

Regeneration and establishment of whole plants from kava (*Piper methysticum* Forster) meristems in tissue culture

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ABSTRACT

In the present study the regeneration and establishment of whole plants free from pathogenic contaminants from kava (*Piper methysticum* Forster) using meristems tissue culture has been established. Four treatments for initiating kava in tissue culture were evaluated for their efficiency in eliminating contaminants and optimizing recovery and growth. One of the treatments, using shoot-tip meristems resulted in 84% decontamination with 72% recovery and another, using nodal bud meristems, resulted in 80% decontamination rate with only 20% recovery. Shoot tips were found to be the ideal explants for kava tissue culture. The minimum time taken for rooting was one month on Murashige and Skoog medium supplemented with 100 mgL⁻¹ myo-inositol, 0.04 mgL⁻¹ BAP, 0.02 mgL⁻¹ NAA, 0.05 mgL⁻¹ GA, 1.0 mgL⁻¹ thiamine and 20 gL⁻¹ sucrose.

Key words: decontamination, meristem, in vitro, nodal buds, recovery, shoot tips.

1 INTRODUCTION

Kava, (*Piper methysticum* Forster.), is the most important non-food plant in Pacific Island Countries (PICs). Socially, kava is taken to induce relaxation. Pacific Islanders have long recognized kava's healing and analgesic qualities, and have used it to treat many afflictions. It has high social, cultural and economic significance, and has been used in PICs for centuries without any obvious ill effects.

Kava has a high demand in both local and export markets. In Europe, kava herbal products have been widely used for cases of nervous anxiety, stress, and insomnia. In the year 2000, kava products in overseas markets were banned because of allegations that kava consumption caused liver cancer amongst the users. As a result of these reports, export of kava from Fiji as well as other PICs has been severely affected. European ban has an impact on future export markets. For example, India and China were considering importing kava from PICs for pharmaceutical purposes. Thus Fiji would have been earning F\$20 million dollars in revenue. The Australian ban alone is estimated to cost Fiji kava growers AU\$24 million a year (The Sydney Morning Herald 2007).

A kava study (Gruenwald 2002), commissioned by the Centre for the Development of Enterprise (CDE) in July 2002 found that there were no basis for the market recalls or restrictions by health agencies in Europe in 2001. Scientific papers presented at the International Kava Conference (IKC), Suva, Fiji 2004 (Proceedings of IKC 2004), clearly showed that there was no correlation between kava consumption and liver diseases. In the light of this evidence, the kava market is likely to recover, and with interest from countries, such as India and China, demand for kava could significantly increase. An increase in demand will have to be met by increased production by Pacific growers. However, kava production in PICs is constrained by diseases and a limited availability of planting materials (Island Business 2007). In recent years Fiji has been importing kava from Vanuatu to meet the local demand as production is hampered by the factors

mentioned above. The value of kava imports from Vanuatu in 2004 exceeded \$0.5 million (Fiji Government Online-Portal 2005).

Kava plantings in PICs would be increased once market demand for kava increases. However, currently diseases limit production of kava. The most severe disease of kava is *kava wilt* or *kava dieback* disease, where the stem rots from the tip or the node and progresses to the stem base. This disease was first reported in Fiji in 1932 (Parham 1935) and crop loss has been reported to reach 40 – 100% in certain areas of Fiji (Island Business 2007; Mudliar pers. comm.). There is evidence that cucumber mosaic virus (CMV) in association with other viruses or pathogens are the cause of this disease (Davis 2004; Davis *et al.* 1996). If kava production in PICs is to increase, a strategy has to be developed to manage kava dieback. One component of such a strategy is the use of clean planting material. Kava tissue culture has the potential in producing clean planting materials through the combined process of surface sterilization and use of meristem culture. By manipulating the culture medium and the culture conditions, multiplication rates can be improved. The objective of this study is to develop a tissue culture system for kava, which will produce plants, free of external and internal contaminants, to be multiplied for use as planting material by Pacific growers.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

Kava explants used for this study were taken from cultivars (*Matakaro*, *Yalu* and *Dokobana Loa*) of kava established at the Secretariat of the Pacific Community (SPC) Regional Germplasm Centre (RGC) screen house. The kava plants established in the RGC screen house had originally been brought from Naduruloulou Research Station (NRS) and propagated using stem cuttings in potting mix (Yates). The RGC screen house has an aphid proof screen. All plants held at the RGC screen house are watered with tap water and treated with fungicide Benlate (Du Pont New Zealand Ltd) 0.5g L⁻¹ on a monthly basis.

