

Anthocyanin accumulation in the hypocotyl and petal of Red Agati (*Sesbania grandiflora*), an ornamental legume

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Abstract

Seeds of Red Agati (*Sesbania grandiflora*), an ornamental leguminous tree, were germinated *in vitro* under both light and dark conditions for 7, 10, 15, 20 and 25 days. The localization of anthocyanin-containing cells and level of total anthocyanin content of hypocotyl from several developmental stages were determined. In the hypocotyl of light-grown seedlings, anthocyanin-containing cells were observed in epidermal and sub-epidermal layer and peripheral cortex while none was found in that of dark-grown seedlings. On day 7, the hypocotyl of light-grown seedlings had the highest anthocyanin content (290 µg/g FW). Moreover, Red Agati's petal at various developmental stages was also examined for the total anthocyanin content. It was found that the petal of 3 cm length had the highest total anthocyanin level (455 µg/g FW). It is concluded that the hypocotyl of light-grown Red Agati seedlings is an attractive alternative source of anthocyanins to the petal as the seedlings can be raised and be made available throughout the year.

Key words: Red Agati, anthocyanin, *Sesbania grandiflora*, histology, development

Introduction

Red Agati (*Sesbania grandiflora*), a perennial nitrogen-fixing ornamental tree, belongs to the family Fabaceae. The origin of this leguminous plant is not known but it is considered native to Southeast Asian countries. In Thailand, it is commonly grown in backyard gardens. This plant and a closely related species, Agati (*Sesbania grandiflora*), are useful for reforestation as they have extremely fast growth rate. They are also used for forage, firewood, pulp and paper, food, green manure and landscape decoration (National Research Council, 1979; Thiengburanatham, 1993; Bray, 1994; Gutteridge, 1994 and Jensen, 2001). The petal of Red Agati is large, distinctively rose pink or red in color while that of a related species, Agati, is white or yellowish, reflecting the differing anthocyanin content in the vacuole (Markham *et al.*, 2000).

Anthocyanins are plant phenolic compounds in the flavanoid group. They generally are responsible for the red, purple and blue colors in plant parts such as fruits, vegetables, flowers, leaves, roots and tubers. All natural anthocyanins are glycosides called anthocyanidin. Anthocyanins are receiving renewed attention for their potential health benefits as antioxidants and anti-inflammatory agents (Kalt and Dufour, 1997; Wang *et al.*, 1997; Wang *et al.*, 1999).

Plant tissue culture is one of the important biotechnological tools which has been applied in various aspects, such as, micropropagation (Bodhipadma and Leung, 2002a; Haw and Keng, 2003), *in vitro* flowering and fruiting (Bodhipadma and Leung, 2002b; Bodhipadma and Leung, 2003) and secondary

metabolite, especially anthocyanin production (Rao and Ravishankar, 2002; Filippini *et al.*, 2003).

Though there were some publications on tissue culture of *Sesbania*, the objective of most of them was to propagate this plant (Shanker and Ram, 1990; Shanker and Ram, 1993; Sinha and Mallick, 1993; Detrez *et al.*, 1994). Unlike Agati, Red Agati has a remarkably red petal that would be a good source of anthocyanin. However, the hypocotyl of a Red Agati seedling is also red. This could be a more attractive and convenient source for anthocyanin production than petal. With this goal in mind, the present study was undertaken to compare the amount of anthocyanin extracted from the hypocotyl and petal of Red Agati and investigate the time during their development when maximal accumulation of anthocyanin might occur in the appropriate organ.

Materials and methods

Plant materials: Mature dry seeds of Red Agati were obtained from a local garden of Nonthaburi province. To avoid possible complications from microbial contamination during raising seedlings from seeds for the present experiments, the Red Agati seeds were germinated under aseptic conditions: they were immersed in sterile distilled water overnight before they were surface-sterilized by soaking in 70% (v/v) ethanol for 30 seconds, 1.5% (v/v) sodium hypochlorite for 15 minutes and rinsing them three times with sterile distilled water. After seeds germinated *in vitro* on the medium (see Medium) under both light and dark conditions for 7, 10, 15, 20 and 25 days, hypocotyl from these seedlings were cut and examined for total anthocyanin content

and its localization. Flowers of Red Agati were collected from local garden of Nonthaburi province. Only petal parts 3, 6 and 9 cm in length were used to analyze for total anthocyanin content. The collection was made after the opening of the petals in 4, 7 and 12 days, respectively.

