

Structural development and bioactive content of red bulb plant (*Eleutherine americana*); a traditional medicines for local Kalimantan people

EVI MINTOWATI KUNTORINI¹, LAURENTIUS HARTANTO NUGROHO²

¹Biology Program, Faculty of Mathematics and Natural Sciences, Lambung Mangkurat University (UNLAM), Jl. Ahmad Yani Km 35.8, Banjarbaru 70714, South Kalimantan, Indonesia, Tel./Fax.: +62-511-4773868, ✉e-mail: evimintowati@yahoo.com
²Faculty of Biology, Gadjah Mada University (UGM), Yogyakarta 55284, Indonesia

Manuscript received: 23 September 2009. Revision accepted: 21 October 2009.

ABSTRACT

Kuntorini EM, Nugroho LH (2010) Structural development and bioactive content of red bulb plant (*Eleutherine americana*); a traditional medicines for local Kalimantan people. *Biodiversitas* 11 (2): 102-106. Red bulb plant or “bawang dayak” (*Eleutherine americana* Merr.) is commonly used as a traditional medicine especially for anti breast cancer of Kalimantan people, because it contains bioactive naphthoquinone-derivatives. Naphthoquinones is usually used as antimicrobial, antifungal, antiviral and antiparasitic agents. This study was aimed to complete the investigation of Red bulb plant as a traditional medicine from anatomical and phytochemical point of view, especially the anatomical structure of bulb and leaf of red bulb plants during their development and their naphthoquinone derivative contents. The anatomical structures of bulb and leaf were analyzed by paraffin embedding method and naphthoquinone derivative contents were analyzed using HPLC. During plant development, the diameter and the length of bulbs as well as the thickness of mesophyll and epidermis layers of the leaves increased significantly. The size of vascular bundles increased and so did phloem and xylem components. The content of bioactive compounds in bulbs increased significantly as the size of parenchymal cells increased. There was no significant difference in the content of bioactive compounds of leaves during development, despite the rise in the thickness of mesophyll and the constituting cells as the location of synthesis.

Key words: development, bulb, leaf, *Eleutherine americana*, naphthoquinone.

INTRODUCTION

Red bulb plant or “bawang dayak” (*Eleutherine americana* Merr.) has been widely used as traditional medicine. Bulbs of the plants have been used against breast cancer by local people of Kalimantan, against heart diseases, and function as immunostimulant, anti-inflammation, anti-tumor and, anti-bleeding agent (Sa'roni et al. 1987; Saptowalyono 2007). Studies demonstrated that bulbs of *Eleutherine* (*E. bulbosa* and *E. americana*) contain naphthoquinones (elecanacine, eleutherine, eleutherol, eleutherinone) (Hara et al. 1997; Alves et al. 2003; Jinzhong et al. 2006; Nielsen and Wege 2006; Han et al. 2008). Naphthoquinones, recognized to exhibit antimicrobial, antifungal, antiviral, and antiparasitic properties, and to demonstrate bioactivity as anti-cancer and antioxidant, are usually located within cell vacuoles in the form of glycosides (Herbert 1995; Robinson 1995; Babula et al. 2005).

Biosyntheses of secondary metabolites may take place in any cell and tissue. However, the syntheses usually occur in specific cell or tissue and depend on the level of differentiation and development of the plant. Consequently, plant structures play important roles in various aspects of life (Wink 1990; Utami 2007). During a preliminary study, microscopic examination of leaves and bulbs of red bulb

failed to reveal any secretory cell. Presumably, the bioactive compounds were hidden within vacuoles or cytosol.

This study aimed to learn more about the use of red bulb plant as a traditional medicine especially in the structure and development of leaves and bulbs of red bulb, describe and quantitatively analyze bioactive compounds in leaves and bulbs during plant development, and reveal the relationship between structural development and bioactive content in leaves and bulbs of red bulb.

