Optimizing Growth Conditions for Green Grass Jelly (*Cyclea barbata* Miers) Derived in Vitro Culture: Greenhouse Acclimatization and Field Growth

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Abstract. *Cyclea barbata* Miers, a species within the Menispermaceae family, holds significance in medicinal and beverage applications. In vitro propagation is a common practice for conserving this plant; however, a comprehensive investigation of its growth characteristics within controlled greenhouse and natural field environments is essential. This study aims to determine the optimal planting media for acclimatization and the subsequent growth of *Cyclea barbata* Miers propagated from in vitro cultures. The experimental design encompasses two phases: 1) greenhouse acclimatization and 2) field growth. Various planting media, including combinations of sterile soil, manure, and husk charcoal, were rigorously evaluated during the acclimatization phase. Following the acclimatization period, the plants were transferred to the field. Multiple parameters were assessed: plant growth percentage, branch and node counts, leaf dimensions (length and width), and leaf wet and dry weights. Our findings reveal that a planting medium comprising sterile soil, manure, and husk charcoal provided the best performance growth outcomes during acclimatization. Furthermore, we observed that plant age exerts a discernible influence on the growth dynamics of *Cyclea* barbata Miers.

1 Introduction

Green grass jelly (*Cyclea barbata* Miers) belongs to the Menispermaceae family and is native to Southeast Asia, including Indonesia, particularly on the island of Java. It has a history of traditional use for treating ailments such as fever, diarrhea, stomachaches, inflammation, and hypertension [1],[2].

The habitus of green grass jelly is a liana, which twines stem or vines to the other plant. The plant has an incomplete leaf because it only has stalks, leaf blades, and no plant sheath. The plant leaves are single, shield-shaped, curved with fingers, blunt leaf tips, flat-leaf edges, and grooved leaf bases. The upper and lower leaf surfaces are hairy. The color of the young leaves at the top is green, and the bottom is light green. The leaf bone is fingered. The average leaf length reached 7.41 cm, and the leaf width was 6.27 cm [3].

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Green grass jelly contains various chemical components such as fats, polyphenols, flavonoids, chlorophyll, and saponin [4]. Research indicates its pharmacological effects, including anti-inflammatory, antioxidant, anti-malaria, anti-hypertensive, antibacterial, and anti-cancer properties [1]. Moreover, ethanol extract from green grass jelly leaves has demonstrated the ability to inhibit the growth of *Salmonella typhi* biofilm [5].

Green grass jelly propagation mainly employs stem cuttings, which are more readily available than tuber. In response to high demand, plant leaf supply is on the rise. At the Indonesian Spices and Medicinal Crops Research Institute (ISMCRI), germplasm collection for green grass jelly is conducted in a greenhouse. Additionally, efforts were conducted in the lab to propagate the plant in vitro through culture on an MS medium supplemented with 0.1 mg/l of BA.

Successful tissue culture plant propagation relies on explant sources, media composition, and precise application of plant growth regulators. Another critical factor is the effectiveness of the acclimatization process in the greenhouse, where plantlets adapt from in vitro culture to their natural environment, ensuring their successful establishment in the field [6]. Each plantlet's successful acclimatization in the greenhouse depended on suitable growing media and environmental conditions [7]. However, the acclimatization process, growth, and yield of *C. barbata* derived from in vitro culture have never been reported. This research aims to determine the effect of planting medium on the growth and yield of green grass jelly derived from in vitro culture.

2 Materials and Methods

2.1 Study site and plant materials

The research was carried out in the greenhouse of the Indonesian Spices and Medicinal Crops Research Institute Bogor from 2020 to 2021. The plant material used was plantlets of green grass jelly from in vitro culture (Fig 1).

Fig 1. Plantlet of green grass jelly in vitro

2.2 Research implementation

2.2.1 Acclimatization in greenhouse

Following in vitro culture, the plantlets underwent a meticulous process that included thorough washing to eliminate residual agar before being transplanted into plastic containers measuring 12 x 20 cm in preparation for acclimatization. Diverse planting media treatments

were scrutinized, encompassing sterilized soil mixed with manure in a 2:1 ratio, sterilized soil combined with manure and husk charcoal in a 2:1:1 ratio, non-sterilized soil blended with manure in a 2:1 ratio, and non-sterilized soil mixed with manure and husk charcoal in a 2:1:1 ratio. Each green grass jelly plantlet was meticulously placed in an isolated container, nourished with distilled water, and safeguarded beneath plastic coverings to sustain optimal humidity levels within the greenhouse throughout the two-month acclimatization phase. After this period, the gradual removal of the plastic coverings commenced. Upon achieving the requisite vigor, the plantlets were translocated into 20 x 30 cm polybags containing a mixture of soil and manure in a 2:1 ratio. At the same time, additional support in the form of climbing poles was provided for root attachment.