Pesticides Mavrik (Yates) 10mLL⁻¹ and ATTACK (Crop Care) 2mLL⁻¹ are used alternately on a monthly basis. Five to 16 explants from kava cultivars shoot tips (ST) and nodal buds (NB) were used in this study. Care was taken in the selection of explants so that only shoot tips (STs) with fully enclosed outer leaf sheaths and top four to five nodes were used. ST explant sizes varied from 3 – 6 cm in length and 0.5 – 1 cm in diameter. The final meristem (with one leaf primordial) measured 2 – 5 mm. The meristems were pale green and slightly translucent in appearance.

2.2 DECONTAMINATION TREATMENTS

An out line of decontamination treatments carried out to initiate kava into tissue culture is presented in Table 1. Four different decontamination treatments, A, B, C and D, described in Table 2, were evaluated for initiating kava into tissue culture. The explants were dissected under a binocular (Olympus) microscope with a fine scalpel blade (No. 11) until meristems of sizes 2 – 5 mm were obtained. The dissection and meristem washes were carried out in

the sterile Laminar Airflow Cabinet. All instruments were treated to 250°C in a dry bead sterilizer for at least 20 sec. Great care was taken in the handling of these meristems.

As shown in Table 2, treatment D (also described in Table 1), proved to be the optimum initiation protocol. Treatment D was therefore used as a standard protocol for kava initiation into tissue culture. However, with treatment D, smaller meristems of sizes 0.5 – 1.5 mm died. Therefore, experiments were carried out to modify this treatment to cater for the smaller meristems. It was considered important to establish cultures from the smaller meristems because of a greater chance of virus elimination. The modifications were:

1. Step 10 of treatment D was modified with reduction of the final bleach concentration from 10% (or 0.056 M NaOCl) to 5% (or 0.023 M NaOCl) (Table 3).
2. As (1) above but in addition, the meristems were kept moist after dissection by placing them in a sterile tube that contained a few drops of sterile distilled water before the bleach treatment (Table 3).

Table 1 An outline of the decontamination treatments A – D used to initiate kava into tissue culture

Step	Procedure	Treatments	A	B	C	D
1	Explants gently scrubbed with toothbrush and detergent under running water. Loose brown skin was removed off the nodes.	→	+	+	+	+
2	Explants soaked in a beaker of about 200mL water with a few drops of detergent. The beaker was swirled occasionally.		+	-	-	-
3	Explants soaked in a beaker of about 200mL water with a few drops of detergent and were magnetically stirred for 30 min or more before they were rinsed with tap water.		-	+	+	+
4	Explants soaked in 70% alcohol for 1 min and were swirled occasionally		+	+	+	+
5	Explants soaked in 15% bleach (or 0.11 M NaOCl) + Tween (1drop ml ⁻¹) for 15 min and were swirled occasionally		+	-	-	-
6	Explants soaked in 15% bleach + Tween (1drop ml ⁻¹) and were magnetically stirred for 15 min		-	+	+	+
7	Explants were put on a sterile filter paper (SFP) to dry in Airflow Cabinet before trimming		+	+	+	+
8	Outer leaf sheath of STs and NBs were trimmed once and put in a sterile tube. 10% bleach (or 0.056 M NaOCl) was poured into the tube and swirled occasionally for 10 min, rinsed with sterile distilled water (SDW), trimmed to final explant size (2 – 5 mm) and put into media		+	+	-	-
9	Outer leaf sheath of STs trimmed 2 – 3 and nodal buds trimmed 4 – 5 times depending if the meristem could be trimmed further. Meristems (2 – 5mm) were put in a sterile tube. 10% bleach was poured into the tube and swirled occasionally for 5 min. Meristems were transferred from bleach to SDW using forceps, dried on SFP before they were inoculated on media.		-	-	+	-
10	As step 9 except the meristem were transferred from bleach to sterile distilled water using sterile dropper.		-	-	-	+