MATERIALS AND METHODS

Red bulb plant used in the study originated from Banjarbaru, South Kalimantan, and was grown in a glass house. Leaf-less seedlings with bulb diameter of 0.3-0.5 cm were individually transferred to a polybag containing 3 kg of soil. Seedlings were also grown on open site, directly on the soil without polybag. Samples of leaves and bulbs were taken from glass-house plants every two weeks for six times: 2-12 weeks after planting (wap), representing treatments (observations) T₁-T₆. Control plants on open site were sampled at 4 (T₂) and 12 (T₆) wap. Samples T₂ and T₆ from open site and glass house were compared for bioactive contents. Single-stain paraffin embedding method

(Ruzin 1999) was applied in preparation of microscopic slides for examination of leaf mesophyll thickness, number of stomata, lower and upper epidermis thickness, as well as xylem and phloem of bulbs. Leaf clearing method of Berlyn and Miksche (1976) was applied in slide preparation for observation of the number of stomata. Bulb diameter and length and number of leaf were also recorded as supplementary data. The quantity of overall naphthoquinone-derived compounds was analyzed with High Performance Liquid Chromatography (HPLC), using vitamin K (phyloquinone) as a model. Qualitative data was described and displayed as figures, while quantitative data was exposed to ANOVA, followed by Duncan's Multiple Range Test (DMRT) with 5% significance level, or its equivalent nonparametric test.

RESULTS AND DISCUSSION

Leaf structure and development

On average, new shoots appeared within 14 days (Table 1). Insignificant difference between T_3 (3.4) and T_4 (3.8) was possibly due to inappropriate environmental condition which hindered the initiation of new leaves and delayed leaf initiation process. In a constant environment, leaf primordia appeared on the stem tip in a constant rate for a certain genotype. Time interval between successive leaf primordia is called plastochron. Temperature, light, and other factors have been demonstrated to affect plastochron development (Gardner et al. 1985).

Among treatments for mean thickness of both upper and lower epidermis revealed significant differences with Kruskal Wallis test (Table 1). They could be inferred the increase of the thickness on upper and lower epidermis layer during plant development. Leaf cross section showed uneven thickness in the two layers, thicker in some part but thinner in other part (Figure 1). Mauseth (1988) suggested that in Monocotyledoneae, e.g. Iridaceae and Liliaceae, leaves need longer time to develop. While the distal part of the leaf has fully developed and showed active photosynthesis, the basal part was still growing and developing. That indicates that during observation the epidermis was still growing, increasing in thickness as the plant getting older.

Mean number of stomata per mm^2 in upper epidermis showed significant difference (i.e. a decrease), during development (Table 1). This was related to the elongated shape of the cells of upper epidermis and to the cell growth. Cells of upper epidermis increased more in length rather than thickness (Akan et al. 2007; Satil and Selvi 2007). During cell extension up to T_4 , no stoma was formed, and consequently the mean number of stomata per mm^2 was altered. However, during T_4 - T_6 cell length was constant and accordingly the number of stomata was not significantly different. Cell elongation is a part of cell extension in which growth tends to occur towards one direction, usually along the plant axis (Fosket 1994). The number of stomata in lower epidermis differed significantly only between T_1 ($177.78/\text{mm}^2$) and T_2 ($262.22/\text{mm}^2$), while later observations did not show significant difference (Table 1). This indicated that as T_1 stomata were formed, subsequently epidermal cells grew through division and extension.

The mean number of stomata in lower epidermis was higher than that in upper epidermis with univariate t-tests, except at T_1 . Leaves of terrestrial plants usually have stomata only in lower epidermis. In herbs stomata can be found in both upper and lower surfaces, but higher quantity occurs in the lower epidermis. This represents an adaptation for prevention of excessive water loss due to exposure to sunlight. To control water loss through transpiration, plants tend to have fewer stomata in upper surface and more in lower surface (Tjitrosomo 1985; Salisbury and Ross 1995).

