
The effect of peptone and silver nitrate on *In vitro* shoot formation in *Hevea brasiliensis* Muell Arg.

Yupaporn Sirisom and Sompong Te-chato*

Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Hatyai, Songkhla 90112, Thailand.

Yupaporn Sirisom and Sompong Te-chato (2012) The effect of peptone and silver nitrate on *In vitro* shoot formation in *Hevea brasiliensis* Muell Arg. Journal of Agricultural Technology 8(4): 1509-1516.

The effects of Peptone and silver nitrate (AgNO_3) on *in vitro* shoot formation from cultured shoot tips of *Hevea brasiliensis* seedlings raised *ex vitro* were investigated. The addition of silver nitrate at concentrations of 3-5 mg/l to the shoot induction medium (SIM; Murashige and Skoog (MS) + 5 mg/l 6-Benzyladenine (BA) + 0.5% activated charcoal + 3% sucrose + 0.75% agar), induced multiple shoot formation in all the explants at mean rates of between 2 and 3 shoots per explant. However, the mean numbers of shoots per explant were not significantly different ($p < 0.05$) among all the concentrations tested nor were they significantly different from the mean number of explants raised in a control medium without silver nitrate supplementation. When the concentration of silver nitrate was decreased to 0-2 mg/l, the number of shoots per explant improved. The best results were achieved using a medium containing 1 mg/l silver nitrate in which a mean number in excess of 5 shoots per explant was obtained, the shoots having dark green leaves and vigorous growth, while those obtained from the medium without silver nitrate supplementation were pale green, and senescence of leaves was found after they had been cultured for 4 weeks. The addition of peptone (0-2%) was not successful in inducing multiple shoots. The number of shoots per explant decreased in comparison with the control treatment. Some shoots developed in the peptone-containing medium were small with senescence of leaves occurring after being cultured for 4 weeks.

Key words: Peptone, Silver Nitrate, Multiple shoot formation, *Hevea brasiliensis*

Introduction

Hevea brasiliensis Muell Arg., belonging to the Family Euphorbiaceae, is an economically important perennial tree grown in Thailand and Southeast Asia as the source of natural rubber. *H. brasiliensis* is still propagated by grafting clonal axillary buds onto unselected seedlings to maintain intracultural heterogeneity for both vigour and productivity (Hua *et al.*, 2010). Recently, however, there have been several reports of *Hevea* micropropagation using

* Corresponding author: Sompong Te-chato e-mail: stechato@yahoo.com

explants raised in different culture media selected according to the objective of the study. For clonal improvement, most experiments use somatic embryogenesis. Successful explants have been produced from cells derived from the anther (Jayasree *et al.*, 1999; Hua *et al.*, 2010) and from the immature inner integument (Te-chato and Chartikul, 1993; Sushamakumari *et al.*, 2000; Montoro *et al.*, 2003; Lardet *et al.*, 2007). In order to propagate true-to-type clones, the microcutting technique is always used. This technique begins by culturing axillary buds or cotyledonary nodes and then inducing plantlets from them. However, an efficient protocol for the large scale micropropagation of elite *Hevea* clones has not yet been developed (Nayanakantha and Seneviratne, 2007).

There have been several reports of peptone being used to improve multiple shoot formation in, for instance orchids (Seeni and Latha, 1992; Chen and Chang, 2002) and avocado (Nhut *et al.*, 2008). Silver nitrate (AgNO₃) has also been shown to be effective in improving plantlet regeneration during somatic embryogenesis in a number of crop species, including Brussels sprouts (Williams *et al.*, 1990), cassava (Zhang *et al.*, 2001), *Paspalum scrobiculatum* L. (Vikrant and Rashid, 2002), *Ziziphus jujuba* Mill. (Feng *et al.*, 2010), achiote (Parimalan *et al.*, 2010) and turnip (Cogbill *et al.*, 2010). However, the use of peptone and silver nitrate have not yet been reported in *H. brasiliensis*. Therefore, in the present study, we report on the *in vitro* shoot multiplication of *H. brasiliensis* using peptone and silver nitrate in the mass propagation of uniform plantlets from this tree species.

