerants from MSCs in field experiment.

**Table 1** Effect of hormone concentration on the formation of MSCs derived from shoot apices in tiller of dwarf napiergrass

<table>
<thead>
<tr>
<th>Hormone concentration</th>
<th>No. of inoculated shoot apices</th>
<th>No. of MSCs formation</th>
<th>% of MSCs formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D (mg L⁻¹) BAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>60</td>
<td>0³</td>
<td>0</td>
</tr>
<tr>
<td>0.01</td>
<td>2</td>
<td>60⁴</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>2</td>
<td>60⁵</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 2** Effect of CuSO₄ concentration on proliferation of MSCs from shoot apices in dwarf napiergrass

<table>
<thead>
<tr>
<th>Hormone concentration</th>
<th>No. of inoculated clumps</th>
<th>No. of proliferated clumps</th>
<th>% of proliferated clumps</th>
<th>Proliferation rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP CuSO₄ (μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>2</td>
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*Different letters following each value within a column indicated significant difference by Tukey’s Test (P < 0.05).*

The addition of 2.0 mg L⁻¹ BAP and 0.1 mg L⁻¹ 2,4-D to MS medium was the effective treatment for MSCs formation (29%). The MSCs derived from a single shoot apex were maintained on the same medium. Repeated subculture at 15 days interval resulted in a lower frequency of shoot tip formation throughout the period. In order to promote the proliferation of clumps, several concentrations of CuSO₄ (0.0, 5.0 and 50 μ M) were added to MS medium containing 2.0 mg L⁻¹ BAP and 0.1 mg L⁻¹ 2,4-D (Table 2). The density of multiple shoots increased dramatically and showed high density of apical meristem on MS medium containing 2.0 mg L⁻¹ BAP, 0.1 mg L⁻¹ 2,4-D and 50 μ M CuSO₄ (Figure 1c). For proliferation of clumps, a MSCs derived from a single shoot apex was visually selected based on criteria being prolific growth and adventitious shoot structure. It was observed that high levels of CuSO₄ (50 μ M) promoted the proliferation capacity of MSCs and increased the regeneration rate into plantlets.

Table 3 shows the percentage of regenerated plants from MSCs on regeneration medium. After 15 days, MSCs were germinated and regenerated (Figure 1f). The efficiency of plant regeneration was ranged from 22.2-84.1%. Medium with 0.1 mg L⁻¹ NAA and 2 mg L⁻¹ BAP was the best medium to induce regeneration of clumps (Figure 1g). In each shoot apex, more than 100 green shoots grew during culture initiation. All shoots developed the roots on half-strength MS medium treated without hormone (Figure 1h), which indicated that this system has a high potential of nursery plant production. The MSCs showed high regenerative potential with more than 100
green shoots recovered within 12 weeks of regeneration of the original shoot apex. Moreover, the regeneration frequency of clumps was high. The cultures of MSCs have been reported in other species (Zong et al. 1992; Zhang et al. 1996; Sharma et al. 2004; Gondo et al. 2007, 2009). This study indicated that CuSO4 can improve not only proliferation, but also the number of shoot buds in multiple-shoot, which are important findings for plant propagation in this study. Similar result has been reported on embryogenic callus culture in barley (Cho et al. 1998), oat (Cho et al. 1999) and bahiagrass (Gondo et al. 2005). The MSCs could show the vigorous regeneration and capacity for plant formation, which was maintained for more than 1 year.

**Estimation of DNA content using flow cytometry analysis**

The healthy plantlets were transferred in the greenhouse. The tillers from regenerated plants and control plants (dwarf napiergrass from field) were grown well under protected environmental condition (Figure 1t). Figure 2 showed that the mean 2C DNA contents of regenerated plants and control plants were 4.7±0.33 pg 2C⁻¹ and 4.5±0.36 pg 2C⁻¹, respectively. No differences were observed (P > 0.05) in 2C DNA content between regenerated plants and control plants. The stability in DNA content of *in vitro* regenerants from MSCs was analyzed using FCM. As a fast and accurate technique for estimating nuclear DNA content, FCM had been successfully used to evaluate the stability in DNA content of *in vitro* regenerated plants (Sliwińska and Thiern 2007; Obae and West 2010). It was inferred that regenerated plants from MSCs produced from this system maintained the same DNA content compared to control plants.

**Evaluation of morphological characteristics**

To assess somaclonal variation from MSCs regenerants, their morphological characteristics are shown in Table 4. Plant height of the regenerants ranged from 115.2 to 145.2 cm, whereas those of the control plants varied from 118.2 to 143.4 cm. Similarly, plant length of the regenerants varied from 116.3 to 148.1 cm, whereas those of the control plants varied from 123.4 to 150.4 cm. Leaf blade length of the regenerants ranged from 62.0 to 81.3 cm, whereas those of the control plants ranged from 63.6 to 79.9 cm. Similarly, leaf blade width of the regenerants varied from 2.4 to 3.4 cm, whereas those of the control plants ranged from 2.8 to 3.0 cm. Number of tillers

![Graph](image)

**Figure 2** Fluorescent intensity measures of dwarf napiergrass and *Oryza sativa* cv Nipponbare by flow cytometry. (A) Peak 1 corresponds to *O. sativa* cv Nipponbare, peak 2 corresponds to the control plant (native dwarf napiergrass). (B) Peak 1 corresponds to *O. sativa* cv. Nipponbare, peak 2 corresponds to regenerant plant from MSCs of dwarf napiergrass.

<table>
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<tr>
<th>Plants (Regenerants/ control)</th>
<th>Plant height (cm)</th>
<th>Plant length (cm)</th>
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<th>Leaf blade width (cm)</th>
<th>Tiller number</th>
<th>Fresh yield (g)</th>
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**Average**

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**Table 4** Morphological characteristics and fresh yield of regenerants from MSCs of dwarf napiergrass and control plants (*native dwarf napiergrass*) cat at 90 days after transplanting.

*: Not significant (P > 0.05)
of the regenerant plants varied from 16.0 to 32.0, whereas those of the control plants varied from 20.0 to 23.0. Fresh biomass yields of the regenerants varied from 561.8 to 876.8 g/pot, whereas those of the control plants varied from 661.2 to 871.4 g/pot. Statistical analysis showed no significant differences in morphological characters between regenerant and control plants. Regenerated and control plants used in morphological characteristics evaluation were survived and grew well.

Conclusions

The effective MCSs formation system in dwarf napiergrass was established. The most effective phytohormone treatment for MCSs induction was 0.1 mg L^{-1} 2,4-D plus 2.0 mg L^{-1} BAP. The addition of 50 μM CuSO4 could increase the percentage of MCSs proliferation. Plant regeneration frequency was achieved up to 84% by culturing the MCSs on solid MS medium containing 0.1 mg L^{-1} NAA and 2.0 mg L^{-1} BAP. The regenerant plants had similar morphological characters and DNA content as the control plants, which implies that this system is stable and suitable for nursery plant production on dwarf napiergrass in future. Attempts to establish a transformation system of napiergrass using particle bombardment has been taken and are now in progress. While this system established in this study is a method effective in producing many nursery plantlets of dwarf napiergrass, it can consider that it is applicable also to transgenic trait conversion on this species.

References


Sharma VK, Hänsch R, Mendel RR, Schulze J 2004. A


