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Propagation of *Vatica diospyroides* Symington: An Endangered Medicinal Dipterocarp of Peninsular Thailand by Cultures of Embryonic Axes and Leaf-derived Calli

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Abstract: Embryonic axes and young leaves of *Vatica diospyroides* Symington were excised sterilely and then cultured on MS medium supplemented with 0-20 mg L^{-1} of a growth regulator and 0-0.3% Activated Charcoal (AC). The growth regulators tested were 2, 4-Dichlorophenoxyacetic acid (2,4-D), α-Naphthaleneacetic acid (NAA) and 3, 6-Dichloro-2-methoxybensoic acid (Dicamba). Vigorous shoot development was observed with 0-20 mg L^{-1} of each plant growth regulator and 0.1-0.15% AC. Shoot-derived calli were obtained 6 months after culturing embryonic axes on MS medium, with 15 mg L^{-1} dicamba and 0.1% AC. Interestingly, after culturing young leaves for 2 months, the highest weight of compact calli (0.40 g) was achieved with a medium containing 20 mg L^{-1} dicamba and 0.1% AC, in darkness. These were successfully multiplied by renewing and culturing in the same medium and transfer to shooting induction in MS medium supplemented with 0-20 mg L^{-1} 6-benzyladenine (BA). Unfortunately, shoot induction from calli was unsuccessful and despite initiated roots being induced. A successful *in vitro* propagation protocol of *V. diospyroides* should be thus investigated more extensively.

Key words: Callus, Dipterocarpaceae, embryo, tissue culture, *Vatica diospyroids*

INTRODUCTION

The plant family Dipterocarpaceae has many medicinal tree species such as *Dipterocarpus alatus* Roxb., *Shorea roxburghii* G. Don, *Hopea odorata* Roxb. and *Vatica diospyroides* Symington (found in Malaysia and Vietnam and endemic in peninsular Thailand (Smitinand, 1966). They are valuable sources of significant curative compounds namely dimer, oligomer and tetramer derivatives of resveratrol (Seo *et al*., 1999; Ito *et al*., 2003; Atun *et al*., 2008) which play an important role in cardiovascular prevention and have cytotoxicity against human cancer cell lines (Kinghorn, 2000; Zain *et al*., 2011; Wu *et al*., 2011; Srisawat *et al*., 2013). Interestingly, flowers of plants in this family are usually sweet scented because of aromatic resins and oils. Particularly, the flowers of *V. diospyroides* have the best lingering strong and pleasant fragrance (Pooma, 2002): they are difficult to replace as raw material of perfumes. Because of such demand, *V. diospyroides* trees are at the highest risk level due to their flowers being collected and whole plants being stolen.

Based on data from The International Union for Conservation of Nature and Natural Resources (IUCN), *V. diospyroides* has been given the Critically Endangered (CR A1cd, C2a) status of threatened species (Ashton, 1998): its habitat is fragile, it is overexploited and its wild population is decreasing. Although flowering and fruiting of this plant are typically regular for a short period from February to April, the plant did not bloom during 2009 to 2011 in Thailand, due to severe and long droughts (Srisawat *et al*., 2012). On the other hand, *V. diospyroides* lacks natural dormancy of seeds (Smith *et al*., 2002) which cannot be stored for long periods (Abdulhadi *et al*., 1981). Since it is propagated by seed, growths of seedling are threatened by insects, wildlife and also by humans. This species faces a high risk of extinction in the wild, in the immediate future. Thus, it is necessary to develop vegetative propagation, to artificially supplement large numbers of *V. diospyroides* trees to the field. Unfortunately, only limited success has been achieved with vegetative propagation of dipterocarpaceae (Moura-Costa and Lundoh, 1994).