No significant difference was found between T_1 and T_2 in terms of mesophyll thickness ($43.9 \mu\text{m}$ and $46.7 \mu\text{m}$, respectively) (Table 1). This implies that cell division at the leaf basal meristem was still on going and more dominant than leaf cells enlargement. Mesophyll thickness did not differ significantly either between T_3 ($52.3 \mu\text{m}$) and T_4 ($52.2 \mu\text{m}$). During leaf development, red bulb plants could take some time to proceed from active division at basal meristem to enlargement of the cells. On the other hand, an increase in mesophyll thickness was observed between T_5 ($58.5 \mu\text{m}$) and T_6 ($66.9 \mu\text{m}$) indicating cell enlargement. In Monocotyledoneae, such as Iridaceae and Liliaceae, leaf growth takes a relatively long time. While distal part of leaf reaches maturity and hosts active photosynthesis, the basal part is still growing and meristematic (Mauseth 1988; Westhoff et al. 1998).

Table 1. Mean number of leaves, thickness of upper and lower epidermis, number of stomata on upper and lower epidermis, and thickness of mesophyll during red bulb plant development in glass house.

Weeks after planting (wap)	Number of leaves	Thickness of upper epidermis (μm)*	Thickness of lower epidermis (μm)*	Number of stomata on upper epidermis per (mm^2)	Number of stomata on lower epidermis per (mm^2)	Thickness of mesophyll (μm)
T_1	2	1.2 ^a	20.0	137.38 ^a	177.78 ^a	43.9 ^a
T_2	4	2.2 ^b	18.8	115.56 ^{ab}	262.22 ^b	46.7 ^a
T_3	6	3.4 ^c	17.7	93.33 ^{bc}	235.56 ^b	52.3 ^b
T_4	8	3.8 ^c	17.5	66.67 ^{cd}	244.44 ^b	52.2 ^b
T_5	10	4.6 ^d	21.5	62.22 ^d	240.00 ^b	58.5 ^c
T_6	12	5.8 ^e	24.3	57.78 ^d	244.44 ^b	66.9 ^d

Note: Numbers followed by similar alphabet within the same column are not significantly different in DMRT with = 5%; *: nonparametric Kruskal Wallis test

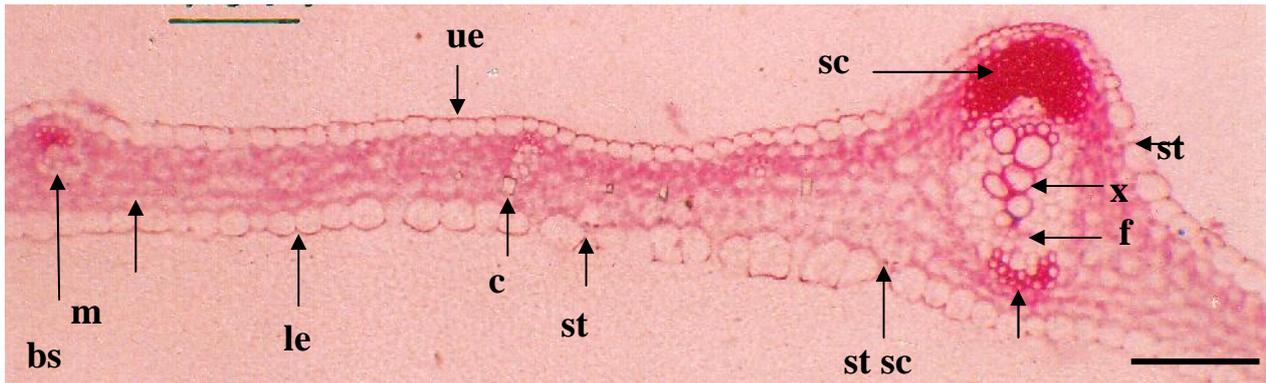


Figure 1. Cross section of a leaf of 12 wap plant grown in glass house. bs: bundle sheath; ue: upper epidermis; le: lower epidermis; f: phloem; c: crystal; st: stomata; sc: sclerenchym; x: xylem; m: mesophyll. Bar = 100 μ m.