Medium: MS basal medium (Murashige and Skoog, 1962) in this study were adjusted to pH 5.7, gelled with 0.8% (w/v) agar, and autoclaved at 121 °C and 15 psi for 20 minutes. All the cultures were kept in a growth room at 25 °C under 16 hours illumination from white fluorescent lamps (3,180 Lux) or in the dark.

Determination of total anthocyanin content: Total anthocyanin content was measured using a method modified from that of Fuleki and Francis (1968). 1.5 g of hypocotyl from seedlings grown *in vitro* under both light and dark conditions after 7, 10, 15, 20 and 25 days, or petal (3, 6 and 9 cm in length) were blended with a mixture of 95% (v/v) ethanol and 1.5 N HCl (1.28: 0.23 mL). One mL of the extraction mixture was mixed with 95% ethanol and 1.5 N HCl (10.75: 1.88 mL) in a 20-mL vial wrapped with aluminum foil. This was gently shaken, kept overnight at 4 °C and then centrifuged at 6,870 x g for 10 min. The absorbance

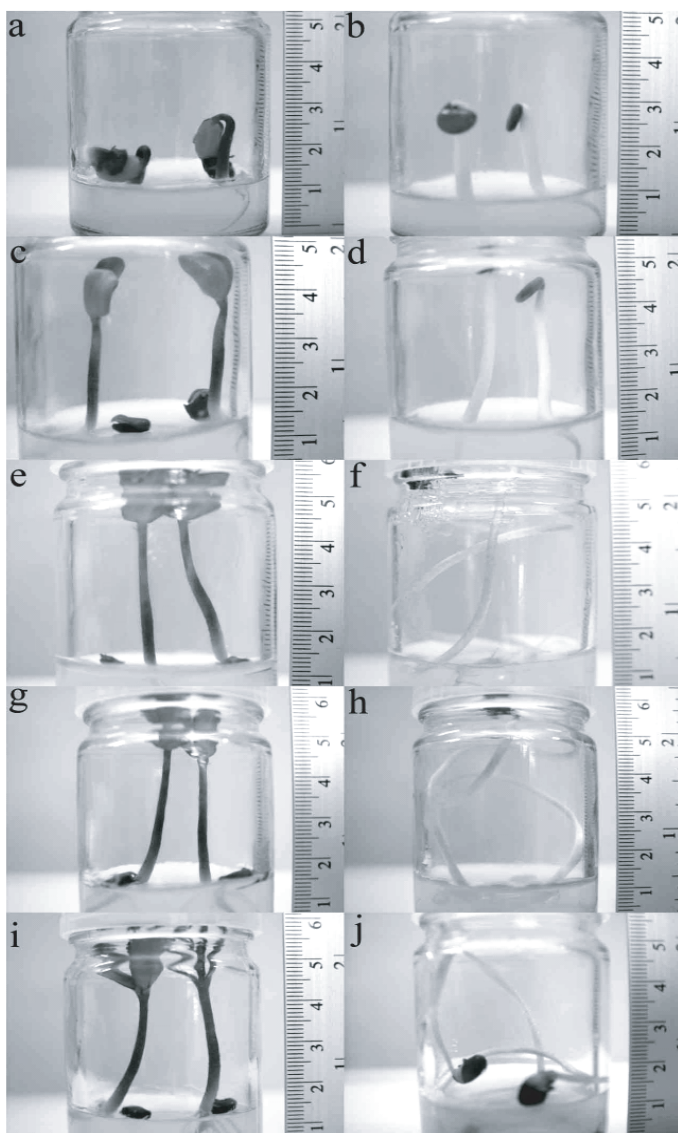


Fig. 1. Red Agati grown *in vitro* on 7, 10, 15, 20 and 25 days under light (a, c, e, g and i, respectively) and dark (b, d, f, h and j, respectively) conditions.

of the supernatant was read at 535 nm. Total anthocyanin content was calculated according to Fuleki and Francis (1968).

Anthocyanin localization: Hypocotyls of Red Agati seedlings grown *in vitro* under both light and dark conditions for 7, 10, 15, 20 and 25 days were cut independently with a razor blade. The free hand sections were then placed in a drop of water on a glass slide, covered with a cover slip and examined under a light microscope to localize the anthocyanin-containing cells.

Results

Morphology of Red Agati seedlings: Red Agati seeds, germinated *in vitro* developed normally into healthy seedlings in the light but not in the dark (Fig. 1). On day 7 in the light, the cotyledons started expanding and emerging from the seed coat while in the dark by day 25, the cotyledons still remained inside the seed coat. On day 10, true leaves could be found on some seedlings under light conditions. It was clearly seen that plant height was greater in the dark than in the light throughout the experiment (Fig. 2). Hypocotyls of dark-grown seedlings were white while those grown in the light were red. However, the red color appeared lighter as the hypocotyls grew.

