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BIO-POTENTIAL OF FERMENTED FRUIT WASTE SOLUTIONS ON *IN VITRO* SEED GERMINATION AND REGENERATION OF *Lycium barbarum* AND *Aquilaria malaccensis* Lamk.

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ABSTRACT

There are many synthetic growth media for plant tissue culture available in the market such as Murashige and Skoog (MS) Medium, Woody Plant Medium (WPM), Schenk and Hildebrandt (SH) Medium and Gamborg's B-5 Medium. The aim of this study was to substitute the synthetic media used in the plant tissue culture by organic additives which are pineapple, banana, papaya, calamansi lime, kaffir lime and key lime peels. Two formulated fermented fruits waste solutions composed of these organic additives were prepared in different concentrations (Formula A- calamansi lime, kaffir lime, and key lime peel; Formula B -banana, pineapple, and papaya peels) to study their effects on *in vitro* seed germination and regeneration of *Lycium barbarum* and *Aquilaria malaccensis* Lamk. Statistical results showed that they were significantly different in interaction effects ($p<0.05$) in promoting the plant growth in the formulated media as compared to control medium determined by ANOVA test. Application of this formulated fermented fruits waste solutions should be considered since it is found to be responsive in *in vitro* seed germination and regeneration of *L. barbarum* and *A. malaccensis* Lamk and will potentially minimize the operational cost.

Key words: Fermented fruits waste solutions, organic additives, *in vitro* seed germination

INTRODUCTION

In vitro micropropagation of plant has played a vital role in the production of large numbers of healthy plants in a relatively short period as compared to conventional techniques (Ahmadian *et al*., 2013). The cost of commercializing plant tissue culture techniques is often very expensive. Therefore, many researches were conducted to substitute any materials that can minimize the cost of production without compromising the quality of produced plants (Savangikar, 2002 and Demo *et al*., 2008). According to previous study by Kaur and Bhutani (2012), addition of coconut water and banana homogenate produced high number of shoot and early plantlet development of *Cymbidium pendulum*. In addition, Daud *et al.* (2011) demonstrated the effectiveness of coconut water, papaya, tomato and banana juices in regenerating shoots of *Celosia* sp.

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Lycium barbarum or commonly known as goji or wolfberry is a species classified under the family of Solanaceae. Goji has been prescribed as herbal medicines that contain various medicinal properties of antioxidant, antidiabetic and providing excellent effects on cardiovascular system and cholesterol levels (Osman *et al*., 2013). *Aquilaria malaccensis* Lamk., which is locally known as 'gaharu' or 'karas', is known for its economic importance as source of cosmetic and medicines. Recently, due to the high value of gaharu, the trees are often cut down indiscriminately. Therefore, to conserve the trees and ensuring sustainable supply of the gaharu, methods for propagation had been developed as well.

In recent times, there has been a great deal of interest in utilizing fruit wastes for the production and recovery of many value-added products. Based on review by Anupama and Ravindra (2000), various fruits wastes may serve as raw materials that could be processed into value-added products to overcome the problem of environmental pollutions. Several fruits and mixed fruit wastes have been

reutilized for producing value added products such as jam which was made from watermelon rind (WMR) waste. In addition, wastes from pineapple canneries have been used as the substrate for bromelain, organic acids, ethanol, since these are potential sources of sugars, vitamins and growth factors. However, there is no report on utilization of these wastes for substitution of plant tissue culture media. Therefore, the present study was undertaken to identify the effectiveness of fermented fruits waste solutions formulated from pineapple, banana, papaya, calamansi lime, kaffir lime and key lime to substitute the use of MS medium for seed germination and shoot regeneration of *L. barbarum* and *A. malaccensis* Lamk.

