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Microsporogenesis in *Rauvolfia serpentina* (L.) Benth ex Kurz (Apocynaceae): An evidence for dual cytokinesis in microspore mother cells

Balkrishna Ghimire¹, Bimal Kumar Ghimire² and Kweon Heo¹*

¹Department of Applied Plant Sciences, Kangwon National University, Chuncheon 200-701, South Korea.  
²Department of Applied Life Science, Konkuk University, Seoul 143-701, South Korea.

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*Rauvolfia serpentina* is being used worldwide for its extensive medicinal value. A very few studies were done about microsporogenesis and male gametophyte formations in *R. serpentina* and they lack in accurate information about it. The aim of the present study was to give accurate information regarding microsporogenesis and male gametophyte formations in *R. serpentina*. Results explain the most important characteristic features of *R. serpentina* that was tetrasporangiate anther, dicotyledonous type of anther wall formation, the occurrence of both successive and simultaneous cytokinesis during the meiosis in microspore mother cells, uninucleate and highly vacuolated glandular tapetum, tetrahedral, tetragonal and decussate pollen tetrad and three-celled mature pollen grain during shedding time. These results were compared and discussed with other previous studies about *Rauvolfia*. Some of the studies contradicted with each other in terms of anther development and microsporogenesis, within genus and even in the species *R. serpentina*. Through this study, we clarified all these controversial information of microsporogenesis and concluded that *R. serpentina* undoubtedly follows both successive and simultaneous cytokinesis. Appearance of both types of cytokinesis is common in *Rauvolfia*, even though simultaneous is prevalent.

**Key words:** *Rauvolfia serpentine*, Apocynaceae, microsporogenesis, simultaneous cytokinesis, successive cytokinesis.

**INTRODUCTION**

*Rauvolfia serpentina* (L.) Benth. ex Kurz is an erect, glabrous, perennial shrub, it is a good source of many important alkaloids of medicinal value (Alamgir and Ahamed, 2005). It is one of the approximately known 80 species of *Rauvolfia*, which are mainly found in tropical regions (Mabberley, 2008). Flowers borne in branched cyme and pentameroius except gynoecium, calyx synsepalous with imbricate lobes, corolla sympetalous forming a funnel shape with five limbs, and stamens are epipetalous. Fruit are drupe, tiny, oval, fleshy which turn a shiny purple-black when ripe (Cronquist, 1981; IUCN Nepal, 2004). *R. serpentina* or common serpentine plant has been recognized as antidotes to the stings and bites of insects and poisonous reptiles in different parts of India and Nepal (Vakil, 1955; IUCN Nepal, 2004). It has also been used as a febrifuge, as a stimulant to uterine contractions, for diarrhea, dysentery, insomnia and insanity (Vakil, 1955; Ahamed et al., 2002; IUCN Nepal, 2004).

The noteworthy contributions to the embryological studies of the Apocynaceae were made by Fyre and Blodgett (1905), Guignard (1917a,b), Anderson (1931), Schnarf (1931), Meyer (1938) and Rau (1940). While summarizing the embryological data on Apocynaceae, Davis (1966) mentioned the controversy concerning the nature of division of microspore mother cells and due to the occurrence of different types of embryogeny Apocynaceae termed as heterogeneous group by Maheshwari in 1971.

*Corresponding author. E-mail: laurus@kangwon.ac.kr. Tel: 82-33-250-6412. Fax: 82-33-244-6410.
Microsporogenesis is the production of microspores from microspore mother cells in plants. The existing typological convention for microsporogenesis in angiosperms recognizes two patterns i.e. simultaneous and successive, although intermediate patterns called modified simultaneous can also been seen (Sampson, 1969a,b). The successive division involves the formation of centrifugal cell plates, whereas simultaneous involves the formation of centripetal furrows, although this may not be always the same case (Furness and Rudall, 1999). Early developmental events in microsporogenesis are known to play role in pollen morphology and tetrad shape. Successive cytokinesis, particularly common in monocots (Rudall et al., 1997; Furness and Rudall, 1999) generally leads to tetragonal, decussate, T-shaped or linear tetrad. Simultaneous cytokinesis, on the other hand, is mostly associated with tetrahedral tetrad. This type is found in monocots, but is the rule in eudicots (Rudall et al., 1997; Furness and Rudall, 1999; Furness et al., 2002). Majority of genera in Apocynaceae follow simultaneous pattern of microsporogenesis (Davis, 1966; Johri et al., 1992). However, successive cytokinesis occurs in Apocynum androsaefolium, Holarrhenaa antidysentrica (Lattoo, 1974), Plumeria diffusa and Plumeria rubra (Chauhan, 1979). Simultaneous as well as successive cytokinesis has been reported in Catharanthus pusillus (Bhasin, 1971) and Rauvolfia canescene (Meyer, 1938). Maheshwari (1970) and Lamba (1976) mentioned only simultaneous cytokinesis in R. serpentina and same for Rauvolfia sumatrana by Lakshminarayana (1988). As mentioned above single genus Rauvolfia has been confirmed with diverse mode of microsporogenesis. In the same way, Bhasin (1971) and Lamba (1976) reported two-celled pollen in Catharanthus pusillus and R. serpentina respectively but three-celled pollen grains had been reported in R. serpentina (Maheshwari, 1970) and Lamba (1976) mentioned only simultaneous cytokinesis in R. serpentina and same for Rauvolfia sumatrana by Lakshminarayana (1988).