Localization of anthocyanin in hypocotyl of Red Agati: When free hand sections of Red Agati hypocotyls, cultured under both light and dark conditions *in vitro*, were examined under a light microscope, it was revealed that anthocyanin could not be found in hypocotyls of dark-grown seedlings at all developmental stages. In contrast, anthocyanin was evidently present in the hypocotyls of light-grown seedlings (Fig. 3). The anthocyanin-containing cells were found in the epidermal and sub-epidermal layer. Besides, some could be seen in the peripheral cortex of the hypocotyl. The hypocotyl on day 7 appeared to have the highest number of anthocyanin-containing cells.

Total anthocyanin content in Red Agati hypocotyl: Total anthocyanin content was measured in hypocotyls of germinated seeds *in vitro* under both light and dark conditions for 7, 10, 15, 20 and 25 days. It was revealed that hypocotyls of light-grown seedlings had the highest anthocyanin content (290 µg/g FW) on day 7 (Fig. 4). Then total anthocyanin content decreased sharply. In contrast, anthocyanin were not detectable in hypocotyl of

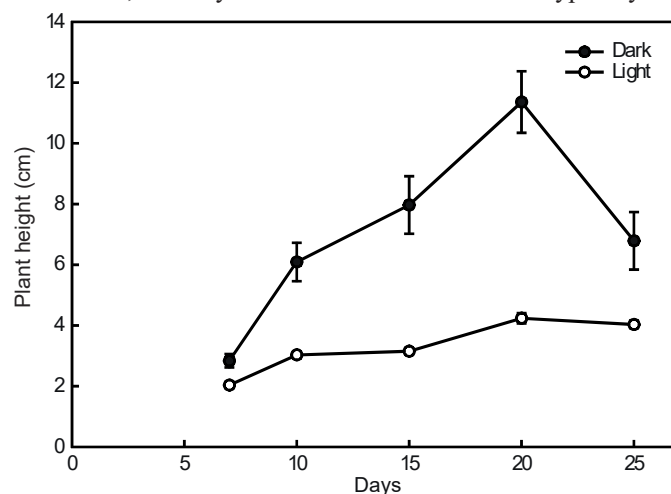


Fig. 2. Height of Red Agati grown *in vitro* at various developmental stages (values are means of 20 replications \pm SD). Value in day 25 dropped because explants mostly died and mean of this stage are included all of those 20 replications.

dark-grown seedlings (Fig. 4).

Total anthocyanin content in Red Agati petal: During Red Agati flower development, the petal increased in size and length. The petal of 3 cm in length had the highest total anthocyanin