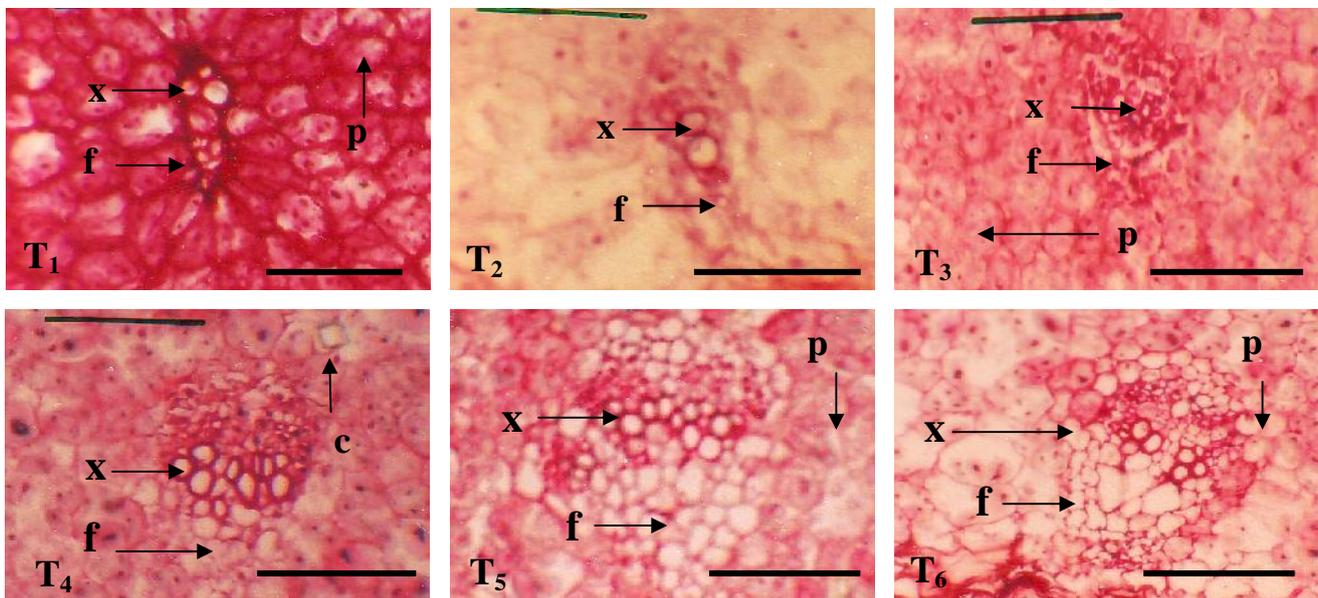


Figure 2. Cross section of vascular bundle in bulbs during plant development in a glass house. x: xylem; f: phloem; p: parenchymal cells; c: styloid crystal. T₁: 2 wap; T₂: 4 wap; T₃: 6 wap; T₄: 8 wap; T₅: 10 wap; T₆: 12 wap. (wap: weeks after planting). Bar = 100 μ m.

Bulb structure and development

Observable development of anatomical structure in bulbs showed differences in the size of vascular bundle and the constituent cells, particularly among T₁, T₂, and T₃ (Figure 2). During T₄-T₆ the size of vascular bundle did not noticeably differ, but there was an increase in the quantity of cells making up xylem and phloem. The vascular bundle was of collateral type where xylems are parallel with phloem without cambium.

Enlargement of cells of bulbs was predominantly towards one direction, i.e. elongation of bulb. Cell elongation is a part of cell extension in which growth proceeds towards one direction, usually along plant axis. Direction of the cell extension is also controlled by the orientation of cellulose microfibrils of the cell wall (Fosket 1994; Westhoff et al. 1998).