Materials and methods

Plant material and culture conditions

Shoot tips derived from a native cloned rubber tree grown naturally on the Hatyai campus of Prince of Songkla University, Songkhla province in Thailand, were collected from a one-month-old seedling and washed in running tap water for 10 minutes. The explants were surface sterilized in 70% ethanol for 30 seconds and in 20% sodium hypochlorite for 20 minutes, followed by three rinses with sterilized distilled water. The sterilized shoot tips were then cut into 0.5 cm. lengths and cultured on a shoot induction medium (SIM; Murashige and Skoog (MS) medium supplemented with 5 mg/l 6-Benzyladenine (BA), 3% sucrose, 0.05% activated charcoal) as described by Te-chato and Muangkaewngam (1992). The medium's pH was adjusted to 5.7 with 0.1 N HCl or KOH before adding 0.75% agar and autoclaved at 1.05 kg/cm², at 121°C

for 15 minutes. The cultures were maintained at $28\pm 0.5^{\circ}$ C under fluorescent lamps at $12.5 \mu\text{mol}/\text{m}^2/\text{s}$ for a 14 hour photoperiod.

Effect of peptone and silver nitrate on multiple shoot formation

The shoot tips were cultured on SIM under the conditions specified above, for 4 weeks. The cultures were routinely subcultured at 4 week intervals to induce multiple shoot formation. After being cultured for 4 weeks, the micro-shoots obtained from the SIM were individually excised and transferred to SIM, supplemented with various concentrations of peptone (0, 1, 1.5, and 2%) or silver nitrate (0, 3, 4 and 5 mg/l). After being cultured for a further 6 weeks, the extent of multiple shoot formation and the number of shoots per explant was recorded.

Improved multiple shoots formation by silver nitrate

The Micro-shoots cultured on the SIM were individually excised and transferred to SIM supplemented with various concentrations of silver nitrate all of which were lower than in previous experiments (0, 0.5, 1 and 2 mg/l). The cultures were maintained under the same conditions as described above. After 6 weeks of being cultured, the extent of multiple shoot formation and the number of shoots per explant were recorded.

Shoot elongation and rooting

Six-week-old multiple shoots derived from the SIM were transferred to a growing medium based on solid MS medium supplemented with 1 mg/l silver nitrate and 0.05% activated charcoal but without the addition of a plant growth regulator to encourage shoot elongation. After 4 weeks of culture, each elongated shoot was excised and transferred to a half-strength MS medium supplemented with 5 mg/l IBA, 1 mg/l silver nitrate, 3% sucrose, 0.05% activated charcoal and 0.75% agar for root induction. Complete plantlets were then hardened and transferred to a greenhouse.

Statistical analysis

Mean values were analyzed using a one-way analysis of variance (ANOVA). Significant differences among treatments were detected using Duncan's multiple range tests (DMRT) at the 0.05 level of probability.

Results

Effect of peptone and silver nitrate on multiple shoot formation

It was found that peptone, and silver nitrate at concentrations of 3 mg/l and above affected the formation of multiple shoots on cultured shoot tips from a rubber tree, reducing the average number of shoots per explant when compared with the control medium, although the effect was only significant in respect of the peptone supplemented medium. The formation of multiple shoots was detected after 3 weeks of being cultured in SIM containing peptone. Between 93 and 100% of the explants cultured in SIM supplemented with all concentrations of peptone produced shoots without significant difference between explants raised in media containing different concentrations of peptone, in either the percentage in which multiple shoot formation occurred or the number of shoots produced per explant (Table 1). There was however a significant difference between the number of shoots produced between the control medium and the peptone-supplemented media. In addition, some shoots were small and wilted after 4 weeks of being cultured (Fig. 1b).

The explants cultured in SIM supplemented with silver nitrate formed multiple shoots at all concentrations. The number of shoots was approximately three per explant and the number was not significantly different among the different concentrations tested (Table 1), nor was there any significant difference between the number of shoots produced in the control medium and the silver nitrate-supplemented media, although in all instances the number was less than that in the control medium. After being cultured for 4 weeks, the shoots had dark green leaves and produced roots in a similar manner to shoots cultured in the control medium consisting of unsupplemented SIM (Fig. 1a, c).