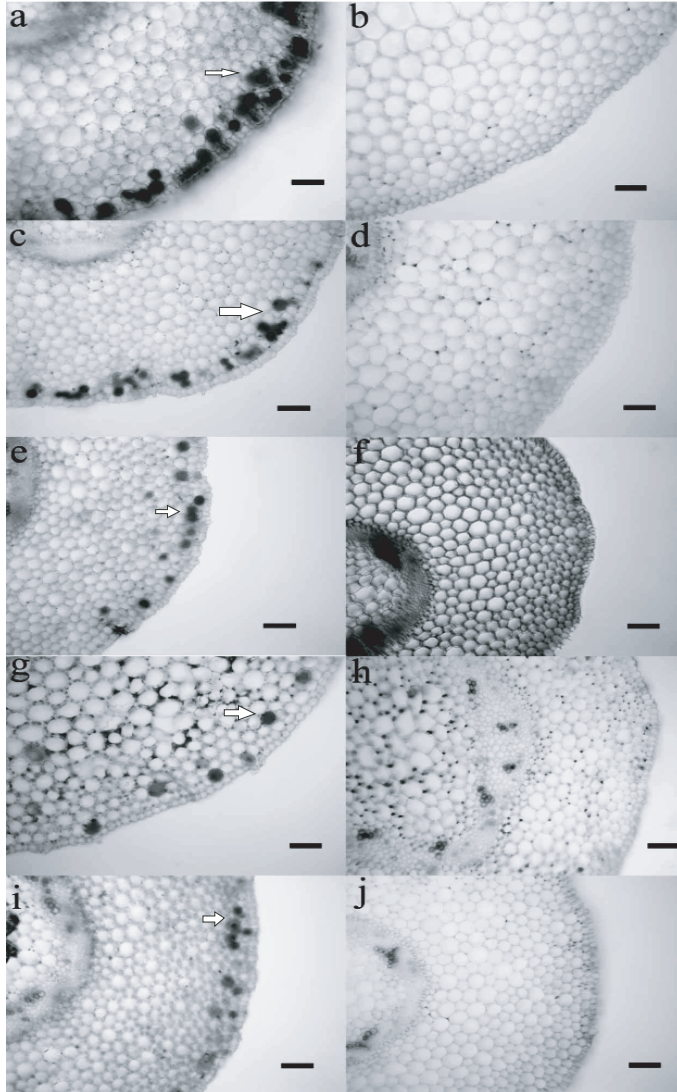


Fig. 3. Cross section of Red Agati's hypocotyl grown *in vitro* on 7, 10, 15, 20 and 25 days under light (a, c, e, g and i, respectively) and dark (b, d, f, h and j, respectively) conditions. Scale bar = 100 μm .

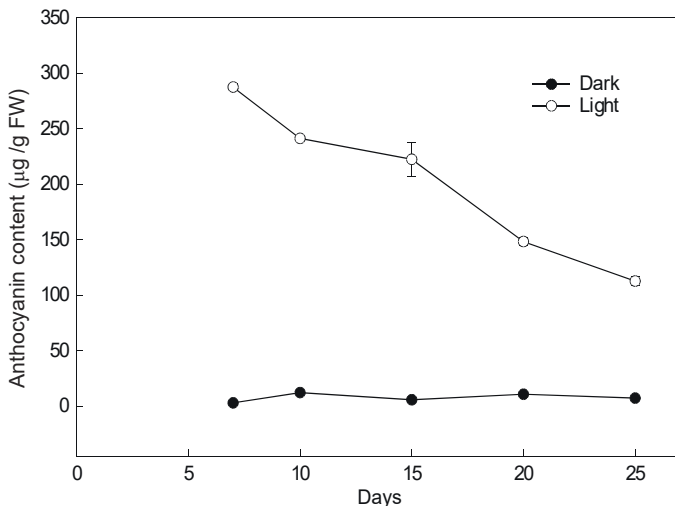


Fig. 4. Total anthocyanin content in hypocotyl of Red Agati at various developmental stages (values are means of 9 replications \pm SD).

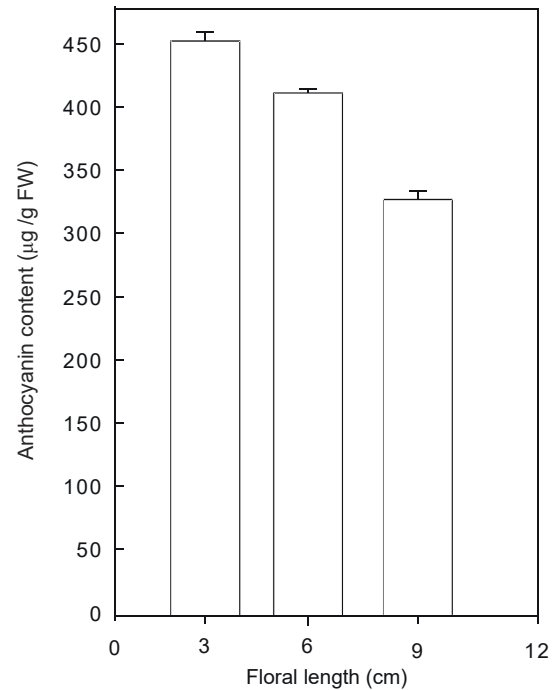


Fig. 5. Total anthocyanin content in petal of Red Agati at various developmental stages (values are means of 9 replications \pm SD).

content (455 $\mu\text{g/gFW}$). Total anthocyanin content obviously decreased when the petal length increased further (Fig. 5).

Discussion

The demand of natural anthocyanins has been growing due to their beneficial attributes for human health and well-being when compared to synthetic dyes (Mayer and van Staden, 1995; Stintzing and Carle, 2004). Though leaves and fruits from other plants are the good source of anthocyanins, such as, 188 and 375 $\mu\text{g/gFW}$ of total anthocyanin content were found in purple basil leaf and acerola fruit, respectively (Simon, *et al.*, 1999; Vendramini and Trugo, 2004), hypocotyl and flower of Red Agati could provide high amount of anthocyanins as well. In this research, total anthocyanin content of Red Agati in reproductive (petal) and nonreproductive (hypocotyl) tissues were investigated for the first time. Reddening was found in hypocotyl of Red Agati seed germinated under light condition. This phenomenon, the presence of a red color in seedling, was also discovered in several *Pinus* species and many dicotyledonous epigeous seedlings, especially hypocotyl part which developed a red pigmentation shortly after it emerges from the soil into light (Nozzolillo *et al.*, 2002).

