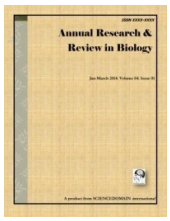

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In vitro Microrhizome Formation in *Kaempferia parviflora*

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript

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ABSTRACT

Plantlets produced *in vitro* from rhizomes of *Kaempferia parviflora* were used as the source of explants for microrhizome development. Sixty percent of the plantlets formed microrhizomes in liquid medium supplemented with1 mg/L BAP+1 mg/L NAA, with 60 g/L sucrose. The treatment gave the highest fresh weight of microrhizomes, at 265 mg/plantlet. This optimized protocol is suitable for the commercial production of disease-free *Kaempferia* microrhizomes that can be stored and transported easily.

Keywords: Kaempferia parviflora; microrhizomes; medicinal plants; in vitro.

1. INTRODUCTION

The increasing demand for many plant species for food and medicine poses a threat to their rapid depletion from their natural habitats. In this regard, the capability to regenerate and propagate plants from cultures of cells and tissues is one of the most exciting and useful aspects of *in-vitro* cell and tissue culture. In many instances, *in vitro* culture can provide a sustainable alternative supply that alleviates the pressure of harvesting from the wild.

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Kaempferia parviflora is an herbaceous species belonging to the *Zingiberaceae* family. Since ancient times, it has traditionally been used as a health-promoting tonic that is both a stimulant and a vitalizing agent. The plant is very popular for its purported ability to enhance sexual performance mostly in males, and for which various presentations of this product are available especially in the Thai market for traditional medicines. This medicinal herb is propagated vegetatively through rhizomes, but mass propagation by this means is very slow. Tissue culture can be an important tool for rapid multiplication of slow propagating species such as this. Propagation through axillary bud multiplication is a relatively easy method to obtain uniform planting material. *In vitro* rhizome formation in ginger has been reported [1,2,3]. *In vitro* ginger rhizome formation in cultures supplemented with high sucrose concentrations (9 to 12%) has been reported [4] and the multiplication of microrhizomes has helped in the conservation of *Zingiber* [5]. Microrhizome formation is affected by many factors such as the concentrations of auxins, cytokinins and sucrose in the culture medium, as well as the temperature and photoperiod [6,7,8].

In recent times, storage organs such as bulbs, corms, tuber sand rhizomes have become a focus of attention in vegetative propagation because such propagules can be directly transferred to the soil with minimal acclimatization or hardening. Microrhizomes are easy to acclimatize and store; they are also less vulnerable to transportation conditions. There are a few reports on propagation success with microrhizomes of turmeric [9,10,11,12,13]. However, there being no publication on *in vitro* microrhizome culture of *Kaempferia parviflora*, we present this report on our study.

2. MATERIALS AND METHODS

2.1 Establishment of Initial Cultures

Cultivated plant of *Kaempferia parviflora* (Fig. 1a-b) were cultivated in the glasshouse at the Malaysian Agricultural Research and Development Institute (MARDI) Serdang, Selangor, Malaysia. After one month in the green house when the rhizomes had sprouted, buds from the shoots were collected and used as the source of explants. The shoots were cleaned under running tap water for an hour, then washed with a commercial laboratory detergent and rinsed thoroughly with water. The explants were then immersed in 1% (v/v) fungicide (Benomyl 50%, Benlate ®) for one hour and again rinsed thoroughly under running tap water. Subsequently, the explants were surface sterilized with Clorox®, followed by several rounds of rinsing with sterile distilled water. The outer layers of the leaf sheaths of the buds were removed with a sterile surgical blade under aseptic conditions. The sterilized explants were then inoculated onto basal Murashige and Skoog's (1962) medium supplemented with 3.0 mg/L benzyl aminopurine (BAP) for the *in vitro* production of plantlets. The pH of the medium was adjusted to 5.8prior to autoclaving (121℃) under 1.05kg/cm^2 pressure for 15 minutes. The cultures were grown under white fluorescent light (3,000 lux) adjusted to a photoperiod of 16 hrs light/8 hrs darkness at 25±2ºC. The plantlets obtained *in vitro* were used for the microrhizome experiments described below.

2.2 Induction of Microrhizomes

Aseptic shoots approximately 4.5 cm long from plantlets of *in vitro* cultured *Kaempferia parviflora* were used as explants for the induction of microrhizome. The explants were sub-cultured in 150 ml flasks on basal solid medium (3 g/L phytogel) consisting of MS medium supplemented with 3% sucrose and varying concentrations of benzyl aminopurine (BAP) (0, 0.5, 1.0, 3.0 and 5.0 mg/L) and napthyleneacetic acid (NAA) (0, 0.5, 1.0, 3.0 and 5.0 mg/L), or in combinations of both the growth regulators. The cultures were then incubated under 16 hrs light/8 hrs darkness. After six months of culture, the percentage and fresh weight of microrhizomes that developed were recorded.

