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Callus Induction and Plant Regeneration of Brachiaria Grass from Immature Inflorescence Explants

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ABSTRACT

This research was aimed to optimize concentration of phytohormone in callus induction and *in vitro* regeneration of three species of Brachiaria grass plant, namely *Brachiaria brizantha, B. decumbens*, and *B. ruziziensis*. Immature inflorescences were used as explant material. To induce callus, explants were inoculated into Murashige and Skoog (MS) basal medium supplemented with phytohormone combination of 2,4-dichlorophenoxyacetic acid (2,4-D), dicamba and kinetin. Observation of callus induction included percentage of callus formation and callus morphology. The embryogenic calli were then transferred into regeneration media, i.e. MS basal medium supplemented with kinetin and 6-benzylaminopurin (BAP). The result showed that 4 mg/L 2,4-D + 0.2 mg/L kinetin showed highest callus induction in *B. brizantha* and *B. decumbens*, namely 76% and 88% respectively. Whereas in *B. ruziziensis*, 3 mg/L 2,4-D + 0.2 mg/L kinetin showed highest callus induction, namely 86%. MS medium supplemented with 4 mg/L kinetin showed highest regeneration in all three grass species, namely 92.5% in both *B. brizantha* and *B. ruziziensis*, and 88.75% in *B. decumbens*.

Keywords: *Brachiaria brizantha*, *B. decumbens, B. ruziziensis,* Callus induction, Phytohormone, Regeneration

Introduction

One of the limiting factors in ruminant animal production is lack of feed availability, both in quality and quantity. Farmers usually just use plants growing in their surroundings as feed. Land decrease in Java because of development affects pasture and forage crops availability. In order to support livestock production it must be introduced with forage species which have high productivity, high nutrient content, and are tolerant to adverse environmental conditions.

Brachiaria grass is one of the tropical forage grass species which has high dry matter production and nutrient, and is more tolerant to drought condition. This grass is originally from Africa (Uganda, Kenya, Tanzania) and widespread to many areas including Asia and Pacific. Brachiaria consist of many species, such as *B. brizantha*, *B. decumbens*, *B. humidicola*, *B. ruziziensis*, *B. dictyoneura*, and *B. distachya*. This forage grass seems has high potential in Indonesia because it can grow well in many parts in the country. Brachiaria has already been used for many usages including fodder plant, land conservation and reclamation, and also cover crop (Fanindi and Prawiradiputra, 2005; Fanindi, 2016). This plant can be grown in marginal land such as peat land, individually or intercropped with legumes (Ali *et al*., 2014). Biomass production and seed production of this grass is quite high (Umami *et al*., 2016; Umami *et al*., 2018). Characterization of the plant is required to know all the potential.

Improvement of forage grass quality especially in nutrient content is important. This can be achieved by plant breeding methods such as hybridization or through genetic engineering. Plant genetic engineering via genetic transformation requires efficient tissue culture method or *in vitro* regeneration. High-frequency *in vitro* plant regeneration protocol is also a prerequisite for clonal propagation of grasses. The regeneration occurs through somatic embryogenesis or organogenesis. Somatic embryogenesis is based on plant regeneration through resembling process with zygotic embryo germination. Somatic embryo can develops in two way, directly or indirectly. Indirect somatic embryogenesis involves dediferentiation and embryogenic callus development process. The callus is then differentiated into plant tissues.

Several research on *in vitro* culture of Brachiaria have been conducted. The explant used is usually seed or plant parts developed from seed germinated *in vitro*. Efficiency of embryogenic callus induction and its regeneration

vary. Source of explants included seed (Thome *et al*., 1996; Cabral *et al*., 2011; Cabral *et al*., 2015; Takamori *et al*., 2015), basal segment, young leaves, and shoot tip of *in vitro* plant (Yaguinuma *et al*., 2018; Cabral *et al*., 2011; Ishigaki *et al*., 2009; 2012; 2014), immature embryo (Rodrigues-Otubo *et al*., 2000), and embryo (Silveira *et al*., 2003).

