The effect of 2,4-dichlorophenoxyacetic acid, benzyl amino purin and cupric sulphate on in vitro propagation system from shoot apices of shoot tiller of hybrid Napier grass (Pennisetum purpureum Schum)

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Abstract. An efficient micropropagation method of hybrid Napier grass (Pennisetum purpureum Schum) for in vitro plant production and material breeding was established from multiple-shoot clumps (MCS) regeneration system. This system was important for forage breeding system. Shoot apices from shoot-tillers produced MSC on Murashige-Skoog (MS) induction medium containing several combinations of BAP and 2,4-D in induction stage. The addition of 5 µM (v/v) and 50 µM (v/v) CuSO4 were added in best medium for inoculation to proliferate the clump in proliferation/multiplication stage. Plant regeneration was achieved by culturing on solid MS with several combination of medium containing NAA and BAP in regeneration stage. The best results for induction were Murashige-Skoog (MS) induction medium containing 2 mgL⁻¹ BAP and 0.1 mgL⁻¹ 2,4-D. The proliferation stage on MS medium containing 5 µM CuSO4 effective for proliferation (50% multiple shoot formation). The regeneration stage using 0.1 mgL⁻¹ NAA and 2.0 mgL⁻¹ BAP (51.6% number of shoot can regenerate). All plantlets were successfully grown up in an acclimatization stage. Based on the results, the hybrid Napier grass regeneration via MSC was a stable tissue culture system (no albino plats), which could be applied either for further genetic transformation assay or for alternative supply of nursery plant in the future.

1. Introduction

Napier or Elephant grass (Pennisetum purpureum Schum) is well-known as one of the extensive grasses grown during warm and cold seasons. This grass has several advantages like being resistant to pests, eyespot disease by fungus Helmintbosporium ocellum [1] and perennial nature and also having high biomass. It also accepts some soil conditions like low fertility acid soils and slightly alkaline soils. In addition, Napier grass tolerates the drought condition and has high photosynthetic and efficient in water use. Napier grass is highly nutritious during the vegetative phase. Therefore, farmers select this grass to feed their cattle due to its high palatability. Moreover, compared to other grasses and legumes, Napier grass can supply more dry matter per time unit. The research result of Ananta et al. [2] showed that Napier grass can produce 39.16% higher dry matter than conventional grass i.e. 22.24% and 13.53%, respectively. Napier grass radiation 39.16% higher dry matter than without radiation i.e. 14.62% and
12.18%, respectively[3]. Due to this fast growth and environment-friendly biomass, Napier grass is considerable for methane production and conversion to alcohol [1,4].

Material planting is crucial for cultivating grass in the field or grazing land. Napier grass produces fewer seeds. Several warm season grasses such as Napier grass has asexual mode of reproduction or apomictic reproduction. Pangtongkan et al. [5] reported that there were different heading period of Napier grass making difficult to have cross pollinated. In some habitats, this seed is also rarely grown. In the meantime, the seeds are grown they often have low viability or germination rate because the type of sexual mode of reproduction. Therefore, the propagation of material plants in Napier grass is performed with the cutting of stem node. The general type of cultivar hybrid Napier grass is higher and contains higher biomass production than the dwarf ones. Thus, this cultivar has high potential as forage in the cut and carry system. Today’s hybrid Napiergrass has recently been grown and examined for its growth in the tropics and subtropics in Southern Kyushu, Japan [6,7]. All of these researches reported that normal Napier grass was propagated vegetatively with stem node cuttings or rooted tillers. The hybrid Napier grass has a limiting factor in lignin content, palatability, and degradation rate. Therefore, it needs breeding activity to enhance the quality. This grass has not to produce seeds. However, it has some limiting factors for breeding, such as an apomictic type of reproduction of tropical grasses [8,9]. Pongtongkan et al. [5] explained that there was not any genetic variation in apomictic grasses because the seed only from mother gamet (apomictic). In normal reproduction type grass can produced seed from pollination between male and female gamet, but it was not happened in apomictic plants.

Researchers have suggested in vitro micropropagation as a possible way to grow material breeding and stock quickly. The main advantages of micropropagation are new varieties, which are rapidly multiplied without depending on the season, improvement of the plant health, and versatile germplasm storage. Its capability in producing similar regenerants with the mother plant qualifies this method as a method for propagation [10]. However, in vitro propagation frequently allows some genetic disruptions that can make variation of plants regeneration of tissue culture method (somatical variation)[11]. Therefore, checking the stability of the genome in vitro regenerants is essential if they will be used for nursery plant sources specifically [12].

Agriculture has extensively used micropropagation for the mass plant propagation such as sugarcane (Saccharum officinarum) [10,13], pineapple (Ananas comosus) [14], Imperata cylindrica [15], and also Brachiaria ruziensis [16]. It is necessary that all plants vegetatively propagated can be established for micropropagation system in the plant tissue culture method. Napier grass dwarf type has been previously regenerated via multiple shoot clumps [17,18]. However, at present studies, there are no identifications about the system of establishment and plant regeneration from multiple-shoot clumps of hybrid Napier grass.