These findings suggest that silver nitrate plays a more significant role in shoot formation than peptone. However, the optimum concentration noted in this phase of the study was not actually optimal for producing the maximum number of shoots, since the unsupplemented control medium resulted in the production of the largest number of shoots. Therefore, a further experiment was conducted to test lower concentrations of silver nitrate (0-2 mg/l) to establish if they would result in an increase in the number of shoots. The results showed that decreasing the concentration of silver nitrate to 1-2 mg/l produced an average of 5 shoots per explant. However, there was no significant difference among the concentrations tested (Table 2). The shoots cultured on SIM containing silver nitrate were dark green and exhibited vigorous growth (Fig. 1e, f) while those cultured on the control medium unsupplemented with silver nitrate were pale green and exhibited senescence of the leaves after 4

weeks of being cultured (Fig. 1d). Silver nitrate at a concentration of 1 mg/l proved to be optimal for multiple shoot induction.

Shoot elongation and root induction

Elongation of shoots was carried out by transferring small clusters of multiple shoots developed in SIM to a solidified MS medium without plant growth regulators in the presence of 1 mg/l silver nitrate and 0.05% activated charcoal. After 4 weeks of being cultured those shoots were ready for root induction (Fig. 1g); elongated single shoots, 2–3 cm long were excised from the multiple shoots and transferred to solidified half-strength MS medium supplemented with 5 mg/l IBA, 1 mg/l silver nitrate, 3% sucrose and 0.05% activated charcoal. The shoots developed roots within 3 weeks of being transferred and there was a 100% rate of root induction (Fig. 1h).

Table 1. Effect of peptone or silver nitrate on multiple shoot formation from cultured shoot tip explants raised on SIM for 6 weeks

Concentration of substance	Multiple shoot induction (mean %)	Average number of shoots/explant
Peptone (%)		
0	100a	3.40 ± 0.55a
1	93.33a	1.60 ± 0.55b
1.5	100a	1.40 ± 0.55b
2	93.33a	1.60 ± 0.55b
Silver nitrate (mg/l)		
0	100a	3.40 ± 0.14a
3	100a	3.00 ± 0.84a
4	100a	2.60 ± 0.89a
5	100a	2.20 ± 0.45a

Mean values followed by the same letter(s) within a column are not significantly different ($P < 0.05$)

Table 2. Effect of various concentrations of silver nitrate on average numbers of shoots per explant on SIM supplemented with 5 mg/l BA, 3% sucrose, 0.05% activated charcoal after being cultured for 6 weeks

Concentration of silver nitrate (mg/l)	Multiple induction (%)	Average number of shoots/explants
0	100a	3.50 ± 1.35a
0.5	100a	4.20 ± 2.68a
1.0	100a	5.60 ± 3.36a
2.0	100a	5.29 ± 1.29a

Mean values followed by the same letter(s) within a column are not significantly different ($P < 0.05$)