Note: For treatments A – D, (+) indicates the step was carried out while (-) indicates a step was not carried out. Only shoot-tips were used in this experiment as they were much easier to dissect and gave a higher recovery rate.

2.3 CULTURE MEDIUM AND CONDITIONS

The culture medium used in the decontamination experiment (section 2.2) was full strength MS medium (Murashigue and Skoog 1962) supplemented with 0.04

mgL⁻¹ benzyl amino purine (BAP), 0.02 mgL⁻¹ naphthalene acetic acid (NAA), 0.05 mgL⁻¹ gibberellic acid (GA), 1.0 mgL⁻¹ thiamine, 100 mgL⁻¹ myo-inositol and 20 gL⁻¹ sucrose. The pH was adjusted to 5.8.

Filter paper bridges were used with this liquid medium in pre-sterilized glass (30 mL Wide Mouth Universal,

Richardsons Ltd.) bottles. Sterilization was done by autoclaving these bottles at 120°C at 103 KPa for 15 minutes. The meristem cultures were maintained at 24°C ± 2°C with no overhead lights, although some light (5.5-11 μmol m⁻² s⁻¹) was available from the adjacent shelves. Establishment of plants were recorded after two weeks following initiation. Once the cultures reached 7 – 10 mm in size, (ca. two to four weeks) they were transferred to shelves with 16-hr photoperiod and a light intensity of 27-46 μmol m⁻² s⁻¹. All meristem cultures were subjected to the same conditions. Results were recorded two to three weeks after initiation of cultures.

3. RESULTS

Figure 1 shows the recovery of kava meristem from kava explant dissection after three months following initiation to full grown kava plants *in vitro*. Table 2 shows the recovery of meristems following two weeks of initiation. Expanded and green meristems were recorded as alive. Black meristems were recorded as dead. With kava, injured meristems became black within a few minutes. Fungal contamination generally appeared within one to four days while bacterial contamination appeared within two to six days. None of the contaminated kava meristem cultures survived and these were recorded as contaminated and dead. Table 3 shows the results of an amended treatment D which was the most successful treatment.

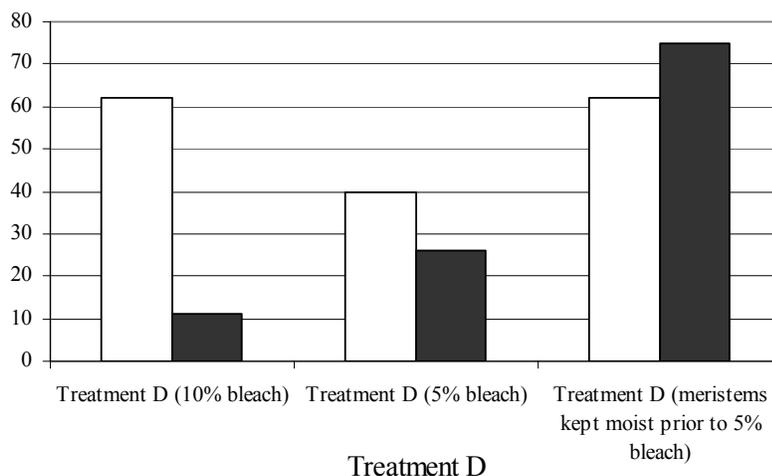


Figure 1 Decontamination treatments for 0.5 mm to 1.5 mm meristems. Bar graph showing the recovery of kava meristems of different sizes with the modification of treatment D – by reducing the bleach concentration and keeping the meristems moist prior to bleach treatment