MATERIALS AND METHODS

Preparations of Fermented Fruits Waste Solutions and Mineral Analysis

The banana, pineapple, papaya, calamansi lime, kaffir lime, and key lime peels were sourced from Shah Alam, Selangor. All fruits' peels were chopped and ground by electric blender. Approximately 100 g of each fruit peel were added into 100 ml of molasses in container. Two different formulae of mixtures were prepared according to the pre-determined formulation in which the Formula A consisted of calamansi, kaffir and key lime peels; meanwhile Formula B contained banana, pineapple and papaya peels. The mixture was then stored at room temperature for four weeks. The initial and final pH values of each sample were recorded. The mineral content of the fermented fruits waste solutions were analysed by using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) instruments

Preparation of media

The culture media were prepared according to the composition mentioned in Table 1. Each treatment was supplemented with 30 g/l sucrose and 8 g/l gelling agent. The medium composed of MS (Murashige and Skoog's) basal salt served as control treatment. Totally, there were 11 treatments including control. All culture media were adjusted to pH 5.8 and they were sterilized by autoclaving at 121ºC.

In vitro Seed Germination of *Lycium barbarum* and *Aquilaria malaccensis* Lamk

L. barbarum berries were purchased from Giant Supermarket, Shah Alam. The goji berries were surface sterilized in sodium hypochlorite solutions with a few drops of Tween 20 and then rinsed thoroughly with sterilized distilled water. Seeds of *A. malaccensis* Lamk (gaharu) were obtained from Alor Gajah, Melaka. The gaharu seeds were washed thoroughly with tap water for 30 minutes. Later, the seeds were soaked in liquid detergent for 15 minutes and then rinsed with distilled water. To surface sterilize, seeds were treated with 70% ethanol for five minutes, followed by 50% sodium hypochlorite solution and a drop of Tween 20 for 15 minutes. Then they were rinsed thoroughly with sterile distilled water. Sterile seeds obtained from the dissected berries were germinated aseptically on the prepared media of different treatments (Table 1). All cultures were incubated at 25ºC under 16-h photoperiod for duration of eight weeks. The percentage of germination, height of the plantlets and the number of leaves were observed and recorded.

In vitro Shoot Regeneration of *Lycium barbarum* and *Aquilaria malaccensis* Lamk

Two month old *in vitro* seedlings of *L. barbarum* and *A. malaccensis* Lamk were used for regeneration purposes. The nodal and stem explants of *L. barbarum* and *A. malaccensis* Lamk were used respectively. The explants were cultured according to the treatments (Table 1) with combination of 0.5 mg/L BAP + 0.5 mg/L NAA for nodal explant of *L. barbarum* while combination of 1.5 mg/L BAP + 0.5 mg/L IBA and 0.5 mg/L NAA were used for stem explant of *A. malaccensis* Lamk. All cultures were incubated at 25ºC under 16-h photoperiod and sub cultured every 4 weeks on the same media.

Experimental Design and Data Analysis

Experiments were performed for eight weeks. The height and number of shoots of each plantlet were recorded. The results were analysed using one way analysis of variance (ANOVA) by SPSS 21 program. The values of $p<0.05$ were considered as significant.

RESULTS AND DISCUSSION

Fermentation and mineral analysis of fermented fruits waste solutions

Fermentation is a natural process in which an organism converts a carbohydrate into an alcohol or an acid. This experiment is a natural fermentation process that took place in container prepared with agricultural waste and molasses. The fermentation process was conducted for four weeks and the initial and final pH values were recorded. Changes in pH are shown in Table 2. Fermentation was found to cause a reduction in pH with time due to increase in acidity. This enhanced the quality of fermented waste. This result agrees with Murdock and Fields (1984), and Nanson and Fields (1984) who reported that fermentation process causes a rapid drop of pH.