Nowadays, propagation in *in vitro* culture can overcome the limitations of conventional propagation, for some dipterocarp species. There have been successes with *in vitro* culture of Dipterocarpaceae

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genera; *D. alatus* and *D. intricatus* embryos were cultured on Woody Plant Medium (Lloyd and McCown, 1980) supplemented with BA for inducing their seedlings (Linington, 1991), whereas seedlings of *S. roxburghii* and *H. odorata* were successfully induced by culturing their embryos on MS medium containing BA (Scott *et al*., 1988; Scott *et al*., 1995). The abundant regeneration of dicotyledonous herbs or shrubs through callus (somatic embryogenesis) is common, but this does not work well for dicotyledonous trees in the family Dipterocarpaceae (Smits and Struycken, 1983). In order to produce a large enough number of, *V. diospyroides* in the field, to meet the demands of ingredients for perfumeries and valuable curative agents, effective propagation of *V. diospyroides* via embryonic axes and callus culture would be useful. In the present study, *V. diospyroides* tissue cultures, starting from embryonic axes and young leaves, to develop micropropagation routines for this species were investigated. This study describes a successful culturing of embryonic axes and a callus culture of *V. diospyroides*, a representative of Dipterocarpaceae.

MATERIALS AND METHODS

Plant materials and surface sterilization: Mature seeds and three-month-old seedlings of *V. diospyroides* Symington type LS were kindly provided by the Director of Nong Thung Thong non-hunting area, Kiansa district, Suratthani province. The tree type had been authenticated previously, as described by Srisawat *et al*. (2012). All seeds were pre-washed by soaking in $KMnO_4$ for 30 min and immediately air dried. Their pericarps with seed coats were removed and the whole cotyledonous embryos were surface sterilized with heat.

Two-week-old leaves of their seedlings were prepared for callus culture. All young leaves were pre-washed by commercial detergent. The sterilization of the explants was performed by soaking in 40% Clorox^{tm}, then rinsing three times with sterile distilled water.

Embryo culture and callus initiation: The axes of embryos were cut from their cotyledons with aseptic new razor blades. The excised embryo axes were then cultured on MS medium (Murashige and Skoog, 1962) containing 0-15 mg L^{-1} of 2,4-D, or NAA, or dicamba and supplemented also with 0-0.3% AC. Culturing at 25±2°C and 2000 lux light intensity for 16:8 L/D was done to induce seedling germination and callus initiation from embryo axes. Callus formation was also attempted starting from young leaves. The surface sterilized leaves were trimmed, cut and divided into 1 cm^2 size by keeping the middle veins of the leaves for all final explants. The explants were cultured on MS medium containing 0-20 mg L^{-1} dicamba supplemented with 0.1 % AC at 25±2°C in darkness, to induce callus formation.

Shoot induction: The vigorous calli were transferred to a shoot induction medium (MS) containing 0-20 mg L^{-1} BA and 0.1% AC. To induce shooting, the explants were separately cultured in darkness and/or 2000 lux light condition with 25±2°C of temperature.

RESULTS AND DISCUSSION

This study is focused on embryonic axis culture and callus initiation of *V. diospyroides* Symington, in order to support efficient propagation of this plant in the near future. The seedling production was dominant in culturing of embryonic axes on MS medium supplemented with 0.10 or 0.15% AC (Fig. 1). Interestingly, supplementing the medium with various auxins (5-20 mg L^{-1} of 2,4-D, NAA, or dicamba) or without regulators, induced vigorous shoot production within the third week of culturing. However, after six months of culture, calli were initiated at the base of seedlings grown on the medium with AC and 15 mg L^{-1} dicamba (data not shown). Callus formation from embryonic axis cultures did not happen in the media containing 2,4-D or NAA which are known to be effective growth regulators for callus and root cultures of other dipterocarp species such as *Shorea curtisii* (Smits and Struycken, 1983), *S. roxburghii* and *S. leprosula* (Vaario, 1996). Hundred percent of the embryonic axes died when cultured on a medium without AC, due to browning exudates. Phenolic compounds were released from the wounds inflicted during excision of embryonic