In Red Agati hypocotyl, anthocyanin was observed in the epidermal and sub-epidermal layer and peripheral cortex. There have been many reports showing that localization of anthocyanin varies depending on the species. Mostly, anthocyanins are found in or just below the epidermis (Chalker-Scott, 1999; Nozzolillo *et al.*, 2002). However, Hara *et al.* (2003) found that the location of anthocyanin-containing cells in radish hypocotyls changed during growth. On day 14, these cells were seen in the peripheral cortex but they were mostly found in the pericycle cells on day 21. This finding differed from the present result as the distribution of anthocyanin-containing cells of Red Agati hypocotyl was still in the sub-epidermal layer and peripheral cortex and remained or unchanged throughout the experiment.

There have been a number of reports showing that light is an important factor for anthocyanin synthesis (Matsumoto *et al.*, 1973; Chalker-Scott, 1999). Our results are also in agreement with these studies. Nevertheless, callus culture of *Vitis* and strawberry produced large amounts of anthocyanin in the dark (Yamakawa *et al.*, 1983; Nakamura *et al.*, 1999).

Total anthocyanin content in Red Agati hypocotyl on day 7 under light conditions was comparable to that of the 3-cm long petal. Furthermore, the developmental changes in their total anthocyanin content appeared similar.

Anthocyanin accumulating pattern in Red Agati hypocotyl of seedlings grown under light and dark condition was totally different. The localization of anthocyanin were mostly found in sub-epidermal layer and peripheral cortex at all developmental stages. The early developmental stage of both hypocotyl and petal had the higher amount of total anthocyanin content than the late developmental stage. Thus from the present results, the suitable time to harvest Red Agati hypocotyls for anthocyanin extraction would be on day 7 after sowing seeds under aseptic conditions. Similarly, petal of 3 cm long would also be ideal. Although total anthocyanin content was less in hypocotyl than in petal, the former is more readily available and would be a more favorable source or an attractive alternative to petal of Red Agati to use for anthocyanin production. Further studies would be needed to examine if the anthocyanin profiles in the hypocotyl and petal of Red Agati are comparable.