Table 1. Composition of each treatment; Formula A (calamansi lime, kaffir lime, and key lime peel) and Formula B (banana, pineapple and papaya peels)

Treatment	Composition
T0	MS0 + Sucrose + Agar + Hormone
T1	1% of formula A + Sucrose + Agar + Hormone
T2	2% of formula A + Sucrose + Agar + Hormone
T3	3% of formula A + Sucrose + Agar + Hormone
T4	4% of formula A + Sucrose + Agar + Hormone
T5	5% of formula A + Sucrose + Agar + Hormone
T6	1% of formula B + Sucrose + Agar + Hormone
T7	2% of formula B + Sucrose + Agar + Hormone
T8	3% of formula B + Sucrose + Agar + Hormone
T9	4% of formula B + Sucrose + Agar + Hormone
T10	5% of formula B + Sucrose + Agar + Hormone

Table 2. pH of fermented fruits waste solutions stored at room temperature for four weeks

Formula	pH	
	Initial	Final
Formula A(calamansi lime, kaffir lime, and key lime peels)	4.27	3.58
Formula B (banana, pineapple and papaya peels)	5.68	4.19

The molasses served as a food source for microbes carrying out the fermentation process. The naturally occurring microbes in the fermentation process produce symbiotic interaction between microbes and plants hence permits the supply of some minerals including nitrogen and phosphate (Kawaguchi & Minamisawa, 2010). Therefore, both formulations of fermented fruits waste solutions contained greater amount of nutrients than MS basal salt. This was proven by the result of nutrient content analysis (Table 3) that was analyzed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) instruments.

Effect of fermented fruits waste solutions on *in vitro* seed germination and regeneration of *Lycium barbarum* and *Aquilaria malaccensis* Lamk

The use of organic compounds from natural resources may influence growth and regeneration of plants in *in vitro* plant tissue culture. Six types of fruits' peels had been used for the development of new plant tissue culture media. Pineapple, papaya and banana are known worldwide as major crops that are primarily consumed as fresh fruits. Meanwhile the calamansi, kaffir and key lime are the citrus species which are usually used for culinary and medicines.

A one way analysis of variance (ANOVA) was conducted to compare the effect of varying treatment of fermented fruits waste solutions on *in vitro* seed germination and regeneration of *L. barbarum* and *A. malaccensis* Lamk. The results obtained from the one way ANOVA (Table 4 and Table 5) indicated that there was a significant effect ($p<0.05$) of varying treatment of fermented fruits waste solutions on *in vitro* seed germination and regeneration of *L. barbarum* and *A.malaccensis* Lamk.

For *in vitro* seed germination, the mean values of the plant height and number of leaves of *L. barbarum* at 2%, 3% and 4% of Formula B (T7, T8, T9) were significantly different from the control (T0). Meanwhile for the mean values of the plant height and the number of leaves of *A. malaccensis* Lamk at 3%, 4% and 5% (T3,T4,T5,T8,T9,T10) of Formula A and Formula B were significantly different from the control (T0).

For *in vitro* regeneration, the mean values of the plant height and the number of shoots of *L. barbarum* at 3%, 4% and 5% of Formula B (T8, T9, T10) were significantly different from the control (T0). Meanwhile for the mean values of the plant height and the number of shoots of *A. malaccensis* Lamk at 5% (T5) of Formula A were significantly different from the control (T0). The growth of both *L. barbarum* and *A. malaccensis* were observed to be different due to dissimilarity of species.

After eight weeks of culture, the *in vitro* regeneration process of both *L. barbarum* and *A. malaccensis* Lamk could be classified as indirect organogenesis because the explants showed callusing response prior to regeneration of shoots. For *L. barbarum*, treatment 10 (T10) which

Table 3. Mineral contents (mg/L) of MS Basal Salt and Fermented Fruits Waste Solutions

Elements	Mineral Contents(mg/L)		
	MS basal salt	Formula A (calamansi lime, kaffir lime, and key lime peels)	Formula B (banana, pineapple and papaya peels)
Nitrogen (N)	253.45	224.55	256.85
Potassium (K)	695.88	1662.83	1618.48
Calcium (Ca)	107.78	246.26	264.63
Magnesium (Mg)	19.38	134.62	118.29
Phosphorus (P)	21.88	34.77	59.10
Sulphur (S)	36.31	199.55	285.05
Sodium (Na)	9.99	256.47	236.73
Copper (Cu)	0.04	0.95	4.34
Manganese (Mn)	4.77	5.18	6.40
Ferum (Fe)	4.34	20.24	22.51