The auxin and cytokinin phytohormones were used for callus induction and plant regeneration in those experiments. The auxins included 2,4-dichlorophnoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), and picloram. The cytokinins included 6-benzylaminopurine (BAP), kinetin, and thidiazuron. Ishigaki *et al.* (2009) showed the optimum growth regulator for embryogenic calli formation in *B. ruziziensis* was 4.0 mg/L 2,4-D + 0.2 mg/L BAP. The plant regeneration via multiple shoot formation was obtained by supplementation of 1.0 mg/L BAP or 2.0 mg/L kinetin + 2.0 mg/L gibberellic acid.

One of the donor material can be used as explant in tissue culture is immature inflorescence. In some plants it is very responsive to *in vitro* treatment because it contains many meristematic cells. Therefore, optimization of somatic embryogenesis and regeneration of Brachiaria grass in this experiment was carried out using immature inflorescence. It was conducted on three species of *Brachiaria,* namely *B. brizantha*, *B. decumbens,* and *B. ruziziensis*. This experiment was aimed to find concentration of phytohormone in induction of callus and regeneration of the three Brachiaria species.

Materials and Methods

The Brachiaria plants used in this experiment were *Brachiaria decumbens* cv. Basilisk, *Brachiaria ruziziensis* cv. Kennedy, and *Brachiaria brizantha* cv. MG5. Explant material were immature inflorescences. Basal medium consisted of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) added with 200 mg/l casein hydrolisate, pH 5.6. Explants were surface sterilized prior to be inoculated into medium. Most upper node of shoot of Brachiaria plants aged 8-9 weeks-old was cut and some laminas were removed, then it was soaked in detergent for 10 minutes and rinsed with tap water until clean. The sterilization process then was to be continued in laminar air flow hood, the explants were soaked in 70% ethanol for 30 seconds. The explants then were soaked in 20% commercial bleach (Bayclean) for 7 minutes, after that those were rinsed with sterile water for 5 minutes 3 times. The explant was put on petri dish and cut longitudinally to pick immature inflorescence. The immature inflorescence then was cut ±0,5 cm in length using scalpel blade.

The sterilized explants then were planted into callus induction media. There were 8 callus induction media, namely MS basal medium supplemented with 2,4-D (1, 2, 3, and 4 mg/L) + 0.2 mg/L kinetin, and dicamba (1, 2, 3, and 4 mg/L) + 0.2 mg/L kinetin. Each medium was planted 5 replicates consisted of 10 explants. The cultures then were incubated at 25℃ in dark condition for 4 weeks. Formation of embryogenic callus and its morphology were observed and calculated. The calli then were proliferated in MS medium supplemented with 2 mg/L 2,4-D and 0.2 mg/L kinetin for 2 weeks. Callus proliferation was carried out twice.

The sub-cultured callus was then transferred into regeneration medium. There were 8 regeneration media, namely MS basal medium supplemented with kinetin (1, 2, 3, and 4 mg/L), and MS basal medium supplemented with BAP (1, 2, 3, and 4 mg/L). Each medium was planted 5 replicates consisted of 16 calli. The cultures then were incubated at 25ºC in 12 hours bright and 12 hours in dark condition for 4 weeks.

There were 8 treatments of phytohormone combination in callus induction experiment and 8 treatments of phytohormone combination in regeneration. Observation data included callus color and texture (morphology), percentage of callus formation, and percentage of regeneration. The experiment was conducted using completely randomized design. Data were analyzed using One Way Anova analysis with Statistical Product and Service Solution (SPSS) series 16 software.