Bulbs are derived from thickening petioles in which the vascular bundles are similar to those in leaves. No

supportive tissue or sclerenchyma was found in bulbs. Between main vascular bundles in each layer of a bulb smaller vascular bundles were found with simpler structure and cell arrangement, parallel to each other in a cross section.

Microscopic examination of bulbs disclosed styloid crystals similar to those in leaves. Various shapes of crystal were found in plant cells. In higher plants, the most common crystal is calcium oxalate. Styloid crystals have long prismatic shape with two ends pointed. In cells, these crystals are solitary. Styloid is usually found in plants of the family Iridaceae, Agavaceae, Liliaceae and several others (Dickison 2000).

Bioactive compounds

The quantity of bioactive compounds in leaves did not differ significantly between T₂ and T₆, but so did in bulbs (Table 3).

Table 3. Mean quantity of bioactive compounds in leaves and bulbs at 4 (T₂) and 12 (T₆) wap

Observation	Bioactive compounds in leaves (area)		Bioactive compounds in bulbs (area)	
	Glass house	Open site	Glass house	Open site
	T ₂ (4 wap)	1528348 ^a	2336205 ^a	2830361 ^a
T ₆ (12 wap)	1722076 ^a	2888872 ^a	6278518 ^b	3876370 ^b

Note: Numbers followed by similar alphabet within the same column are not significantly different with $\alpha = 5\%$.

HPLC analysis confirmed that naphthoquinone-derived bioactive compounds were already detectable in leaves and bulbs since the first observation (T₁) with a retention time of 4.731 min. Biosyntheses of the naphthoquinone-derived bioactive compounds are chloroplast-related. In the present study, vitamin K (phyloquinone) was used as the benchmark compound. Phyloquinone is produced by higher plants and plays important role in photosynthesis (as the second electron acceptor in photosystem I). Photosystem I is located in thylacoid membrane, as a light-dependent system (Salisbury and Ross 1995; Sansuk 2002; Verberne et al. 2007). It is then assumed that phyloquinone synthesis begins during differentiation of chloroplast-forming cell to mesophyll tissue.

Factorial experiment proved that the titer of bioactive compounds in leaves at 4 wap (T₂) and 12 wap (T₆) were not significantly different both in glass house and open site (Table 3). The experiment showed no interaction between planting site and time of observation factors in their effect on the syntheses of the bioactive compounds. That means that during plant development, despite an increase in the thickness of mesophyll tissue, the quantity of the bioactive compounds remains insignificantly different. Then, the naphthoquinone-derived compounds are synthesized in leaves and subsequently transported to storage organs. Sansuk (2002) and Gross et al. (2006) pointed out that phyloquinone biosynthesis takes place in chloroplast membrane. Wink (1990) suggested that in most plant species biosynthesis takes place in a specific organ, and the product is then accumulated in different organs or throughout the plant body. Biosynthesis of secondary metabolites may take place in any tissue and cell; however most occur in a specific tissue or cell and largely depending on the level of differentiation and development. Manitto (1981) pointed out that a substance may be produced and used almost simultaneously, so that the concentration in an organ or organism remains constant.

Descriptive examination on anatomical structure of bulbs revealed that cells of parenchymal tissue at T₁, T₂, and T₃ were somewhat small with few starch granule-containing vacuoles (Figure 2). The structure of xylem and phloem vascular bundles was comparably simpler than that at later observations. That means that bulbs as storage organs were still in their early development. The cell size and the simplicity of vascular tissue components as the transport system for products of assimilation and other compounds explained the low accumulation of bioactive compounds. With increasing age of the plant at T₄, T₅ and

T₆, the size of parenchymal cells of bulbs increased, the size of vacuoles also increased, the vacuolar starch granules were more numerous and more variable, and the number of cells composing xylem and phloem vascular bundles likewise raised (Figure 2).