Fig. 1: Seedlings of *V. diospyroides* germinated from embryo axes, cultured on MS medium containing 0.15% AC, 2 months into culturing

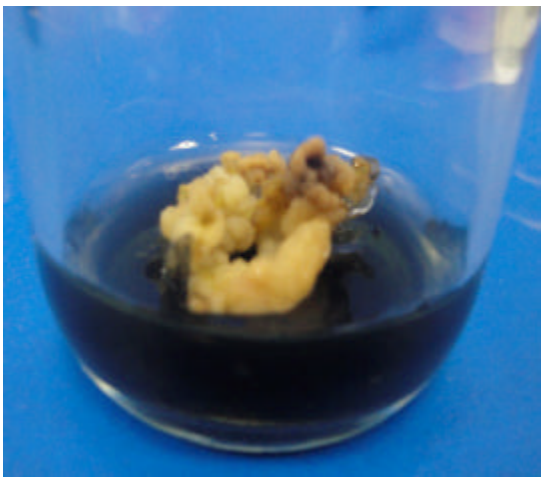

Fig. 2: Callus formation of young *V. diospyroides* leaves cultured on MS medium containing 20 mg L^{-1} dicamba and 0.10% AC, 2 months into culturing

Table 1: Average weights of leaf-derived calli cultured on MS medium containing 0-20 mg L^{-1} dicamba, after 2 months of culturing

Concentrations of dicamba (mg L^{-1})	Average weight of calli (g)
0	0.00[b]
5	0.00[b]
10	0.00[b]
15	0.0469[b]
20	0.4006[a]

Averages sharing the same superscript (a or b) are not significantly (p = 0.05) different, according to Turkey's multiple comparison test

axes from their cotyledons (Srisawat *et al*., 2012); these compounds changed the medium from clear to brownish. Activated charcoal, AC, is an effective absorber of (harmful) compounds: its role in the medium was in absorbing exudates and other harmful compounds released from plant tissue during culture (Thomas, 2008). In our case, AC suppressed the phenolic compounds, while it may have weakened the effects of auxin (Thomas, 2008). In the media containing 0.10-0.15% AC, viability of seedlings was 100% (data not shown). Therefore AC was a key ingredient enabling successful *in vitro* culture of seedlings and callus initiation. The *in vitro* seedling production of dipterocarp obtained by using our protocol, without using plant growth regulators, was considerably better than reported by Linington (1991), Scott *et al*. (1988, 1995), who studied seedling induction from embryo cultures of three genera of dipterocarpaceae by using BA as regulator.

Being an effective auxin for producing calli, dicamba was used to initiate the callus formation from young leaves. The best result in terms of callus fresh weight (0.40 g) was swiftly obtained at 2 months of culturing, compared to seedling-derived calli, with the highest concentration of dicamba (20 mg L^{-1}) and 0.1% AC in darkness (Table 1). Possibly even higher concentrations of dicamba would be useful, on embryogenic callus manipulation and help establish large scale propagation of *V. diospyroides* via somatic embryogenesis. Dicamba appears well suited for *in vitro* micropropagation of *V. diospyroides*, whereas 2,4-D and NAA were found to be not effective. Dicamba, a kind of herbicide, is an auxin-like growth regulator usually used at low concentration (0.2-2.0 mg L^{-1}) for promoting and maintaining embryogenic calli of various monocotyledonous crops. Dicamba, as a promoter of embryogenic callus and somatic embryogenesis, has enabled several successful cultures of calli, with maize (Gorji *et al*., 2011), wheat (Bahieldin *et al*., 2000; Satyavathi *et al*., 2004), barley (Aguado-Santacruz *et al*., 2011), sugarcane (Brisibe *et al*., 1994) and ginger (Kackar *et al*., 1993). In the present study, a higher concentration of dicamba (20 mg L^{-1}) than previously reported was effective in compact callus initiation and multiplication. The size and texture of a compact callus, from culturing young leaves on the medium with the highest concentration of dicamba, are shown in Fig. 2. Clearly the young leaf-derived callus is of homogenous and compact type. After transferring the calli to shooting induction medium (0-20 mg L^{-1} BA, 0.1% AC), 100% of calli were browned during culture in dark or light conditions. By the way, the calli were rooted after 6 months of culturing in darkness.