For treatment D – refer to Table 1 of materials and methods **Legends:** meristem sizes: □ 2 – 5 mm ■ 0.5 – 1.5 mm

Table 2 Effect of different decontamination treatments on kava establishment *in vitro*

Treatment ¹	Explant source for M	Total # Tested	% clean both dead & alive	% recovery	% not recovered	% FC	% BC
A	ST	39	15	0	15	67	* 18
	NB	35	29	0	29	54	* 17
B	ST	15	53	20	33	0	47
	NB	10	0	0	0	0	100
C	ST	25	80	16	64	4	*16
	NB	16	81	6	75	0	19
D	ST	25	84	72	12	8	*8
	NB	10	80	20	60	10	*10

*Actual figure for bacterial contamination may be higher as it was difficult to detect bacterial contamination in fungal infested cultures.

¹: For treatment description: see Table 1

M – meristem, NB – nodal bud, FC – fungal contamination, ST – shoot tip, BC - bacterial contamination.

Table 2 also summarizes results from the four different protocol treatments used to establish kava plants *in vitro*. Treatment D proved to be the optimum of the four protocols investigated. Using treatment D where the final meristems were rinsed in 10% bleach (or 0.056 M NaOCl),

the decontamination rate for shoot tips was 84% with 72% recovery and 80% for nodal bud meristems with 20% recovery. Shoot tips were the ideal explants for kava tissue culture, being easy and quick to dissect, whereas, dissecting meristems from nodal buds was time consuming

and difficult. During nodal bud dissection, it was difficult to remove the layers from the nodal bud due to relatively compact structure as compared to removing leaf sheaths from the shoot tips. In addition, nodal meristems were not very prominent in shape and size compared to shoot-tip

meristems, thereby, reducing visibility. Visibility was further reduced when the surrounding tissues of the nodes released water during dissection. Kava meristems are very susceptible to wounding resulting in necrosis as a result of polyphenol oxidation.

Table 3 The effect of different concentrations of bleach (amended treatment D) and sterile distilled water (SDW) soak on the successful initiation of meristems of different size *in vitro*

Treatment ¹	M size (mm)	total # tested	% clean both dead & alive	% recovery	% not recovered	% FC	% BC
Control (final bleach Conc. – 10%)	2-5	39	72	62	10	15	*13
Final bleach. Conc. 5%	0.5-1.5	28	96	11	86	0	4
Final bleach conc. 5% + SDW soak	2-5	15	53	40	13	20	*27
	0.5-1.5	15	93	26	67	0	7
	2-5	21	62	62	0	24	*14
	0.5-1.5	20	80	75	5	10	*5

*Actual figure for bacterial contamination may be higher as it was difficult to detect bacterial contamination in fungal infested cultures.

¹: For treatment description see 2.3 of methodology. The treatment steps are modifications of treatment D described in table 1 of Methods.

SDW – sterile distilled water, M – meristem, FC – fungal contamination, BC – bacterial contamination

There was a high percentage of fungal contamination in treatment A compared to the rest of the treatments. A major difference between treatment A and the remaining treatments was the absence of constant agitation (using a magnetic stirrer). It would, therefore, seem that constant agitation in the decontamination process is essential to eliminate fungal contaminants. Treatments C and D had low percentage of observable bacterial contamination compared to Treatment B. The major difference between treatment B and treatments C and D was that meristems of

treatment C and D were soaked in 10% bleach for 5 min (Table 3). It was therefore very likely that bacterial strains were present in all the tissue of a kava plant even in the tightly covered shoot tips. Generally, ST meristems larger than 5 mm did not survive as a result of excessive contamination, however, those that did survive grew well (Figure 2), producing leaves and roots within one month. It took, on an average, 1 – 2 months for the meristems to develop into a two-leaf-stage.