These inaccurate and contradicting information about microsporogenesis and number of cells in mature pollen during shedding in genus Rauvolfia facilitate us to re-examine the microsporogenesis and anther characters of R. serpentina. The purpose of this study was to present truthful information about microsporogenesis of R. serpentina and to produce a comprehensive understanding about microsporogenesis and other anther characteristics in Rauvolfia.

The flowers are bisexual, pink pedicels and calyx, white corolla, and inflorescence irregular corymbose cymes (Figure 1). Floral parts are arranged in pentameric except gynoecium (Figure 2B). Calyx synsepalous with imbricate lobes and persistent, corolla sympetalous forming a funnel with five limbs which will be deciduous after fertilization and stamens are epipetalous (Figures 1 and 2A-B). The ovary was superior, bicarpellary and bilocular. Each stamen appears basifixed and tetrastachyous anther (Figures 2A-B). Young flowers have very short style and stamen around the carpel (Figures 2A-B), but in open flowers, style extends its length and corolla grows into a long tube. Therefore, stamen is located in upper part of corolla tube.

Anther wall formation

The anther wall, prior to maturity, was four to five layered: an epidermis, an endothecium, one or two middle layers and a tapetum (Figure 3A-D). The primary parietal cell undergoes periclinal and anticlinal divisions and forms secondary parietal layer. The secondary parietal layer adjacent to epidermis again divides and produces endothecium and middle layers (Figure 3A). The middle layer further divides periclinally and becomes two layered structure (Figure 3B). The secondary parietal layer adjacent to sporogenous cells functions as tapetum without further division (Figure 3A-B). Thus, the anther wall formation is dicytocolous type in which only the outer secondary parietal layer takes part in the formation of middle layer (Figure 3A). Glandular tapetum surrounds sporogenous tissue (Figure 3C). The tapetal cells remain uninucleate throughout and some of them undergo a periclinal division resulting in a two-layered condition (Figure 3C). Their dense cytoplasm become vacuolated by the time tetrad are formed (Figure 3C). The glandular tapetum degenerates only after the pollen reaches the two-celled stage. The middle layer is ephemeral and degenerates soon while the endothecium develops fibrous thickenings (Figure 4F-G). The anther epidermal cells are nearly collapsed at the time of pollen shedding. Thus, the mature anther wall is composed of a sole fibrous endothecium layer (Figure 4G).

RESULTS

Samples ranging from small buds to open flowers of different developmental stages were collected and fixed in FAA (5 parts formalin: 5 parts glacial acetic acid: 90 parts 50% ethanol) for one week and then preserved in 50% ethanol. Preserved buds and flowers were dehydrated by ethanol series (50, 70, 80, 90 and 95% and absolute ethanol). After dehydration, buds and flowers were embedded in Technovite 7100 resin and histo blocks were prepared. Serial sections of thickness 4 to 5 μm were cut by rotary microtome using a disposable knife. Staining was performed by 0.1% Toluidine blue O and stained slides were mounted with Entellan. Some flowers were also dehydrated by tertiary butyl alcohol series and embedded in paraplast of melting point 56°C. Serial sections of thickness 6 to 8 μm were cut and dried on the slide warmer. Slides were stained with Heidenhain’s haematoxylin, safranin and fast green FCF and mounted with Entellan. All prepared slides were observed with the BX-50 light microscope (Olympus Co, Japan). Photographs were taken by digital camera system attached to the microscope.