On the basis of our official assessment, there are fewer than 200 trees of *V. diospyroides* in the Nong Thung Thong non-hunting area (the biggest genetic source of *V. diospyroides* in peninsular Thailand, Srisawat *et al*., 2012). Unfortunately, *V. diospyroides* has been recalcitrant to induced organogenesis or somatic embryogenesis from calli which would be ideal for large scale artificial propagation of this plant. Only one genus of Dipterocarpaceae has been successfully propagated by callus formation: that is genus *Shorea* (Smits and Struycken, 1983; Scott *et al*., 1988; Vaario, 1996). The genera *Dipterocarpus* (Linington, 1991), *Shorea* (Scott *et al*., 1988) and *Hopea* (Scott *et al*., 1995) have been successfully only by *in vitro* embryos cultured. It appears that the *in vitro* propagation of plants in Dipterocarpaceae remains difficult and this aspect warrants more research work. This study has now reported the first successful induction of seedling and callus cultures of *V. diospyroides*, a critically endangered species of Dipterocarpaceae. This success supports establishing propagation via somatic embryogenesis and may help save the endangered tree species.

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REFERENCES

Abdulhadi, R., K. Kartawinata and S. Sukardjo, 1981. Effects of mechanized logging in the lowland dipterocarp forest at Lempake, East Kalimantan. Mala. Forester, 44: 407-418.

Aguado-Santacruz, G.A., A. Velazquez-Ordinola, B. Moreno-Gomez, L.M. Gomez-Torres, L.F. Diaz-Espino and F.P.G. Vazquez, 2011. Development of long-term and reliable *in vitro* plant regeneration systems for elite malting barley varieties: Optimizing media formulation and explant selection. Afr. J. Biotechnol., 10: 19522-19533.

Ashton, P., 1998. Vatica diospyroides. IUCN Red List of Threatened Species. http://www.iucnredlist.org/details/33482/0

Atun, S., N. Aznam, R. Ariamingrum, Y. Takaya and N. Masatake, 2008. Resveratrol derivatives from stem bark of *Hopea* and their biological activity test. J. Phys. Sci., 19: 7-21.

Bahieldin, A., W.E. Dyer and R. Qu, 2000. Concentration effects of dicamba on shoot regeneration in wheat. Plant Breed., 119: 437-439.

Brisibe, E.A., H. Miyake, T. Taniguchi and E. Maeda, 1994. Regulation of somatic embryogenesis in long-term callus cultures of sugarcane (*Saccharum officinarum* L.). New Phytol., 126: 301-307.

Gorji, A.H., M. Zolnoori, A. Jamasbi and Z. Zolnoori, 2011. *In vitro* plant generation of tropical maize genotypes. Proceedings of the International Conference on Environmental, Biomedical and Biotechnology, Volume 16, August 19-21, 2011, Shanghai, China, pp: 52-59.

Ito, T., Y. Akao, H. Yi, K. Ohguchi and K. Matsumoto *et al*., 2003. Antitumor effect of resveratrol oligomers against human cancer cell lines and the molecular mechanism of apoptosis induced by vaticanol C. Carcinogenesis, 24: 1489-1497.

Kackar, A., S.R. Bhat, K.P.S. Chandel and S.K. Malik, 1993. Plant regeneration via somatic embryogenesis in ginger. Plant Cell Tissue Organ Cult., 32: 289-292.