References

- Bodhipadma, K. and D.W.M. Leung, 2002a. Factors important for somatic embryogenesis in zygotic embryo explants of *Capsicum annum* L. *J. Plant Biol.*, 45: 49-55.
- Bodhipadma, K. and D.W.M. Leung, 2002b. *In vitro* flowering of plantlets regenerated via somatic embryogenesis from immature zygotic embryo explants of *Capsicum annum* L. cv. Sweet Banana. *Phyton*, 42: 99-108.
- Bodhipadma, K. and D.W.M. Leung, 2003. *In vitro* fruiting and seed set of *Capsicum annum* L. cv. Sweet Banana. *In Vitro Cell. Dev. Biol. Plant*, 39: 536-539.
- Bray, R.A. 1994. Diversity within tropical tree and shrub legumes. In: *Forage Tree Legumes in Tropical Agriculture*, Gutteridge, R.C. & Shelton, H.M. (eds.), CAB International, Wallingford, UK, pp. 111-119.
- Chalker-Scott, L. 1999. Environmental significance of anthocyanins in plant stress responses. *Photochem. Photobiol.*, 70: 1-9.
- Detrez, C., S. Ndiaye and B. Dreyfus, 1994. *In vitro* regeneration of the tropical multipurpose leguminous tree *Sesbania grandiflora* from cotyledon explants. *Plant Cell Rep.*, 14: 87-93.
- Filippini, R., R. Caniato, A. Piovan and E.M. Cappelletti, 2003. Production of anthocyanins by *Catharanthus roseus*. *Fitoterapia*, 74: 62-67.
- Fuleki, T. and F.J. Francis, 1968. Quantitative methods for anthocyanins: 1. Extraction and determination of total anthocyanin in cranberries. *J. Food Sci.*, 33: 72-77.
- Gutteridge, R.C. 1994. The perennial *Sesbania* species. In: *Forage Tree Legumes in Tropical Agriculture*, Gutteridge, R.C. & Shelton, H.M. (eds.), CAB International, Wallingford, UK, pp. 49-64.
- Hara, M., K. Oki, K. Hoshino and T. Kuboi, 2003. Enhancement of anthocyanin biosynthesis by sugar in radish (*Raphanus sativus*) hypocotyl. *Plant Sci.*, 16: 259-265.
- Haw, A.B. and C.L. Keng, 2003. Micropropagation of *Spilanthes acmella* L., a bio-insecticide plant, through proliferation of multiple shoots. *J. Appl. Hort.*, 5: 65-68.
- Jensen, M. 2001. *Trees and Fruits of Southeast Asia - An Illustrated Field Guide*. Orchid Press, Bangkok, Thailand.
- Kalt, W. and D. Dufour, 1997. Health functionality of blueberries. *Hortic. Technol.*, 7: 216-221.
- Markham, K.R., K.S. Gould, C.S. Winefield, K.A. Mitchell, S.J. Bloor and M.R. Boase, 2000. Anthocyanic vacuolar inclusion – their nature and significance in flower colouration. *Phytochemistry*, 55: 327-336.
- Matsumoto, T., K. Nishida, M. Noguchi and E. Tamaki, 1973. Some factors affecting the anthocyanin formation by *Populus* cells in suspension culture. *Agr. Biol. Chem.*, 37: 561-567.
- Mayer, H.J. and J. van Staden, 1995. The *in vitro* production of an anthocyanin from callus cultures of *Oxalis linearis*. *Plant Cell Tiss. Org. Cult.*, 40: 55-58.
- Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 15: 473-497.
- Nakamura M., Y. Takeuchi, K. Miyanaga, M. Seki and S. Furusaki, 1999. High anthocyanin accumulation in the dark by strawberry (*Fragaria ananassa*) callus. *Biotech. Lett.*, 21: 695-699.
- National Research Council, 1979. *Tropical Legumes: Resources for the Future*. National Academy of Sciences, Washington D.C., USA.
- Nozzolillo, C., P. Isabelle, O.M. Andersen and M. Abou-Zaid, 2002. Anthocyanins of jack pine (*Pinus banksiana*) seedlings. *Can. J. Bot.*, 80: 796-801.
- Rao, S.R. and G.A. Ravishankar, 2002. Plant cell cultures: Chemical factories of secondary metabolites. *Biotech. Adv.*, 20: 101-153.
- Shanker, S. and H.Y.M. Ram, 1990. Plantlet regeneration from tissue cultures of *Sesbania grandiflora*. *Curr. Sci.*, 59: 39-43.
- Shanker, S. and H.Y.M. Ram, 1993. Aberrant chromosome numbers in the callus and regenerated shoot buds in *Sesbania grandiflora* (L.) Pers. *Phytomorphology*, 43: 75-80.
- Simon, J.E., M.R. Morales, W.B. Phippen, R.F. Vieira and Z. Hao, 1999. Basil: a source of aroma compounds and a popular culinary and ornamental herb. In: *Perspectives on new crops and new uses*, Janick, J. (ed.), ASHS Press, Alexandria, VA., pp. 499-505.
- Sinha, R.K. and R. Mallick, 1993. Effect of gamma-radiation on *in vitro* callus growth and regeneration of *Sesbania grandiflora* (L.) Poir. *Cytobios*, 76: 187-193.
- Stintzing, F.C. and R. Carle, 2004. Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. *Trends Food Sci. Tech.*, 15: 19-38.
- Thiengburanatham, W. 1993. *Dictionary of Flowering and Decorative Plants in Thailand*. Suriyaban Publisher, Bangkok, Thailand.
- Vendramini, A.L.A. and L.C. Trugo, 2004. Phenolic compounds in acerola fruit (*Malpighia puniceifolia* L.). *J. Braz. Chem. Soc.*, 15: 664-668.
- Wang, H., G. Cao and R.L. Prior, 1997. Oxygen radical absorbing of anthocyanins. *J. Agric. Food Chem.*, 45: 304-305.
- Wang, H., M.G. Nair, G.M. Strasburg, C.Y. Chang, A.M. Booren and J.I. Gray, 1999. Antioxidant and anti-inflammatory activities of anthocyanins and their aglycone, cyanidin, from Tart cherries. *J. Nat. Prod.*, 62: 294-296.
- Yamakawa, T., S. Kato, K. Ishida, T. Kodama and Y. Minoda, 1983. Production of anthocyanins by *Vitis* cells in suspension culture. *Agr. Biol. Chem.*, 47: 2185-2191.