BIOLOGIA PLANTARUM 52 (1): 110-112, 2008

BRIEF COMMUNICATION

Successful micropropagation protocol of *Piper methysticum*

Z. ZHANG*, L. ZHAO, X. CHEN and X. ZHENG

State Key Laboratory of Tropical Crop Biotechnology, Institute of Tropical Bioscience and Biotechnology, CATAS, Haikou, 571101, P.R. China

Abstract

An efficient *in vitro* propagation of kava (*Piper methysticum*) was established. Utilizing 15-d-old tender shoots from dormant auxiliary buds as explants, significant induction of vigorous aseptic cluster shoots was achieved in Murashige and Skoog (MS) medium containing 0.5 mg dm^{-3} 6-benzyladenine (BA), 0.5 mg dm^{-3} indole-3-acetic acid (IAA), and antibiotics after 30 d. *In vitro* rooting was achieved at 100 % efficiency in MS medium containing 0.75 to 1.00 mg dm^{-3} IAA or indole-3-butyric acid and 3 % sucrose. The most robust and long roots were observed in medium with IBA. Moreover, the embryonic callus was induced from petioles in MS medium supplemented with 1.0 mg dm^{-3} BA and 0.1 mg dm^{-3} IAA, of which 70 % differentiated into shoots in the presence of 1.0 mg dm^{-3} BA and 0.5 mg dm^{-3} IAA.

Additional key words: callus, cluster shoots, dormant auxiliary bud, endogenous contamination, growth regulators.

Piper methysticum Forst., from the family *Piperaceae* and commonly named kava, is an outstanding pharmaceutical shrub that grows throughout South Pacific islands (Singh and Blumenthal 1997).

The major difficulty that hinders successful *in vitro* propagation of *Piper methysticum* is the high occurrence of contamination on induced shoots due to bacterial and fungal infections, which result in great loss of shoots and thus a very slow propagation rate (Taylor and Taufa 1998, Briskin *et al*. 2001). Development of an effective tissue culture approach for *in vitro* propagation of *P. methysticum* thus has great potential for its mass production as herbal remedy, and for the studies of plant germplasm preservation, and genetic manipulations. Our studies have presented a novel procedure, which lead to successful *in vitro* propagation of *P. methysticum*.

Piper methysticum introduced from Cook Island is routinely grown in greenhouse. 1-year-old stem with dormant auxiliary buds were selected as starting materials. The stems were initially submerged in 70 % ethanol solution for several seconds, then surface disinfested with 0.1 % HgCl for 10 min and afterwards washed several times with sterile water. The stems were cultured in half strength Murashige and Skoog (1962; MS) medium supplemented with 6-benzyladenine (BA), indole-3-acetic acid (IAA) and antibiotics at indicated concentrations.

The pH of MS or 1/2 strength MS medium was adjusted to 5.8 prior to sterilization at 120 °C for 20 min. Tissue cultures for shoot induction were maintained in growth chamber at 25 ± 2 °C in 12-h photoperiod with irradiance of 48 μmol m^{-2} s^{-1} provided by white fluorescent tubes. Cultures for callus induction were maintained in dark at 25 ± 2 °C. All medium, supplements, and other chemicals were purchased from *Sigma* (USA).

The tender shoots from 10- to 25-d-old were cut and cultured in MS medium containing 1.0 mg dm^{-3} BA and 0.5 mg dm^{-3} IAA for initial induction of cluster of aseptic shoots. Subsequent experiments, *e.g*. optimization of BA and IAA concentrations, were conducted upon harvest of substantial amount of aseptic shoots.

The sterile petioles were sampled and cultured in MS medium for studies of callus induction and shoot differentiation.

Rooting was induced by incubation of about 10 mm long shoots for 30 d in 1/2 strength MS medium supplemented with various concentrations of IAA or indole-3-butyric acid (IBA), and sucrose as indicated. Regenerated plantlets were then transferred into pots containing *Vermiculite* and sand for full plant establishment.

Received 8 March 2006, *accepted* 5 December 2006.

Abbreviations: BA - 6-benzyladenine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog.

Acknowledgments: We are grateful to Y. Lu for helpful discussions and Y. Zhang for a linguistic revision of the manuscript. This work was supported by a grant from CATAS, China.

* Corresponding author; fax: (+86) 898 65314866, e-mail: zzl_catas@hotmail.com.