Table 4. Mean number of plant height and number of leaves for *in vitro* seed germination of *L. barbarum* and *A. malaccensis* Lamk after eight weeks cultured in fermented fruits waste solutions

Treatment	Plant Height (cm)		Number of leaves	
	Lycium barbarum	*Aquilaria malaccensis* Lamk	*Lycium barbarum*	*Aquilaria malaccensis* Lamk
T0	3.66±0.81	3.53±0.27	5.40±0.25	2.00±0.00
T1	3.50±0.06	2.73±0.24	4.80±0.49	1.33±0.33
T2	3.54±0.25	2.90±0.17	5.00±0.45	1.67±0.33
T3	3.62±0.20	3.67±0.17	5.20±0.20	2.33±0.67
T4	3.54±0.11	4.00±0.40	5.20±0.20	2.33±0.33
T5	3.54±0.13	4.27±0.03	5.40±0.25	3.67±0.33
T6	3.58±0.14	2.10±0.31	5.00±0.45	1.00±0.58
T7	3.78±0.06	3.13±0.19	6.20±0.37	1.67±0.68
T8	3.72±0.04	3.97±0.48	5.80±0.37	2.67±0.33
T9	3.70±0.07	3.67±0.17	5.60±0.55	3.00±0.58
T10	3.62±0.09	4.34±0.14	5.00±0.00	3.67±0.33

Table 5. Mean number of shoot and plantlet height for *in vitro* regeneration of *L. barbarum* and *A. malaccensis* Lamk after eight weeks cultured in fermented fruits waste solutions

Treatment	Plant Height (cm)		Number of shoots	
	Lycium barbarum	*Aquilaria malaccensis* Lamk	*Lycium barbarum*	*Aquilaria malaccensis* Lamk
T0	3.10±0.06	3.53±0.09	2.33±0.33	3.33±0.33
T1	0.00	2.53±0.09	0.00	2.33±0.33
T2	0.00	0.00	0.00	0.00
T3	0.00	3.13±0.03	0.00	2.33±0.33
T4	1.70±0.06	3.10±0.06	0.67±0.33	2.33±0.33
T5	2.23±0.15	4.10±0.06	1.67±0.33	3.33±0.33
T6	0.00	2.90±0.06	0.00	2.33±0.33
T7	0.00	0.00	0.00	0.00
T8	3.70±0.06	0.00	3.33±0.33	0.00
T9	3.80±0.10	0.00	3.00±0.56	0.00
T10	4.01±0.03	0.00	3.33±0.33	0.00

composed of 5% of Formula B medium was identified to give high number of shoots and greater plant height as compared to the control (Fig. 1). Similarly, these findings were found to be in accordance to a study reported by Kong *et al*. (2007), in which the banana extract promoted the organogenesis of in *Dendrobium orchid*. Besides that, the addition of papaya extracts promoted regeneration of shoots in Celoasia sp, and supported vigorous proliferation of *in vitro* Vanda Kasem's Delight (VSD) orchid's protocorm-like bodies (PLBs).

Meanwhile, for *A. malaccensis* Lamk treatment 5 (T5), which composed of 5% of Formula A medium was identified to have high ability in promoting shoot regeneration as compared to control (Fig. 2). To date, no studies on the applications of calamansi lime, kaffir lime, and key lime peels in plant tissue culture medium have ever been documented. The effects exerted on plantlet regeneration could be due to the high concentrations of vitamins, nitrogen, potassium and phosphorus which are among the most important elements needed in supporting the *in vitro* growth of the plants.

However, there were five treatments in both species which had been identified to produce no response at all. In *L. barbarum* T1, T2, T3, T6 and T7 media were found to be ineffective. On the other hand, similar non-responsive effects were also observed from T2, T7, T8, T9 and T10 media in *A. malaccensis*. All explants in those treatments were turned brown and eventually died. Appearance of browning showed that the plantlets secreted phenolic compounds or had undergone oxidation. According to the Sukenda *et al.*, 2008, browning is a restriction in *in vitro* culture due to the phenolic compounds which block enzyme activities and promoting death of culture.