Results and Discussion

Calus induction

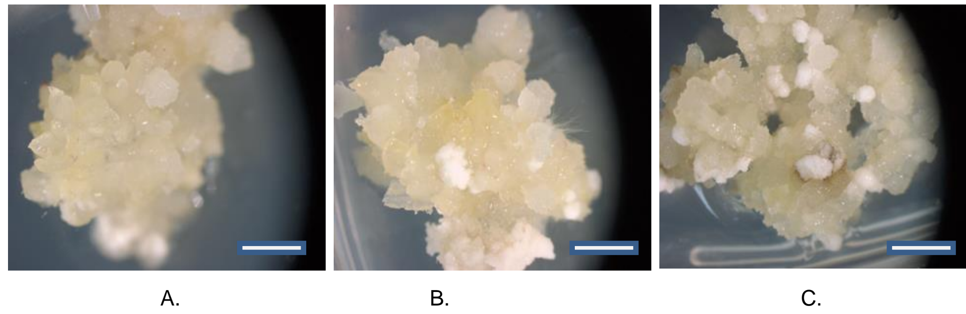
Result of callus induction experiment showed that MS basal medium supplemented with 4 mg/L 2,4-D and 0.2 mg/L kinetin revealed highest percentage of callus formation in *B. brizantha* and *B. decumbens* plants, namely 76±1.02% and 88±0.4% respectively. The highest percentage of callus formation in *B. ruziziensis* was in MS basal medium supplemented with 3 mg/L 2,4-D + 0.2 mg/L kinetin, namely 86±0.49%. Callus morphology in treatment with 2,4-D was generally yellowish in color and compact structure, whereas in medium with dicamba was whitish in color and friable (Figure 1 and 2).

In Table 1 it was shown that higher concentration of auxin resulted in higher callus formation rate. Auxin phytohormone of 2,4-D showed better effect than dicamba. Morphological types of callus formed were yellow and friable, and also white and compact.

The calli then were proliferated in proliferation medium, namely MS medium + 2mg/L 2,4-D + 0.2mg/L kinetin for 4 weeks. After that they were sub-cultured into fresh medium with similar composition.

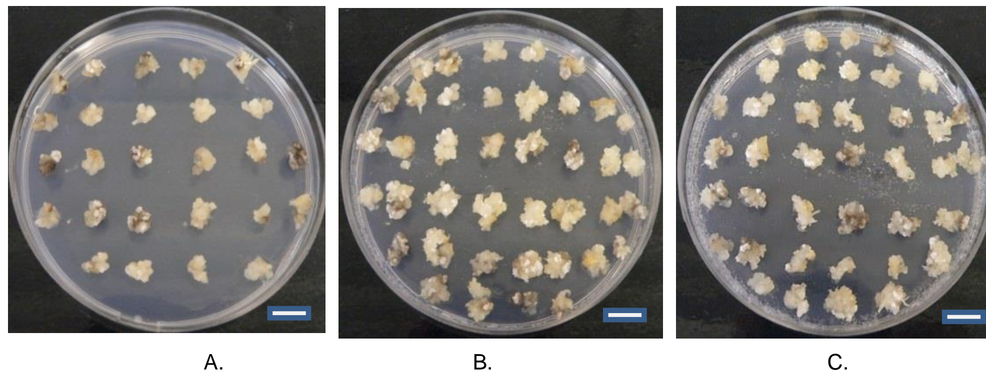
Regeneration

The calli obtained from induction and proliferation were transferred into regeneration medium to initiate shoot and root formation. Result of callus regeneration was presented on Table 2.



A. B. C.

Figure 1. Callus induction in the three species of *Brachiaria*.
A: *Brachiaria brizantha*, B: *B. decumbens*, C: *B. ruziziensis*. Scale bar: 1 cm



A. B. C.

Figure 2. Callus proliferation in the three species of Brachiaria.
A: *Brachiaria brizantha*, B: *B. decumbens*, C: *B. ruziziensis*. Scale bar: 1 cm

Table 1. Percentage of callus induction and morphology in the three Brachiaria species after eight weeks