Piper is an economically and ecologically important genus in the *Piperaceae* family, which includes more than 1 000 species of shrubs, herbs, and lianas. However, few of them (*e.g*. *Piper nigrum*) has been successfully propagated *in vitro* (Ramakrishnan Nair and Dutta Gupta 2006). *P. methysticum*, the very important pharmaceutical shrub plant of this family, naturally grows in highly humid conditions and it is seriously infected by various diseases. This results in severe endogenous contamination that establishment of aseptic cultures is very difficult, and considered as a bottleneck *in vitro* propagation of *P. methysticum* (Bhat *et al*. 1995). Despite the multiple efforts invested in this field of studies (Taylor and Taufa 1998, Briskin *et al*. 2001), propagation of aseptic *P. methysticum* plants was never successful.

Following multiple rounds of sterilization and with inclusion of the antibiotics in the culture medium, tender shoots derived from dormant auxiliary buds were obtained in medium containing 0.5 mg dm^{-3} BA and 0.5 mg dm^{-3} IAA. Despite that almost all induced shoots got re-infected upon incubation longer than 30 d, however, majority of the shoots 15-, 20-, or 25-d-old were partially prevented from being infected. Contamination/infection-free cluster shoots were successfully induced from 15-d-old shoot apex with average length 0.5 ~ 1 mm. The relevance of the age of dormant auxiliary buds-derived shoots to the artificial re-infection was then investigated. It has demonstrated that re-infection rate was markedly reduced to 64 % by use of 15-d-old tender shoots as explants. In contrast, the re-infection occurrence in 20- and 25-d-old shoots reached over 95 and 100 %, respectively. In the group of 10-d-old shoots, re-infection could be cut to 56 %. However, only less than 3 % of the shoots in this group showed induction of cluster aseptic shoots, while the death rate of this group was also significant (more than 41 %).

The studies on the inducing effects of different culture supplements have demonstrated that the combination of 0.5 mg dm^{-3} BA and 0.5 mg dm^{-3} IAA significantly stimulated the rapid regeneration of well-developed shoots, with an average yield of 6 ~ 8 shoots from a single shoot in 30 d (Fig. 1*A*). The combination of 1.5 mg dm^{-3} BA and 0.05 mg dm^{-3} IAA led to rapid production of callus from petioles. However, the callus induced was loose, fragile, and incapable of differentiation into shoots. On the other hand, medium with 1.0 mg dm^{-3} BA and 0.1 mg dm^{-3} IAA resulted in the induction of callus with light yellow green color (Fig. 1*B*). They are compact and 70 % of them differentiated into well-developed shoots. In a 30-d