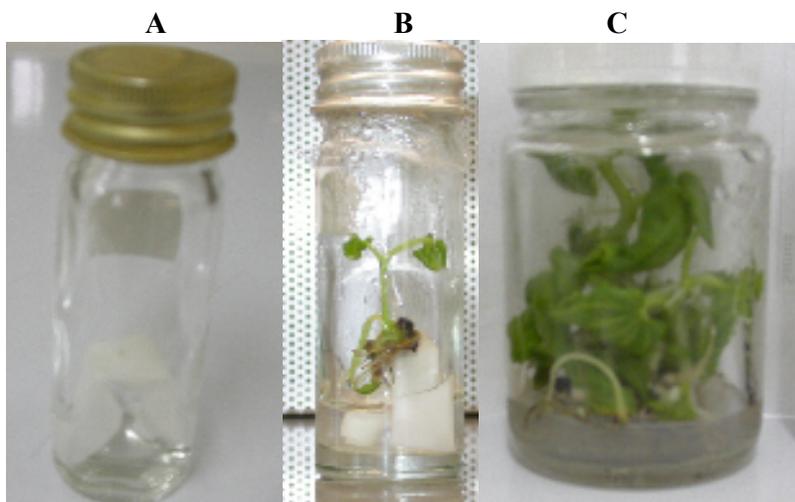


Figure 2 Kava growth and development *in vitro*. A: Kava meristem culture after initiation. B: Kava plant three months following initiation. C: Kava sub-cultured in MS medium

Rooting occurred for all sizes of meristems on the same medium, with 92% of the surviving explants forming roots within two to three months. All cultivars used in this study showed a similar positive response to treatment D. However, observations for each cultivar were not recorded.

Approximately 30% of the live explants developed roots within one month of initiation and 60% developed roots two to three months after initiation. All surviving explants had been rooted by the end of four months following initiation.

4 DISCUSSION

Many workers (Kobyashi *et al.* 2003; Kunisaki *et al.* 2003; Smith *et al.* 2002; Briskin *et al.* 2001; Anand and Rao 2000; Sanxter 1998; Taylor and Taufu 1997; 1998; Bhat *et al.* 1995; Philip *et al.* 1992; Fitchet 1990; Matthews and Rao 1984) have demonstrated in their micropropagation studies on *Piper* species that high levels of exogenous and endogenous pathogens hinder the plant's initiation into tissue culture. In majority of the above studies, decontamination was only effective against fungal contaminants. Despite the high contamination level, some of these species have been successfully micro-propagated. These are *Piper barberi* Gamble, black pepper (*Piper nigrum* Linn.), and *Piper colubrinum* Link (Bhat *et al.* 1995). With all these three *Piper* species, bacterial contamination was observed in more than 90% of the primary cultures. By repeated surface sterilization with the newly emerged shoots during the first two sub-cultures, healthy cultures were eventually established with all three species. It is assumed that the titre of slow-growing bacteria is reduced and ultimately eliminated during successive sterilizations. However, the same could not be achieved with kava (Taylor pers. comm). Often highly toxic chemicals (such as mercuric chloride) have been used to successfully eliminate these contaminants (Taylor and Taufu 1997, 1998), however, such chemicals are extremely toxic, and have serious environmental implications for disposal especially if used in small Pacific Island countries and therefore were not used in this study.

There is little published information on kava tissue culture partly because of proprietary restrictions and partly because of the difficulties encountered in developing a protocol for kava initiation. In the past, high levels of endogenous and exogenous pathogens have hindered the establishment of kava in tissue culture (Kobyashi *et al.* 2003; Kunisaki *et al.* 2003; Briskin *et al.* 2002; Smith *et al.* 2001; Taylor and Taufu 1998; 1997). The only report of successful initiation of kava meristems *in vitro* using organized tissue (nodal bud meristem) comes from Kunisaki *et al.* (2003) but the recovery of the meristems were low (10 – 20%).