Medium	*B. brizantha*		*B. decumbens*		*B. ruziziensis*	
	% of callus induction	Callus morphology	% of callus induction	Callus morphology	% of callus induction	Callus morphology
MS + 1 mg/L 2,4-D + 0.2 mg/L kinetin	32 ± 0.75^{a}	Yellow, friable	34 ± 0.8^{a}	Yellow, friable	36 ± 0.49^{a}	Yellow, friable
MS + 2 mg/L 2,4-D + 0.2 mg/L kinetin	46 ± 1.02^{b}	Yellow, friable	42 ± 0.75^{b}	White, compact	48 ± 0.4^{b}	White, compact
MS + 3 mg/L 2,4-D + 0.2 mg/L kinetin	48 ± 0.75^{b}	Yellow, friable	62 ± 0.75^{c}	White, compact	86 ± 0.49^{d}	White, compact
MS + 4 mg/L 2,4-D + 0.2 mg/L kinetin	76 ± 1.02^{c}	Yellow, friable	88 ± 0.4^{d}	White, compact	62 ± 0.75^{c}	White, compact
MS + 1 mg/L dicamba + 0.2 mg/L kinetin	26 ± 0.8^{a}	Yellow, friable	32 ± 0.4^{a}	Yellow, friable	34 ± 0.49^{a}	Yellow, friable
MS + 2 mg/L dicamba + 0.2 mg/L kinetin	46 ± 1.02^{b}	Yellow, friable	40 ± 0.63^{b}	Yellow, friable	46 ± 0.8^{b}	Yellow, friable
MS + 3 mg/L dicamba + 0.2 mg/L kinetin	52 ± 0.75^{b}	Yellow, friable	62 ± 0.75^{c}	Yellow, friable	66 ± 0.8^{c}	Yellow, friable
MS + 4 mg/L dicamba + 0.2 mg/L kinetin	74 ± 0.8^{c}	Yellow, friable	64 ± 1.02^{c}	Yellow, friable	68 ± 0.75^{c}	Yellow, friable

^{a,b,c,d} Different superscripts at the same column indicate significant differences (P<0.05).

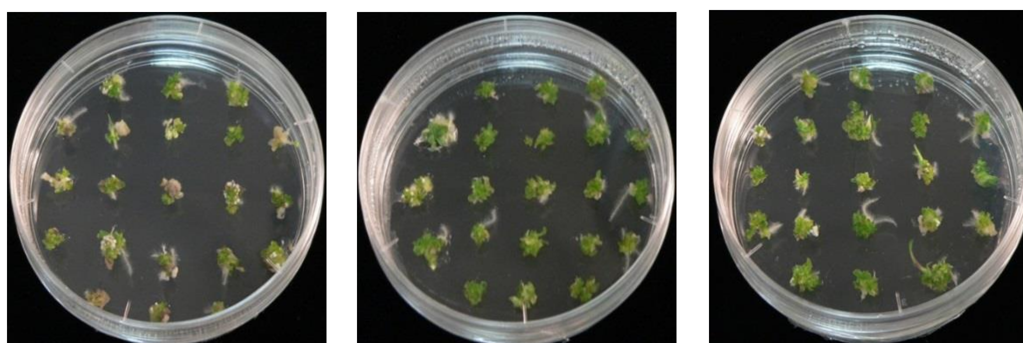
It was revealed that optimal concentration of phytohormone in the three species of Brachiaria was 4 mg/L kinetin. In general, percentage of regeneration in the three grasses was quite high (Table 2). The regenerated callus formed shoots and roots (Figure 3). Both friable and compact type of callus showed regeneration capacity.

In this research using immature inflorescence as the initial explant generally showed high response or efficiency of both embryogenic callus formation and regeneration, up to 90% compared to other research using different explants. Silveira *et al*. (2003) reported 73% frequency of embryogenic callus and 67% of regeneration in *B. brizantha* cv. Marandu using embryo as explant. Ishigaki *et al.* (2009) developed somatic embryogenesis system in *B. ruziziensis* using explants of seedling's shoot apical tissues derived from the seeds germinated *in vitro* added with 4mg/l 2,4-D and resulted in 17% somatic embryogenesis frequency. Experiment conducted by Cabral *et al.* (2011) using explants of *B. brizantha* seeds cultured on MS medium supplemented with 3 mg/l 2,4-D and 0.2 mg/l BA showed 74% yellowish white friable and compact embryogenic callus. Its callus regeneration grown on MS medium supplemented with 0.5 mg/l NAA, 1 mg/l BA, and 2.5 mg/l kinetin showed 54% regeneration rate.