In a separate experiment, the effects of the sucrose and agar concentration were examined to test their effects on rhizome formation over three months. Explants were cultured on medium containing 1 mg/L BAP+1 mg/L NAA and different concentrations of agar. The treatments of agar were solid 3g/L, semi-solid 1.5 g/L and liquid no agar that cultured on orbital shaker, with supplementation of sucrose in varying concentrations (0, 10, 30, 45, 60, 90 and 120 g/L). After three months of culture, the percentage and fresh weight of microrhizomes that developed were recorded. For cultures on the solid medium, all the explants were sub-cultured onto fresh medium at one month intervals. In the case of the liquid medium, the medium was replaced with fresh medium at monthly intervals. Ten explants were transferred

into each jar containing 40 ml of the above media, with three replicates for each treatment.

2.3 Statistical Analysis

The data (10x 3 replicates per treatment) were subjected to one way analysis of variance (ANOVA) to assess treatment differences and interaction using SPSS version 11.0 software. Significance of differences between means was tested by Duncan's Multiple Range Test ($p \leq 0.05$).

3. RESULTS AND DISCUSSION

Microrhizome formation induced directly on cultured explants is influenced by factors such as growth regulators [14] and sucrose [15,16]. These factors are commonly studied in combination with one another. In the present study, *Kaempferia parviflora* plantlets regenerated *in vitro* were cultured in the presence of different concentrations of sucrose and the growth regulators BAP and NAA. After five month of culture in solid media (Fig. 1c), microrhizomes were produced in five treatments, with the highest success rate of 15% obtained using the culture medium containing 1mg/L BAP+ 1mg/L (Table 1). This treatment gave 79 mg fresh weight of microtubers/flask. In comparison, the culture medium that contained 3mg/L BAP+ 3mg/L NAA, and another medium that contained 3 mg/L BAP+1mg/L NAA registered only 10% and 5% respectively in successful microrhizome production, producing 46 mg and 21 mg fresh weight of microrhizomes/flask respectively (Table 1). The number of microrhizomes produced after five months of culture varied according to the growth regulators employed. It was evident that microrhizome production was more efficient when cultured in the presence of both plant growth regulator, BAP and NAA than single application.

The results of the present investigation supported the report that obtained a positive response on microtuberization of yams when BAP was incorporated into the culture medium together with NAA [17]. In that report, the combination of NAA and BAP (0.2- 0.2 mg/L) stimulated the formation of microrhizomes in *Dioscorea cayennins-rotundata* and *D. alata*. This finding was further supported by a scientist who made similar observations when they combined BAP (3 mg/L) and NAA (2 mg/L) to achieve 98% formation of microtubers that subsequently differentiated into shoots [18]. In the present study, the percentage of plantlets producing microrhizome varied according to the concentration of BAP and NAA used in combination, as noted [17]. Nevertheless, the concentration that gave the optimum response would depend on the plant genotype and growth regulators used. According to a report [19], an understanding of the relationship between the aerial parts of the plantlet and microrhizome initiation is important in appreciating the transport mechanisms that allow assimilates to be transferred to the rhizome.

The factors controlling microrhizome initiation are different from those that control microrhizome induction [20]. A genetic component comes into play, and increasing BAP concentration could result in a decrease in the number of genotypes responding positively. In their study on *Solanum tuberosum*, [21] found that that 0.75 mg/L BAP gave the highest microtuber weight. It is relevant also to note the observation of [22] who found relatively high concentrations of BAP (0.75 and 1 mg/L) increased microtuber weight. The role of cytokinin and auxins, individually or in combination, in raising the frequency of *in vitro* tuberization potato has been observed [6,23].

The effects of sucrose and agar to improve microrhizome production were next evaluated. Different concentrations of sucrose (10-120 g/L) were tested using solid, semi-solid or liquid media containing 1 mg/L BAP and 1 mg/L NAA (Fig. 1d). Microrhizomes were produced after 3 months when the explants were cultured in a liquid medium supplemented with 30-60 g/L of sucrose (Fig. 1e-h). Even more microrhizomes were produced after 90 days, with the basal medium supplemented with 45 to 60 g/L sucrose.

The best result (with 60% of the plants producing microrhizomes) was obtained with 60 g/L sucrose added to the liquid culture medium. Good results were also obtained using 45 g/L sucrose in the liquid medium (35% success), or with 60 g/L sucrose in the semi-solid medium (25% success) (Table 2). In the solid and semi-solid media, treatment with 10-30 g/L of sucrose did not produce any mizrorhizomes. The medium with sucrose concentrations of 90 – 120 g/L also produced rhizome however, the plant become more rapidly retarded and died.