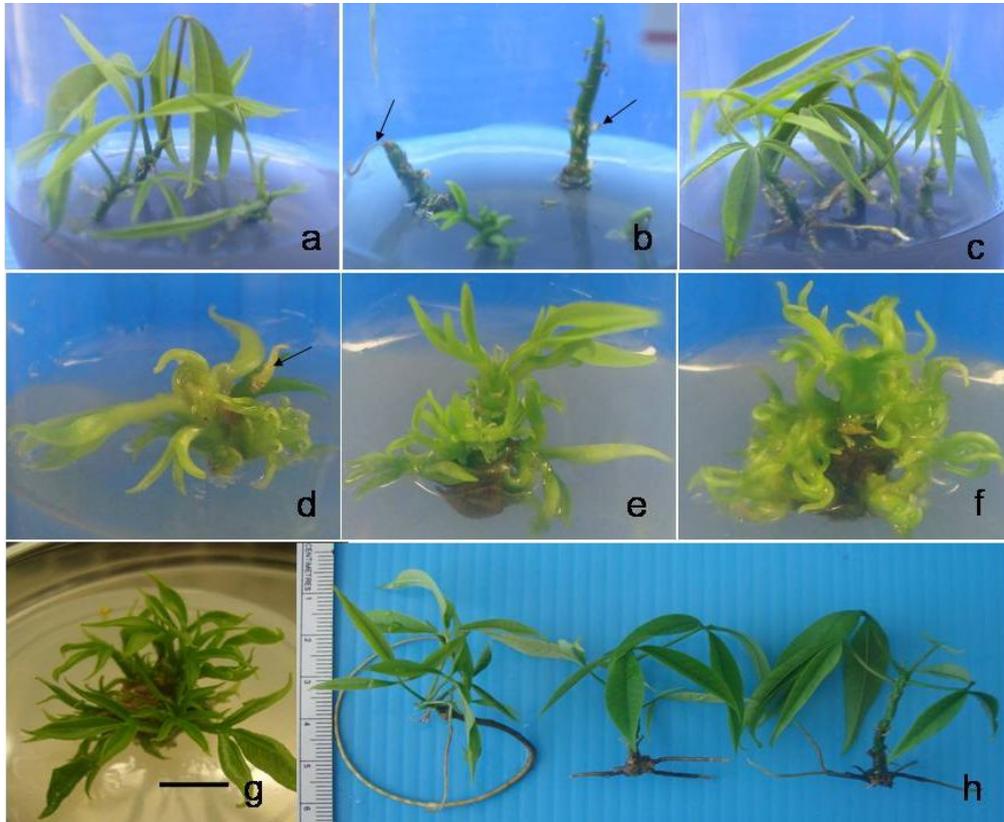


Fig. 1. Effect of peptone and silver nitrate on the formation of multiple shoots: (a) control treatment without peptone or silver nitrate, (b) SIM with 2% peptone showing small wilted shoots (arrow), (c) SIM with 5 mg/l silver nitrate showing healthy shoots with roots, (d) SIM without silver nitrate showing the senescence of leaves (arrow) after being cultured for 4 weeks, (e) SIM with 0.5 mg/l silver nitrate, (f) 1 mg/l, (g) elongation of shoots cultured on MS medium without plant growth regulators for 4 weeks (bar = 0.5 cm), and (h) complete plantlets after being cultured on $\frac{1}{2}$ MS medium supplemented with 5 mg/l IBA for 4 weeks.

Discussion

A number of authors have reported the micropropagation of *Hevea* using somatic embryogenesis induction from immature anther tissue (Jayasree *et al.*, 1999; Hua *et al.*, 2010) as well as from tissue from the immature inner integument (Te-chato and Chartikul, 1993; Sushamakumari *et al.*, 2000; Montoro *et al.*, 2003; Lardet *et al.*, 2007). Using the Microcutting technique, the shoots and nodes of seedlings can be successfully used for *in vitro* multiplication on the induction medium as earlier described by Te-chato and Muangkaewngam (1992). In exceptional cases, the multiple shoots produced from a node were more vigorous than those produced from a shoot tip culture.

Peptone has been used as the source of carbon and nitrogen for plant tissue culture (Nhut *et al.*, 2008) and some reports have shown a positive effect on the growth of explants, including embryo production in *Oncidium* (Chen and Chang, 2002) and shoot multiplication in avocado (Nhut *et al.*, 2008). However, in the present study, the number of multiple shoots produced in the presence of peptone after being cultured for 4 weeks in SIM was small with senescence of the leaves, suggesting that peptone might inhibit shoot formation in this plant.

The mode of action of silver nitrate in plant tissue culture is assumed to be associated with the physiological effects of ethylene, silver ions acting as a competitive inhibitor of ethylene action rather than inhibiting ethylene synthesis (Zhang *et al.*, 2001). Many reports have demonstrated the positive effect of silver nitrate on plant tissue culture (Zhang *et al.*, 2001; Vikrant and Rashid, 2002; Feng *et al.*, 2010; Parimalan *et al.*, 2010; Cogbill *et al.*, 2010). In the present study, the addition of silver nitrate in the induction medium at concentrations ranging from 0.5 to 2 mg/l was successful in promoting shoot multiplication in all the explants with an average numbers of 5 shoots per explant. In addition, silver nitrate provides silver ions which may interact with polyamines, leading to the promotion of organogenesis and embryogenesis (Zhang *et al.*, 2001). Normally, ethylene inhibits S-adenosyl methionine (SAM) decarboxylase, which in turn promotes polyamine (Parimalan *et al.*, 2010). Thus, the present study suggests that it is possible to improve the frequency of shoot organogenesis in *H. brasiliensis* by supplementing the growth medium (SIM) with silver nitrate.

Acknowledgements

We would like to thank the National Research University Project of Thailand's Office of the Higher Education Commission, the Graduate School of Prince of Songkla University and the Center of Excellence in Agricultural and Natural Resources Biotechnology, Postgraduate Education and Research Development Office, Commission on Higher Education, Ministry of Education for financial support.