Besides the nutrient content of organic extract, the regeneration pattern of both *L. barbarum* and *A. malaccensis* Lamk were also determined by hormonal treatments. This is consistent with Juan *et al.* (2010) who stated that, the *in vitro* plant growth is physiologically affected by plant hormones and they are even being among the important factors. Plant growth regulators (PGRs) are substances that profoundly affect the growth and development of plants even though they occur in small concentrations (Ahmadian *et al*., 2013). The five groups of PGRs are auxins, cytokinins, gibberelins, abscisic acids and ethylene, whereby the auxins and cytokinins are by far the most important hormones used in plant tissue culture media. Skoog and Miller (1957) reported that, the major differences in the response of different plants and explants to plant tissue culture conditions depend on the ratio of auxins to cytokinins.

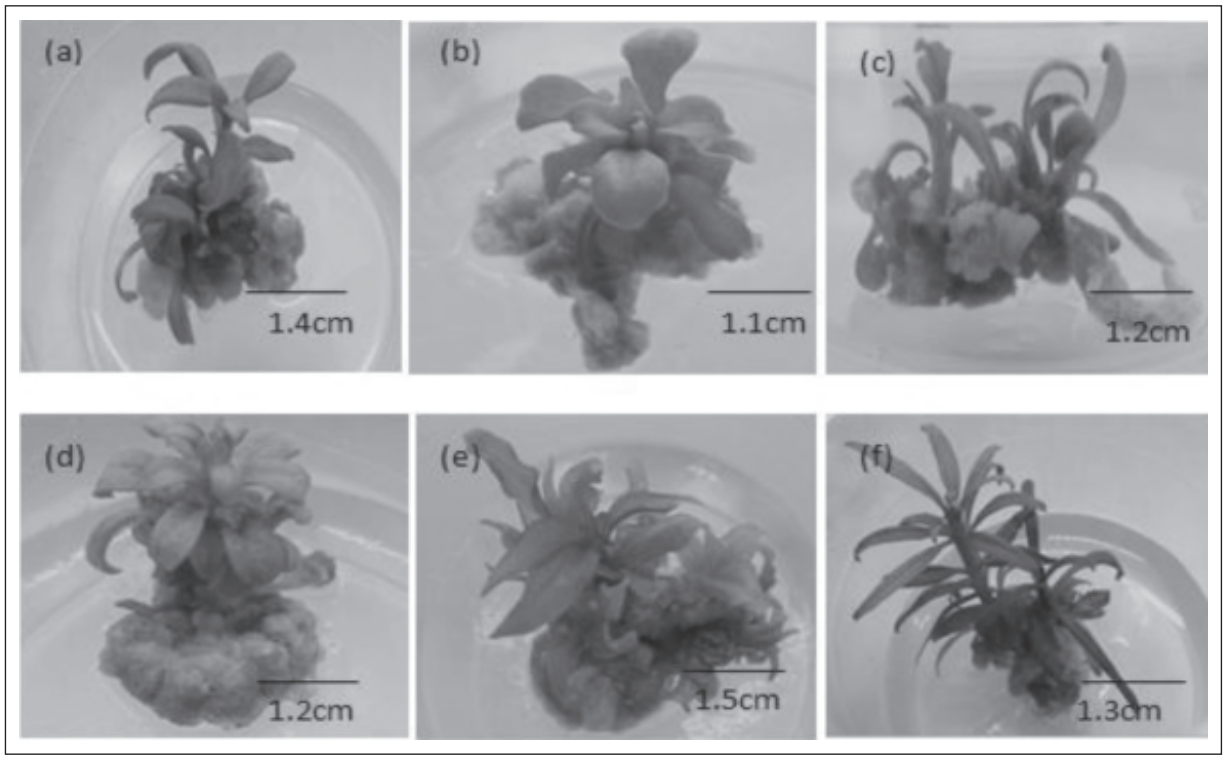

Fig. 1. Nodal explants of *L. barbarum* after 8 weeks cultured on different media a) Control b) 4% of formula A medium c) 5% of formula A medium d) 3% of formula B medium e) 4% of formula B medium f) 5% of formula B medium.