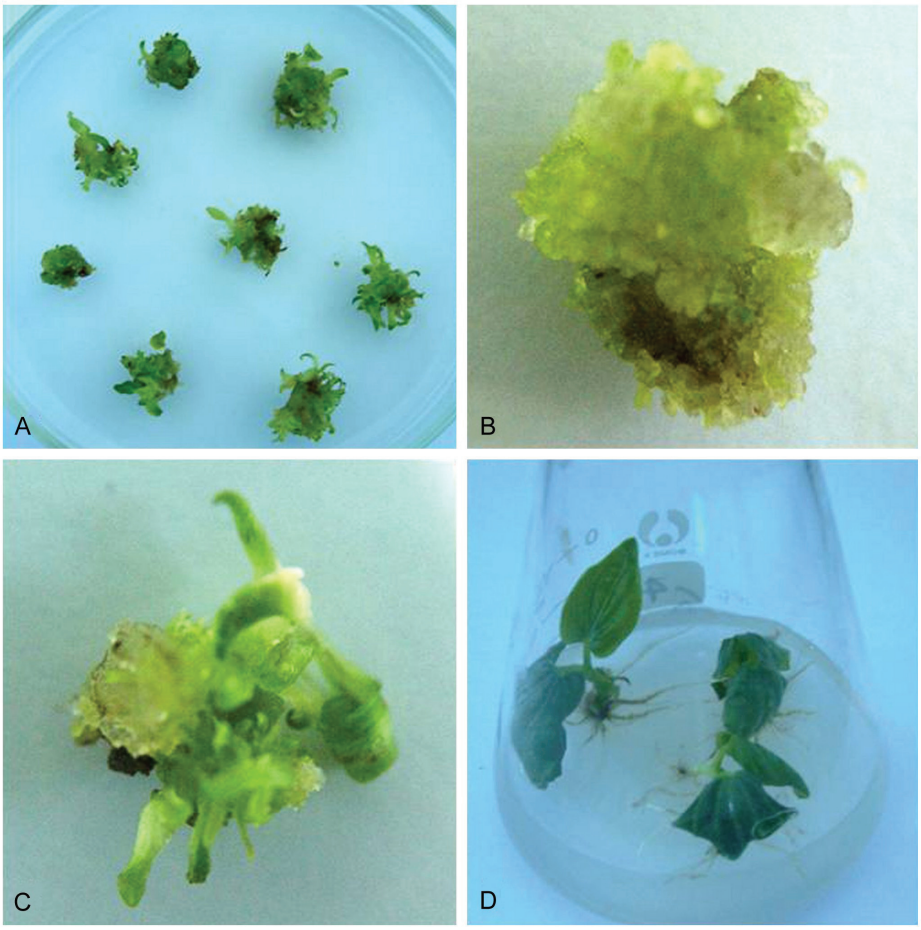

Fig. 1. Cluster shoots induced from individual shoot on MS medium supplemented with 0.5 mg dm^{-3} BA and 0.5 mg dm^{-3} IAA (*A*), callus derived from petioles (*B*), shoots differentiated from callus (*C*), full plants propagated by *in vitro* tissue culture (*D*).

Table 1. Effect of different BA and IAA combinations on cluster shoot induction (shoot number per explant) in MS medium. Data recorded after 30 d of culture from 3 experiments with 15 replicates each; mean ± SE, $n = 45$.

IAA [mg dm^{-3}]	BA [mg dm^{-3}] 0.1	0.5	1.0	1.5
0.1	-	callus	callus	callus
0.5	-	7.6 ± 0.4	5.5 ± 0.3	2.3 ± 0.4
1.0	-	3.4 ± 0.3	5.8 ± 0.4	4.2 ± 0.2
1.5	-	2.8 ± 0.4	2.3 ± 0.3	2.7 ± 0.4

period, a piece of well-developed callus generally produced 4 ~ 5 shoots in medium with 1.0 mg dm^{-3} BA and 0.5 mg dm^{-3} IAA (Fig. 1*C*). In contrast, 2.0 mg dm^{-3} BA in combination with various concentrations of IAA only induced much more fragile callus. Half or full strength MS medium with different concentrations of IAA or IBA (0.05 ~ 1.2 mg dm^{-3}) and sucrose (2, 3, 4, and 5 %) were further compared with hormone-free medium on their rooting stimulation. The results have illustrated that rooting did not occur in the hormone-free medium. Half and full strength MS medium supplemented with 0.75 ~ 1.0 mg dm^{-3} IAA and 3 % sucrose had the best effect in stimulation of all the shoots for root development (Fig. *D*). No significant difference was showed between 1/2 and full strength MS medium, but 3 % sucrose displayed enhanced stimulatory effect on root production while IBA induced much more and longer roots than IAA.

Taken together, this protocol will be of great use in mass multiplication of *P. methysticum* from limited donor plants in limited time, with perspective of its commercial production due to its pharmaceutical significance.

References

Bhat, S.R., Chandel, K.P.S., Malik, S.K.: Plant regeneration from various explants of cultivated *Piper* species. - Plant Cell Rep. **14**: 398-402, 1995.

Briskin, D.P., Kobayashi, H., Mehta, A., Gawienowski, M.C., Ainsworth, L., Smith, M.A.L.: Production of kavapyrones by kava (*Piper methysticum*) tissue cultures. - Plant Cell Rep. **20**: 556-561, 2001.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tabacco tissue cultures. - Physiol Plant. **15**: 473-497, 1962.

Ramakrishnan Nair, R., Dutta Gupta, S.: High-frequency plant regeneration through cyclic secondary somatic embryogenesis in black pepper (*Piper nigrum* L.). - Plant Cell Rep. **24**: 699-707, 2006.

Singh, Y.N., Blumenthal, M.: Kava: an overview. - Herbal. Gram. **39**: 33-55, 1997.

Taylor, M., Taufa, L.: Decontamination of kava (*Piper methysticum)* for *in vitro* propagation. - Acta. Hort. **461**: 267-274, 1998.