References

- Chen, J.T., Chang, W.C. (2002). Effects of tissue culture conditions and explant characteristics on direct somatic embryogenesis in *Oncidium* 'Gower Ramsey'. *Plant Cell, Tissue and Organ Culture* 69: 41-44.
- Cogbill, S., Faulcon, T., Jones, G., McDaniel, M., Harmon, G., Blackmon, R. and Young, M. (2010). Adventitious shoot regeneration from cotyledonary explants of rapid-cycling fast plants of *Brassica rapa* L. *Plant Cell, Tissue and Organ Culture* 101: 127-133.

- Feng, J.C., Yu, X.M., Shang, X.L., Li, J.D. and Wu, Y.X. (2010). Factors influencing efficiency of shoot regeneration in *Ziziphus jujuba* Mill. 'Huizao'. *Plant Cell, Tissue and Organ Culture* 101: 111-117.
- Hau, Y.W., Huang, T.D., Huang, H.S. (2009). Micropropagation of self-rooting juvenile clones by secondary somatic embryogenesis in *Hevea brasiliensis*. *Plant Breeding* 129: 202-207.
- Jayasree, P.K., Asokan, M.P., Sobha, S., Ammal, L.S., Rekha, K., Kala, R.G., Jayasree, R. and Thulaseedharan, A. (1999). Somatic embryogenesis and plant regeneration from immature anthers of *Hevea brasiliensis* Mull. Arg. *Current Science* 76: 1242-1245.
- Lardet, L., Martin, F., Dessailly, F., Carron, M.P. and Montoro, P. (2007). Effect of exogenous calcium on post-thaw growth recovery and subsequent plant regeneration of cryopreserved embryogenic calli of *Hevea brasiliensis* Mull. Arg. *Plant Cell Reports* 26: 559-569.
- Montoro, P., Rattana, W., Pujade-Renaud, V., Michaux-Ferriere, N., Monkolsook, Y., Kanthapura, R. and Adunsadthapong, S. (2003). Production of *Hevea brasiliensis* transgenic embryogenic callus lines by *Agrobacterium tumefaciens*: roles of calcium. *Plant Cell Reports* 21: 1095-1102.
- Nayanakantha, N.M.C. and Seneviratne, P. (2007). Tissue culture of rubber: past, present and future prospects. *Ceylon Journal of Science (Biological Science)* 36: 116-125.
- Nhut, D.T., Thi, N.N., Khiet, B.L.T. and Luan, V.Q. (2008). Peptone stimulates in vitro shoot and root regeneration of avocado (*Persea americana* Mill.). *Scientia Horticulturae* 115: 124-128.
- Parimalan, R., Giridhar, P. and Ravishankar, G.A. (2010). Enhanced shoot organogenesis in *Bixa orellana* L. in the presence of putrescine and silver nitrate. *Plant Cell, Tissue and Organ Culture* 105: 285-290.
- Seeni, S. and Latha, P.G. (1992). Foliar regeneration of the endangered Red Vanda, *Renanthera imschootiana* Rolfe (Orchidaceae). *Plant Cell, Tissue and Organ Culture* 29: 167-172.
- Sushamakumari, S., Asokan, M.P., Anthony, P., Lowe, K.C., Power, J.B. and Davey, M.R. (2000). Plant regeneration from embryogenic cell suspension-derived protoplasts of rubber. *Plant Cell, Tissue and Organ Culture* 61: 81-85.
- Te-chato, S. and Chartikul, M. (1993). Tissue culture of rubber: Certain factors affecting callus formation from integument seed. *Songklanakarin Journal of Science and Technology* 15: 227-233.
- Te-chato, S. and Muangkaewngam, A. (1992). Tissue culture of rubber 1. *In vitro* Micropropagation of rubber. *Songklanakarin Journal of Science and Technology* 14: 123-132.
- Vikrant, Rashid A. (2002). Somatic embryogenesis from immature and mature embryos of a minor millet *Paspalum scrobiculatum* L. *Plant Cell, Tissue and Organ Culture* 69: 71-77.
- Williams, J., Pink, D.A.C. and Biddington, N.L. (1990). Effect of silver nitrate on long-term culture and regeneration of callus from *Brassica oleracea* var. *gemmifera*. *Plant Cell, Tissue and Organ Culture* 21: 61-66.
- Zhang, P., Phansiri, S. and Puonti, K.J. (2001). Improvements of cassava shoot organogenesis by the use of silver nitrate *in vitro*. *Plant Cell, Tissue and Organ Culture* 67: 47-54.

(Published in July 2012)