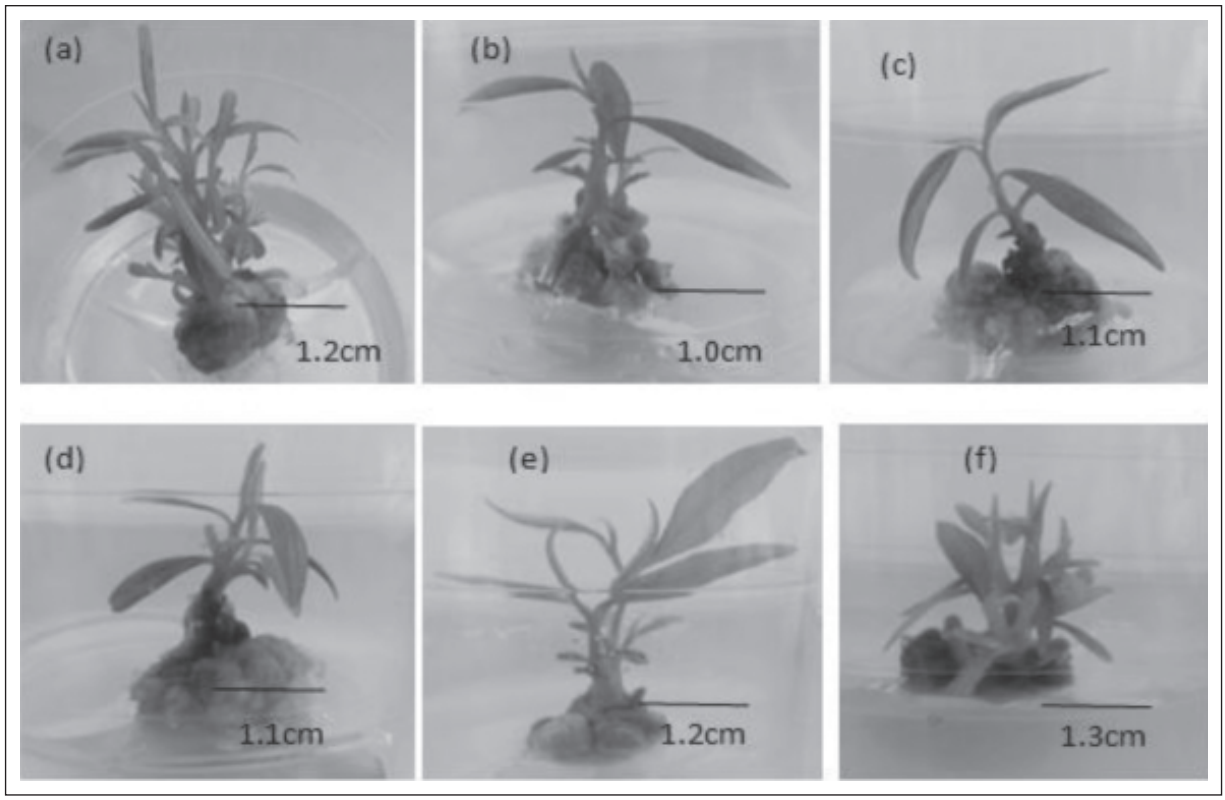

Fig. 2. Stem explants of *A. malaccensis* Lamk after eight weeks cultured on different media a) Control b)1% of formula A medium c)3% of formula A medium d) 4% of formula A medium e) 5 % of formula A medium f) 1% of formula B medium.

CONCLUSIONS

This research had proven that the use of fermented fruits waste solutions was successfully promoting the growth of both *L. barbarum* and *A. malaccensis* Lamk *in vitro* plantlets. The naturally-derived medium hence could be used to formulate a simple and low cost medium for plant tissue culture without using its synthetic counterparts.

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