This paper reported methods that are effective for plant regeneration by inducing multiple-shoot clumps (MSC) formation from shoot-apices of shoot-tillers and applying morphological characters evaluation in hybrid Napier grass (Pennisetum purpureum Schum).

2. Materials and methods
The shoot-tillers of hybrid type Napier grass (hybrid Napier grass) were collected from the field as explants. Then, the sand and dust particles on the shoot-tillers were removed by washing them in 3 hours under running water. Next, we sterilized the tillers by submerging them in 70% (v/v) ethanol in 2 minutes, continued soaking them in a 2% (v/v) sodium hypochlorite solution (NaOCl). After that, we agitated the solution of 3-5 mm shoot-tips in 15 minutes. We continued the process by washing the shoot-tips three times with sterilized water in two minutes. Next, stage one was induction stage, we executed the shoot apices from shoot-tillers 60 shoots every treatment and placed them in tubes of Murashige Skoog (MS) media [19] contained various concentrations of 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D). Murashige Skoog (MS) media contained 0.3% of phytagel, 3% of sucrose, and 0.1% (v/v) of preservative tissue culture media, added with concentration of 2,4-dichlorophenoxyacetic acid (2,4-D: 2.0; 0.01; 0.1; and 0.5 mgL−1) and 6-benzylaminopurine (BAP: 2.0;
0.01; 0.1; 0.5 mg L⁻¹) in the pre-experiment as a culture media. After 30–40 days in culture, only the media containing 2 mg L⁻¹ BAP could produce multiple-shoot clumps. The parameters to get the best result, we used to use 2 mg L⁻¹ BAP with several concentrations of 2,4-D (0; 0.01; 0.1; and 0.5 mg L⁻¹). The apical of shoot tiller become multipleshoot and showed the greeny clumps. To multiply of clumps, in stage two MSC were transferred to MS induction media enriched with few combinations of CuSO₄ (0, 5 and 50 µM). We calculated the clump proliferation capacity with diameter of clumps was -.5-1.0 cm after 21 days of culture.

Multiple-shoot clumps were transferred into stage three in regeneration MS basal medium containing 3% sucrose and 0.3% phytagel supplemented with 2 mgL⁻¹ α-naphthalene acetic acid (NAA) in combination with 0; 0.01; 0.1; and 0.5 mgL⁻¹ BAP, or hormone-free medium. After 21 days of culture, we measured the percentage of plant regeneration.

Further, we moved the germinated shoots to MS medium as rooting medium. The pH of all media should be adjusted to pH 5.6 to 5.8 before being autoclaved using Hirayama sterilizing machine HVE50, with 50 liter capacity, for sterilization at 121°C in 15 minutes. The shoots were placed under fluorescent lights Fultrum grow light white full spectrum of 3500 lux at 27°C for 16 hours for the incubation process. Subsequently, we took the in vitro regenerated plants out of the tube carefully and washed them under the water to clear away nutrients and agar. Next, we planted the shoots in the soil directly in polybags for acclimatization. The plants were placed in the nursery protected with agro-net for 30 days after transfer in polybag for acclimatization. Every day, the soil condition must be controlled to check the water contain of soil. Watering the plants every day and twice a day if the soil is dry. In our study 100% of transplanted plants were survive in acclimatization. After that, we transferred the plants to the field under environmental conditions. We analyzed the data by analysis of variance (ANOVA) and Tukey’s test using SPSS 10 software (version 10, SPSS inc, Chicago, IL. USA).

### 3. Results and discussion

This study proved that a high frequency of multiple-shoot clumps from shoot apices of Pennisetum purpureum cv Mott. could be formed followed by efficient rooting. There were several established methods to produce regenerated plants in Pennisetum purpureum [20]. However, all studies had been observed on the use of embryogenic calli from shoot tiller Napier grass. Embryogenic calli is respond explant in tissue culture media become callus formation contain embryogenic part. This callus can be formed because high auxin content, usually the colour is yellowish colour and compact. The chlorophyl contain is lower than multiple-shoot clumps. Embryogenic calli can developed for transformation material [21]. This paper is the first report which explains multiple-shoot clumps formation using shoot-apices in Pennisetum purpureum. In previous studies, the researchers used inflorescence segments [22], leaf tissue and anther [23], and immature seeds [20], also used shoot apical meristem.