Kinghorn, A.D., 2000. Plant secondary metabolites as potential anticancer agents and cancer chemopreventives. Molecules, 5: 285-288.

Linington, I.M., 1991. *In vitro* propagation of *Dipterocarpus alatus* and *Dipterocarpus intricatus*. Plant Cell Tissue Organ Culture, 27: 81-88.

Lloyd, G. and B. McCown, 1980. Commercially feasible micropropagation of mountain laurel, *Kalmia latifilia*, by use of shoot-tip culture. Proc. Int. Plant Propagators, 30: 421-427.

Moura-Costa, P. and L. Lundoh, 1994. method for vegetative propagation of *Dryobalanops lanceolata* (Dipterocarpaceae) by cuttings. J. Trop. Forest Sci., 6: 533-541.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15: 473-497.

Pooma, R., 2002. Further notes on Thai Dipterocarpaceae. Thai Forest Bull. Bot., 30: 7-27.

Satyavathi, V.V., P.P. Jauhar, E.M. Elias and M.B. Rao, 2004. Effects of growth regulators on *in vitro* plant regeneration in durum wheat. Crop Sci., 44: 1839-1846.

Scott, E.S., A.N. Rao and C.S. Loh, 1988. Production of plantlets of *Shorea roxburghii* G. Don. from embryonic axes cultured *in vitro*. Ann. Bot., 61: 233-236.

Scott, E.S., A.N. Rao and C.S. Loh, 1995. Preliminary studies of micropropagation of *Hopea odorata*, a dipterocarp tree. Plant Cell Tissue Organ Culture, 41: 193-196.

Seo, E.K., H. Chai, H.L. Constant, T. Santisuk and V. Reutrakul *et al*., 1999. Resveratrol tetramers from *Vatica diospyroides*. J. Org. Chem., 64: 6976-6983.

Smith, M.T., B.S.P. Wang and H.P. Msanga, 2002. Dormancy and Germination. In: Tropical Tree Seed Manual, Vozzo, J.A. (Ed.). U.S. Department of Agriculture, New York, pp: 149 -176.

Smitinand, T., 1966. The distribution of the Dipterocarpaceae in Thailand. Proceedings of the 11th Congress on Pacific Science, August 22-September 10, 1966, University of Tokyo, Tokyo, pp: 67-76.

Smits, W.T.M. and B. Struycken, 1983. Some preliminary results of experiments with *in vitro* culture of dipterocarps. Netherlands J. Agric. Sci., 31: 233-238.

Srisawat, T., S. Thipnetr and C. Maknoi, 2012. A preliminary study of leaf morphology and flow cytometry in the *Vatica diospyroides* Symington, endangered medicinal plant of Peninsular Thailand. J. Med. Plant Res., 6: 3681-3688.

Srisawat, T., P. Chumkaew, W. Heed-Chim, Y. Sukpondma and K. Kanokwiroon 2013. Phytochemical screening and cytotoxicity of crude extracts of *Vatica diospyroides* Symington Type LS. Trop. J. Pharm. Res., 12: 71-76.

Thomas, T.D., 2008. The role of activated charcoal in plant tissue culture. Biotech. Adv., 26: 618-631.

Vaario, L.M., 1996. Establishment of advanced tissue culture techniques in *Betula platyphylla* var. japonica and in Dipterocarpaceae Species. Bull. Tokyo Univ. Forests, 96: 51-118.

Wu, J.M., T.C. Hsieh and Z. Wang, 2011. Cardioprotection by resveratrol: A review of effects/targets in cultured cells and animal tissues. Am. J. Cardiovasc. Dis., 1: 38-47.

Zain, W.Z.W.M., N. Ahmat, N.H. Norizan and N.A.A.M. Nazri, 2011. The evaluation of antioxidant, antibacterial and structural identification activity of trimer resveratrol from Malaysia's Dipterocarpaceae. Aust. J. Basic Applied Sci., 5: 926-929.