Factorial experiment involving two times of observation (T₂ and T₆) in glass house and open site disclosed significant difference in overall level of bioactive compounds in bulbs (Table 3). That indicated that in both glass house and open site there was an increase in bioactive compound contents of bulbs during plant development, although the maximum level was still unknown. The increase in the size of parenchymal cells of bulbs might be related to their function as the storage place for naphthoquinone-derived bioactive compounds. As no secretory cell was found in leaves and bulbs, it can be assumed that the bioactive compounds are synthesized in cytosol and subsequently stored in vacuoles. Babula et al. (2005) suggested that naphthoquinone is usually stored in vacuoles of plant cells in the form of glycoside, and can be found in a number of plants of the family Plumbaginaceae, Juglandaceae, Ebenaceae, Boraginaceae, and Iridaceae. Difference in planting site did not affect the synthesis of the bioactive compounds as the content was similar between those grown in glass house and open site at T₂ and T₆. Robbers et al. (1996) highlighted that synthesis of bioactive compounds in plants is influenced by 3 main factors: heredity (genetic make up), ontogeny (developmental stages) and environment. The hereditary factor causes quantitative and qualitative changes, while the other two factors cause mainly quantitative change. Synthesis of secondary metabolites is not a simple process; rather it is a complex process involving interaction among biosynthesis, transport, storage, and degradation processes. Those processes are gene-regulated. The influence of ontogeny is clearly visible on secondary metabolite syntheses in plants; usually there is an increase in the content with age of the plant. However, that also depends on the stage of the plant development. Finally, environmental conditions that may have an effect on secondary metabolite productions among others are climate, growing site, other plants, and planting method.

During plant development bioactive compounds are formed in leaf chloroplasts. The bioactive compounds are used in photosynthesis process at photosystem I. A certain amount of the photosynthesis product is used as precursors in syntheses of naphthoquinone-derived bioactive compounds which are accumulated in bulbs during development of the plant. It can be concluded that the syntheses and storage of secondary metabolites take place in different locations.

CONCLUSIONS

Between 2 and 12 weeks after planting, anatomical structure of leaves and bulbs of red bulb showed an increase in the diameter and the length of bulb, the thickness of leaf mesophyll as well as upper and lower epidermis layers, and the size of cells of parenchyma and

vascular bundles of bulbs. In addition to an increase in size, vascular bundles also showed an increase in the number of cells composing xylem and phloem. Upper epidermis of leaves hosts fewer stomata than the lower layer. The level of naphthoquinone-derived bioactive compounds in bulbs increased significantly during plant development, as the size of parenchymal cells increased. Meanwhile the level of naphthoquinone-derived bioactive compounds in leaves remained stable during plant development, despite the increase in thickness of mesophyll and the composing cells

ACKNOWLEDGEMENTS

The authors wish to thank Prof. Dr. Issirep Sumardi and Abdul Gafur for their supervision during the experiment and useful comments on the manuscript.