In this study, a high bacterial contamination percentage was observed when the meristems were not treated with bleach, indicating that microbes were abundant even in the shoot tips. It would seem that the tissue of most *Piper* species contain endogenous micro organisms, hence the difficulty in trying to establish them into tissue culture. At the University of Auckland, in microbial contamination studies on kava, 15 different fungi and 21 different bacterial strains were isolated from leaf and stem tissue samples of Samoan kava (Taylor and Taufu 1997). This suggests the high level of infection present within kava tissue. Many kava cultivars contain symbiotic bacterial strains within their vascular systems. It is anticipated that these microorganisms often are in balance in the tissue under field conditions. However, when placed under artificial stresses of laboratory conditions, one bacterial race may out-compete and erupt as latent contamination (Sanxter 1998). Fungal contamination can be eradicated using standard decontamination techniques of alcohol, bleach and sterile water treatments. Bacterial and viral contaminants are more difficult to remove. However, the

repeated excision of a small meristem has been successful with some ornamental plant species (Holdgate and Zandvoort 1997). An excision of smaller meristem sizes of 1 – 1.5 mm significantly contributed to the elimination of bacteria and fungi from plant tissue. Similar results have been obtained in the present study. Kava meristems of sizes 0.5 – 1.5 mm showed lower levels of contamination as compared to the bigger meristem sizes of 2 – 5 mm.

High levels of contaminations in kava tissues (Sanxter 1998; Taylor and Taufu 1997; 1998) even in small shoot tip meristems as explant emphasized the need for even smaller meristem culture in kava. Kava meristems are only protected from the external environment by two to three leaf sheaths. Observations under the dissecting microscope showed that the shoot tips are hairy. Presence of hair in the shoot tips contributes to trapping dust and with it fungal spores and bacterial strains). Thus hairy trait could be responsible for the high contamination rate in kava. The results obtained in this study showed that constant agitation during decontamination treatment facilitates greater penetration of the sterilizing agents and thus more effective decontamination. This study confirmed that kava meristems must be treated with dilute (10%) bleach in order to get rid of the endogenous bacterial contaminants and fungal spores. If bacteria are not removed, they either overgrow the explant or grow into the explant ultimately killing it. In most of the previous studies meristems treatment with bleach was not done probably because meristems are very delicate and fragile and the thought of exposing them to bleach never occurred to researchers.

In the present study, it was also observed that some meristems turned dark, usually within a few minutes following dissection or bleach treatment. In cultures where the meristems grew larger than 7 – 10 mm in size in the medium and did not form roots; it was noticed that such growing meristems turned black within a day when transferred to new medium. The kava explants seem to be very sensitive to touch and the less handling involved increases its chance of survival. Fitchet (1990) had also reported the sensitivity of the black pepper shoot tips to touch. The very-gentle handling of kava explants during the decontamination steps cannot be overemphasized. Shoot tips were found to be far better explant source compared to nodal buds. Not only did shoot tip meristems result in less decontamination and higher recovery compared to nodal bud meristems, but they were also much easier to dissect, extract and handle in culture medium.

The medium used by Kunisaki *et al.* (2003) to establish kava meristems was half strength MS supplemented with 15% coconut water. A six-month period was required for the meristems to grow into a two-leaf-stage (Kunisaki pers comm.). In contrast, a commercial laboratory in Hawaii has succeeded in propagating Hawaiian kava using one quarter strength woody plant medium (WPM) supplemented with MS vitamins and growth regulators (Taylor pers comm.). With the WPM, the roots formed in six months. In this study, full strength MS medium supplemented with growth regulators 0.04 mgL⁻¹ BAP, 0.02 mgL⁻¹ NAA, 0.05 mgL⁻¹ GA, 1.0 mgL⁻¹ thiamine and 100 mgL⁻¹ myo-inositol facilitated good shoot and root growth. It took only 1 – 2 months for the meristems to

develop into a two-leaf-stage. Root growth was particularly improved with roots forming in one to three months, compared to six months with the quarter strength WPM and half strength MS media mentioned in the studies above. The different response to the culture media obtained for this experiment compared to the response achieved in Hawaiian labs (where half strength MS and quarter strength WPM were used to initiate kava, albeit at a slower rate) could be due to the differences in varieties being investigated. Differences in varietal response had been observed contributing to the *in vitro* establishment explants in cultures in other crops (Taylor pers comm.). Therefore it is also possible with kava varieties.

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