2.2.2 Plantation in field

Throughout the acclimatization phase, all surviving plants were transitioned to the field environment without any differential treatment. The parameters encompassed the percentage of plant growth, counts of branches and nodes, measurements of leaf length and width, and the determination of wet and dry leaf weights. These parameters were meticulously recorded during the second and third months following the transfer to the field.

2.3 Data analysis

The data obtained were analyzed using the Excel program. Therefore, the LSD test with an accuracy of 1% will be for further tests.

3. Result and Discussion

3.1 Acclimatization in Greenhouse

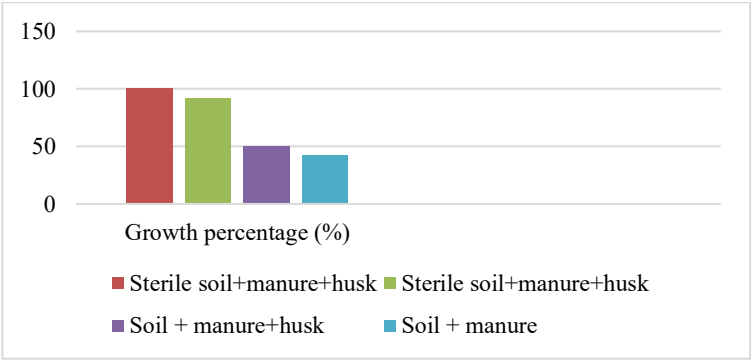

Fig 2. Growth percentage of green grass jelly on acclimatization at the greenhouse

The best growth of green grass jelly during greenhouse acclimatization was achieved with sterile growing media, specifically soil + manure + husk (2:1:1) and soil + manure (2:1) (Fig 2,3). Each plantlet's successful acclimatization in the greenhouse depended on suitable growing media and environmental conditions. The use of sterile growing media can reduce mortality rates during this phase, consistent with findings in the acclimatization of *Encyclia cordigera* using sterile peat moss [7]. Conversely, unsterile media may contain contaminants like bacteria and fungi, which can hinder plant growth, as in vitro-cultured plantlets are more vulnerable compared to their in vivo counterparts in a greenhouse. Notably, *Eucalyptus*

bosistoana plantlets showed different responses, with a mixture of garden soil + sand + compost (1:1:1) supporting successful growth under greenhouse conditions [8].

In vitro-cultivated plants have differed from field-grown ones, often experiencing high mortality upon transfer. Common issues included non-functioning stomata, underdeveloped root systems, and immature cuticles [9]. The response to acclimatization varied among plants. For instance, *Curcuma caesia* Roxb. was successfully acclimatized in the potting medium by combining cocopeat and peat moss, producing the highest survival rate of 77.78% [10]. Success in acclimatizing tissue-cultured plants depends on factors like plantlet size and root condition, significantly affecting nutrient absorption [11]. Careful medium selection is crucial to minimize plantlet mortality and ensure successful acclimatization. When placed in polybags with an optimal sterile growing medium (soil + manure + husk), plant growth increased, resulting in new branches and leaves (Fig 4).

Fig 3. The plant performance on the growth medium two months after acclimatization: a) sterile soil + manure + husk, b) sterile soil + manure, c) soil + manure, d) soil + manure + husk

Fig 4. The plant performance at the polybag four months after acclimatization

3.2 Growth in the field

The number of branches, nodes, and leaf lengths increased significantly after planting in the field for two and three months (Table 1).

Table 1. Growth components of green grass jelly two and three months after planting in the field

Plant age (month)	Number of branches	Number of nodes	Leaf length (cm)	Leaf width (cm)
2	5.1 [b]	35.0 [a]	8.5 [b]	4.2 [a]
3	10.7 [a]	46.5 [b]	9.2 [a]	4.5 [a]
LSD	1.59	5.30	0.27	0.39

Note: The number followed by the same letters is not significantly different at 1% LSD

Increasing plant age positively influenced the number of branches, nodes, leaf length, and leaf width in the field conditions. Plants from in vitro culture demonstrated robust adaptation and growth optimization. Organic fertilizer, specifically cow manure, was applied in this study to enrich the soil and enhance plant growth. Cow manure contains nitrogen (0.29%), P2O5 (0.17%), and K2O (0.35%) [12]. This organic fertilizer fulfills the plant's nutrient requirements and enhances soil fertility [13]. Furthermore, cow manure improves soil conditions by raising pH levels in acidic soil, increasing water-holding capacity, hydraulic conductivity, and infiltration rate, and reducing soil bulk density. It is an excellent source of plant nutrients and enhances soil structure [14].