Table 2. Percentage of callus regeneration in the three species of *Brachiaria*

Medium	*B. brizantha*	*B. decumbens*	*B. ruziziensis*
MS + 1 mg/L kinetin	68.75 ± 0.89[a]	41.25 ± 1.02[a]	68.75 ± 0.63[a]
MS + 2 mg/L kinetin	76.25 ± 0.4[b]	42.5 ± 0.75[a]	68.75 ± 0.63[a]
MS + 3 mg/L kinetin	90 ± 0.49[c]	67.5 ± 0.75[b]	88.75 ± 0.4[b]
MS + 4 mg/L kinetin	92.5 ± 0.75[c]	88.75 ± 0.4[c]	92.5 ± 0.75[b]
MS + 1 mg/L BAP	77.5 ± 0.49[b]	42.5 ± 0.75[a]	76.25 ± 0.4[c]
MS + 2 mg/L BAP	80 ± 0.75[b]	43.75 ± 0.89[a]	78.75 ± 0.8[c]
MS + 3 mg/L BAP	88.75 ± 0.4[c]	67.5 ± 0.4[b]	90 ± 0.8[b]
MS + 4 mg/L BAP	88.75 ± 0.4[c]	68.75 ± 0.63[b]	91.25 ± 0.8[b]

[a,b,c] Different superscripts at the same column indicate significant differences (P<0.05).



A. B. C.

Figure 3 . Callus regeneration in the three species of *Brachiaria*.
A: *Brachiaria brizantha*, B: *B. decumbens*, C: *B. ruziziensis*.

Immature inflorescences have been known as important explant source in many cereal and grass species. They have been used in cereals such as *Paspalum scrobiculatum* millet (Vikrant, 2001), *Setaria italica* (Vishnoi and Kothari, 1996), wheat (Barro *et al*., 1999; Caswell *et al*., 2000, He and Lazzeri, 2001, Kavas *et al*., 2008), barley (Barro *et al*., 1999; Havrlentová *et al*., 2001), tritordeum (Barro *et al*., 1999), and *Sorghum bicolor* sorghum (Brettell *et al*., 1980; Jogeswar *et al*., 2007). In addition, in forage grasses including buffel grass *Cenchrus ciliaris* (Yadav *et al*., 2009), *Bothriochloa* and bermuda grass (*Cynodon* spp.) (Artunduaga *et al*., 1988), miscanthus grass (Głowacka *et al*., 2010), ryegrass *Lolium multiflorum* (Dale *et al*., 1981), kallar grass (*Leptochloa fusca*) (Praveena and Giri, 2012), zoysia turfgrass (Dhandapani *et al*., 2008), seashore paspalum *Paspalum vaginatum* (Neibaur *et al*., 2008). In the research on those plants showed that several factors affecting induction of somatic embryogenesis and regeneration using explant of immature inflorescence included: genotype, age and size of immature inflorescence, media (nutrient composition), and also phytohormone type and concentration.

Conclusions

From this research it can be concluded that callus induction and regeneration rate of Brachiaria grass plants using explant of immature inflorescence was quite high. Phytohormone combination of 4 mg/L 2,4-D + 0.2 mg/L kinetin showed optimum concentration of callus induction in *B. brizantha* and *B. decumbens* , whereas in *B. ruziziensis* was 3 mg/L 2,4-D + 0.2 mg/L kinetin. Regeneration media supplemented with 4 mg/L kinetin showed highest regeneration in the three Brachiaria grass species.

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