Table 1. Effect of BAP and NAA on microrhizome development on Solid MS medium supplemented with 3% sucrose

BAP (mg/L)	NAA (mg/L)	% of plantlets producing microrhizomes	Fresh weight of microrhizoes (mg/flask)
0	0	0	0
0.5	0	0	0
1	0	0	0
3	0	0	0
5	0	1	-
0	0.5	0	0
0	1	0	0
0	3	1±0.5	-
0	5	0	0
0.5	0.5	0	0
1	0.5	2±0.7	25±9
3	0.5	0	0
0.5	1	0	0
1	1	15±2.3	79±11
3	1	5±0.5	21±3
0.5	3	0	0
1	3	0	0
3	3	10±3.1	46±7

Results are mean values ± standard errors after 5 months of culture

The fresh weight of the microrhizomes produced was reflected in its percent success in production. Thus, 60 g/L sucrose in the liquid culture medium resulted in the highest fresh weight of microrhizomes (256 mg/flask). This was followed by the liquid medium containing 45 g/L sucrose which produced 145 mg of microrhizomes per flask.

The results from the present investigation showing the best result with 60 g/L (i.e. 6%) sucrose are consistent with the reports [9,10,11] who found that optimum microrhizome formation in *Curcuma* were obtained in culture media containing 6 - 9% sucrose.Similar results have been reported [24] in their study on *Curcuma longa*. They observed that media containing 60 g/L sucrose and 3 mg/L BAP resulted in the most successful rhizome formation. Their study also found that further increase in sucrose concentration from 60g/L to 90 g/L decreased the percentage response in rhizome formation. In the present study, increasing sucrose concentration to 90-120 g/L resulted in death of the plantlets, or their plant becoming retarded.

Previous work has emphasized the importance of sucrose being present in the culture medium for microrhizome formation. The turmeric plantlets cultured in a medium containing 80 g/L sucrose, and supplemented with low concentrations of growth regulators produced rhizomes under an 8 h illumination period [11]. Areported in agreement, stating that the induction of microrhizomes in media containing either 80g/L or 90g/L sucrose elicited the best response in *Zingiber officinale* after three months of culture [25]. On the other hand, microrhizomes obtained from the treatment with 100g/L sucrose were retarded and they exhibited symptoms of vitrification together with the decaying of buds. In a separate study on ginger, however, [4] found that microrhizomes of plantlets developed *in vitro* in MS medium enriched with sucrose at 90 g/L or 120 g/L. A recent investigation [26] showed that microhizomes of *Costus pictus* were successfully initiated on ½ strength MS medium supplemented with 0.44 mg/L NAA+ 7.2 mg/L BAP, together with 50-130 g/L sucrose. It was observed that the media containing 90 g/L sucrose produced the largest microrhizomes, in terms of size and average fresh weight. A report of the effect of sucrose on microtuber induction in potato. They concluded that tuberization could be induced by an addition of 8% sucrose when kept under short (8 h) photoperiods [27]. Where ginger (*Zingiber officinale*) is concerned, [4] found that temperature and photoperiod had no effect on rhizome induction, but sucrose – as an energy source - was the major determinant of rhizome formation.

Fig. 1. Production of microrhizomes through *in vitro* process (a) Cultivated plantlets, (b) Cross section of underground rhizomes, (c) Five month of culture in solid media, (d) liquid media containing 1 mg/L BAP and 1 mg/L NAA cultured on orbital shaker, (e) In vitro grown shoot showing induction of microrhizomes at its base after 3 months of culture, (f-g) sprouting microrhizomes with shoots (h) Cross section of microrhizomes

Table 2. Effect of sucrose and type of support medium on the production of microrhizomes

Support medium	Sucrose (g/L)	% of plantlets producing microrhizomes	Fresh weight of microrhizomes (mg/flask)
Solid	0	0	0
	10	0	0
	30	0	0
	45	0	0
	60	5+1	13+3
	90	+	0
	120	++	0
Semi solid	0	0	0
	10	0	0
	30	0	0
	45	5+1	15+2
	60	25+5	71+11
	90	+	0
	120	++	0
Liquid	0	0	0
	10	0	0
	30	20+4	45+8
	45	35+4	145+23
	60	60+8	256+34
	90	+	0
	120	++	0

Results are mean values ± standard errors after 3 months of culture
+ 50% showing signs of plant retardation; ++ 75% showing signs of plant retardation

4. CONCLUSION

The *in vitro* microrhizome formation system for *Kaempferia parviflora* was optimized in the present study. Liquid MS medium supplemented with 1mg/LBAP+1mg/L NAA and 60g/L sucrose was the most effective for microrhizome formation. Production of *in vitro* microrhizomes would provide a suitable source of disease-free seed rhizomes that could be stored and transported easily. In addition, *in vitro* microrhizomes do not require acclimatization in the field. The present protocol contributes towards an improved commercial propagation system for *Kaempferia parviflora,* rendering its cultivationmore efficient and productive. The protocol also can be utilized by commercial growers for large-scale production of disease-free ginger and thus provide a sustainable supply for the pharmaceutical sector.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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