Applying organic materials enhances soil's physical, chemical, and biological properties. Organic materials play a crucial role in improving soil's chemical properties by supplying essential macro and micronutrients required by crops [15]. Interestingly, even without inorganic fertilizers, the plants exhibited optimal growth, particularly in their leaves (Fig 5). Fertilizers, which supply necessary plant nutrients, improve soil's physical and chemical characteristics and enhance soil fertility.

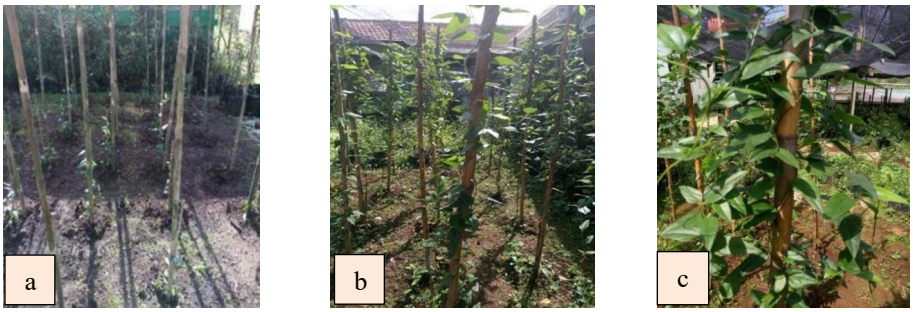

Fig 5. The growth of green grass jelly in the field: a) two weeks after planting, b,c) on three months

The average yield of green grass jelly from in vitro was around 84.9 g (wet weight) and 18.5g (dry weight) after five months of planting in the field (Fig. 4). The production of green grass jelly in the first year of in vitro plants is low. In the first year, the plant adapts to its natural habitat. Several studies have demonstrated the production of *Curcuma aeruginosa* rhizome from in vitro culture, and its rhizome production is still limited [11].

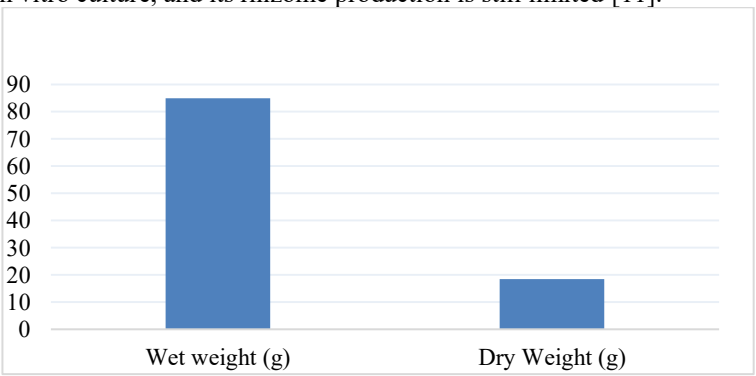

Fig 6. The yield of green grass jelly in five months in the field

In this experiment, we harvested green grass jelly leaves five months after field cultivation. These leaves closely resembled the mother plant, with no significant differences. Chemical compounds found in green grass jelly leaves include alkaloids, saponins, tannins, phenolics, flavonoids, triterpenoids, steroids, and glycosides (Table 2).

Table 2. The quality of green grass jelly leaf

Treatments	Contents
Alkaloid	+
Saponin	+
Tanin	+
Phenolic	+
Flavonoid	+
Triterpenoid	+
Steroid	+
Glycoside	+
Antioxidant IC 50% (ppm)	1.059.70

Conclusion

Propagation of Green Grass Jelly through in vitro culture, followed by acclimatization in greenhouse conditions, has proven viable. Notably, a sterile growth medium comprised of soil, manure, and husk exhibited a remarkable success rate during acclimatization. Growth parameters, such as branch and node counts, and leaf length, showed notable increases with plant age. However, it is worth mentioning that the initial yield in the first year was relatively low.

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