REFERENCES

- Akan H, Ismail E, Satil F (2007) The morphological and anatomical properties of endemic *Crocus leichtlinii* (D. Dewar) Bowles (Iridaceae) in Turkey. Pak J Bot 39: 711-718.
- Alves TMA, Helmut K, Carlos LZ (2003) Eleutherinone a novel fungitoxic naphthoquinone from *Eleutherine bulbosa* (Iridaceae). Mem Ins Oswaldo Cruz Rio de Janeiro 98: 709-712.
- Babula P, Mikelova R, Patesil D, Adam V, Kizek R, Havel L, Sladky Z (2005) Simultaneous determination of 1,4 naphthoquinone, lawsone, juglone and plumbagin by liquid chromatography with UV detection. Biomed Paper 149: 25.
- Berlyn GP, Miksche JP (1976) Botanical microtechnique and cytochemistry. Iowa State University Press, Ames.
- Dickison WC (2000) Integrative plant anatomy. Harcourt Academic Press, New York.
- Fosket DE (1994) Plant growth and development, a molecular approach. Academic Press, San Diego.
- Gardner FP, Pearce RB, Mitchell RL (1985) Physiology of crop plants. Susilo H (transl). UI Press, Jakarta.
- Gross J, Cho WK, Lezhneva L, Falk J, Krupinska K, Shinozaki K, Seki M, Herrman RG, Meurer J (2006) A plant locus essential for phylloquinone (vitamin K₁) biosynthesis originated from a fusion of four eubacterial genes. J Biol Chem 281: 17189-17196.
- Han AR, Min HY, Nam JW, Lee NY, Wiryawan A, Suprpto W, Lee SK, Lee KR, Seo EK (2008) Identification of a new naphthalene and Its derivatives from the bulb of *Eleutherine americana* with inhibitory activity on lipopolysaccharide-induced nitric oxide production. Chem Pharm Bull 56: 1314-1316.
- Hara H, Maruyama N, Yamashita S, Hayashi Y, Lee KH, Bastow KF, Chairul, Marumoto R, Imakura Y (1997) Elecanacin, a novel new naphthoquinone from the bulb of *Eleutherine americana*. Chem Pharm Bull 45: 1714-1716.
- Herbert RB (1995) Biosintesis metabolit sekunder. edisi dua. Srigandono B. (transl). IKIP Semarang Press, Semarang.
- Jinzhong X, Feng Q, Wenjuan D, Gexia Q, Naili W, Xinsheng Y (2006) New bioactive constituents fom *Eleutherine americana*. Chem J Chin 26: 320-323.
- Manitto P (1981) Biosynthesis of natural products. Ellis Horwood, London.
- Mauseth JD (1988) Plant anatomy. Benjamin/Cummings, Menlo Park.
- Nielsen LB, Wege D (2006) The enantioselective synthesis of elecanacin through an intramolecular naphthoquinone-vinyl ether photochemical cycloaddition. Org Biomol Chem 4: 868-876.
- Robbers JE, Speedle MK, Tyler VE (1996) Pharmacognosy and pharmacobiotechnology. Williams & Wilkins, Baltimore.
- Robinson T (1995) Kandungan organik tumbuhan tinggi. Penerbit ITB, Bandung.
- Ruzin SE (1999) Plant microtechnique and microscopy. Oxford University Press, Oxford.
- Sa'roni P, Nurendah, Adjirni (1987) Penelitian efek anti inflamasi beberapa tanaman obat pada tikus putih. Kongres Biologi Nasional VIII. Purwokerto, 8-10 Oktober.
- Salisbury BF, Ross CW (1995) Fisiologi tumbuhan. Jilid 2. Diah L, Sumaryono (transl). Penerbit ITB, Bandung.
- Sansuk K (2002) Method development for determination of phylloquinone in tobacco plants [Thesis]. Division of Pharmacognosy, Leiden University, Leiden.
- Saptowalyono CA (2007) Bawang dayak, tanaman obat kanker yang belum tergarap. www.kompas.com. [15 June 2007].
- Satil F, Selvi S (2007) An anatomical and ecological study of some *Crocus* L. taxa (Iridaceae) from the west part of Turkey. Acta Bot Croat 66: 25-33.
- Tjitrosomo SS (1985) Botani umum 2. Angkasa, Bandung.
- Utami D (2007) Menjadikan struktur dan perkembangan tumbuhan sebuah kajian yang menarik. Pidato Purna Tugas Guru Besar Anatomi Tumbuhan. Universitas Jenderal Soedirman, Purwokerto.
- Verberne MC, Bol JF, Lithorst HJ, Verpoorte R, Sansuk K (2007) Vitamin K1 accumulation in tobacco plant overexpressing bacterial genes involed in the biosynthesis of salicylic acid. J Biotech 128: 72-79
- Westhoff P, Jeske H, Jurgen G, Kloppstech K, Link G (1998) Molecular plant development from gene to plant. Oxford University Press, Oxford.
- Wink M (1990) Physiology of secondary product formation in plant. In Charwood BV, Rodes MJC (eds) Secondary products from plant tissue culture. Clarendon Press, Oxford.