The 2,4-D and BAP’s effects on multiple-shoot clumps formation from shoot apices of hybrid Napier grass are presented in Table 1.

| Hormone concentration (mg L⁻¹) | No. of inoculated shoot apices | No. of multiple-shoot clumps formation | % of multiple shoot-clumps formation |
|-------------------------------|-------------------------------|--------------------------------------|-----------------------------------|
| 2,4-D BAP                    |                               |                                      |                                   |
| 0                             | 0                             | 60                                   | 0α                                |
| 0.01                          | 2                             | 60                                   | 1α                                |
| 0.1                           | 2                             | 60                                   | 30c                               |
| 0.5                           | 2                             | 60                                   | 10b                               |

abc different letters following each value within a column indicate a significant difference by Tukey’s Test (P<0.05) on experiment of multiple-shoot clumps formation.
In inoculation stage, we did not find shoot multiplication in MS media without addition of hormone (0 mg L⁻¹ 2,4-D and 0 mg L⁻¹ BAP). We obtained multiple-shoot formation with elongation of the shoot on MS induction media that contained 2 mg L⁻¹ BAP without 2,4-D and 0.01 mg L⁻¹ 2,4-D. The size of the basal shoot apices enlarged. Those shoot apices produced several shoots after 60 days. By removing leaf and subculture in the same induction MS media contained 2 mg L⁻¹ BAP without 2,4-D and 0.01 mg L⁻¹ 2,4-D, every 14 days, adventitious shoots were performed from the enlarged shoot apices but did not transform into multiple-shoot clumps. Induction media with 2 mg L⁻¹ BAP and 0.01 mg L⁻¹ 2,4-D produced 30 shoots on average in the initial stage. MS media with 2 mg L⁻¹ BAP and 0.1 mg L⁻¹ 2,4-D grew multiple-shoot clumps directly from the basal of shoot apices (50%). Along with this, shoot apices also performed elongation of multiple-shoot from the explants in the same media. We also found the multiple-shoot clumps in MS media with 2 mg L⁻¹ BAP and 0.5 mg L⁻¹ 2,4-D, but sometimes they grew with callus.

Several variations of CuSO₄ concentrations were added in the MS induction media to promote the proliferation or multiply of multiple-shoot clumps. In this stage the total number of multiple shoot clumps will increase. The induced proliferation in the low concentration of CuSO₄ 5 µM was better than that in MS medium without CuSO₄ (Table 2).

### Table 2. Hormone and CuSO₄’s effect on proliferation multiple-shoot clumps from shoot apices of hybrid Napier grass.

| Hormone concentration | No. of inoculated clumps | No. of proliferated clumps | % of proliferated clumps | Proliferation rate† |
|------------------------|--------------------------|---------------------------|-------------------------|---------------------|
| 2,4-D (mg L⁻¹) | BAP (mg L⁻¹) | CuSO₄ (µM) | | | |
| 0.1 | 2 | 0 | 60 | 1a | 1.66 | + |
| 0.1 | 2 | 5 | 60 | 22a | 36.67 | +++ |
| 0.1 | 2 | 50 | 60 | 11a | 18.33 | ++ |

ab different letters of each value within a column indicate significant difference by Tukey’s Test (P<0.05).

The cultured multiple-shoot clumps were created from a single shoot apex delivered to MS media with several concentrations of NAA and BAP (Table 3).

### Table 3. Effect of hormone concentration on plant regeneration from multiple-shoot clumps derived from shoot apices in tiller of hybrid Napier grass.

| Hormone concentration (mg L⁻¹) | NAA | BAP | No. of inoculated clumps | No. of regenerated clumps (%) | No. Of Shoot formation |
|-----------------------------|-----|-----|-------------------------|------------------------------|-----------------------|
| 0 | 0 | 0 | 60 | 4(6.6)a | 8a |
| 0 | 2 | 60 | 9(15.0)a | 20a |
| 0.1 | 2 | 60 | 20(33.3)b | 47b |
| 0.1 | 2 | 60 | 31(51.6)b | 66b |

ab different letters following each value within a column indicate significant difference by Tukey’s Test (P<0.05).

Our result also corresponds to Devi [24] who uses shoot apical meristem from seeds as explant in the culture on MS medium containing 0.125 mg L⁻¹ 2,4-D and 2.0 mg L⁻¹ BAP. This study used MS medium containing 0.1 mg L⁻¹ 2,4-D and 2.0 mg L⁻¹ BAP but in this report, we use shoot apices from shoot tillers. We found that a low level of CuSO₄ (5µM) promoted the proliferation capacity of multiple-shoots clumps and 84% could regenerate into plantlets. These findings are similar to reports of Kalpana [25] on enhancing shoots buds number per explants by using CuSO₄ of pharmaceutical plants. We kept the healthy plantlets in the greenhouse in light condition, the temperature in the greenhouse between 25-30°C. The soil for media acclimatization were sterilized from bacteria and fungi.

In conclusion, we have established multiple-shoot clumps formation system until 50% formation rate from shoot apices of hybrid Napier grass. Multiple-shoot clumps formation system can produce more
regenerated shoots and has a high potential of mass production for nursery plants. We can also use this system for the regenerable target tissue for to transform the genetic traits by utilizing particle bombardment in the future.

4. Conclusions
The hybrid Napier grass regeneration via MSC was a stable tissue culture system because there was no albino regenerants from multiple shoot clumps, which could be applied either for further genetic transformation assays or for alternative supply of nursery plant in the future.

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