MATERIALS AND METHODS

References:

Bhasin (1971) and Lamba (1976) mentioned only simultaneous cytokinesis in R. serpentina and same for Rauvolfia sumatrana by Lakshminarayana (1988). As mentioned above single genus Rauvolfia has been confirmed with diverse mode of microsporogenesis. In the same way, Bhasin (1971) and Lamba (1976) reported two-celled pollen in Catharanthus pusillus and R. serpentina respectively but three-celled pollen grains had been reported in R. serpentina (Maheshwari, 1970) and Lamba (1976) mentioned only simultaneous cytokinesis in R. serpentina and same for Rauvolfia sumatrana by Lakshminarayana (1988).

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Microsporogenesis and male gametophyte

The primary sporogenous cells directly function as microspore mother cells. The microspore mother cells undergo two consequent meiosis divisions and form microspore tetrad. Cell division of microspore mother cell begins with early prophase I and ends with late telophase II passing through metaphase I, anaphase I, telophase I, prophase II, metaphase II and anaphase II (Figures 3E-L and 4A-D). During meiosis II, the spindles oriented parallel or right angles to each other (Figure 4A) forming microspore tetrads (Figure 4D). Cytokinesis in microspores follows both successive (Figures 3J-K) and simultaneous (Figure 4B-C) types although later one was more frequent than former. In successive cytokinesis, cell plate was laid down immediately after the telophase of first meiotic division and another in each of the two daughter cells after the second meiotic division (Figure 3J-L). On the other hand, no cell plate was laid down after meiosis I, and the spindle fibers of this division disappear during the metaphase of meiosis II (Figure 4A). After the four daughter nuclei have become organized, they assumed a tetrahedral arrangement and spindle was reformed between every two nuclei (Figure 4B). It is followed by formation of constriction furrows which start at the periphery and proceed inward until they meet at the center, so that there is simultaneous division of protoplast into four cells microspores (Figure 4C). The resultant microspores arranged themselves in tetrahedral, tetragonal and decussate fashion (Figure 4D). The microspores soon separated from each other and individually developed into pollen grains. The mature pollen grains are triporate surrounded by exine and intine (Figure 4H) and they are three-celled, one large vegetative cell and a central generative cell with two nuclei during shedding (Figure 4I). The dehiscence of
Figure 2. Young flower of *R. serpentina*. (A) Longitudinal section of young flower showing sepal, petal, stamens and carpel (arrow indicates the epipetalous stamen). (B) Transverse section of young flower with different parts arranged in whorl. Abbreviations: ca, carpel; pe, petal; se, sepal; st, stamen. Scale bars indicate 100 µm in Figure 2A and 50 µm in Figure 2B.

Figure 3. Anther wall formation and meiosis I in microspores: (A) Transverse section of young anther of *R. serpentina* showing dicotyledonous type of wall formation. (B) Anther wall showing periclinal division of middle layer. (C) Uninucleate tapetal cells with large vacuole. (D) Five-layer anther wall with glandular tapetum. (E-F) Prophase I in microspore mother cell. (G) Metaphase I. H-I Anaphase I early and late, respectively. J Telophase I. K-L Successive cytokinesis in microspore. Abbreviations: et, endothelium; ep, epidermis; ml, middle layer; pmc, pollen mother cell; t, tapetum. Scale bars indicate 10 µm in all figures.
anther takes place through a longitudinal slit (Figure 4F). Firstly, suture of between two microsporangia in an anther lobe detaches, soon after pollens released from microsporangium through a common slit formed in between two microsporangia of the same lobe (Figure 4G).

**DISCUSSION**

**A summary of anther and microsporogenesis of *R. serpentina***

Previous studies about *Rauvolfia* lacked in terms of detailed about microsporogenesis and anther wall formation (Maheshwari, 1970; Lamba, 1976; Lakshminarayan, 1988). Present study has clarified many distinguished characteristics features of anther and microsporogenesis, like anther tetrasporangiate; anther wall prior to maturation consists up to five cell layers; anther wall formation dicotyledonous type; anther epidermis nearly collapsed; middle layer degenerated; tapetum glandular, usually tapetal cells uninucleate; cytokinesis in microspore mother cell follows both successive and simultaneous types; microspore tetrads are tetrahedral, tetragonal and decussate in shape; pollen grains are tricolpate, three-celled during shedding time and anther dehiscence by longitudinal slit.

**Comparisons within apocynaceae**

The majority of the anther and microspore characteristics observed in *R. serpentina* are coincides with general features of Apocynaceae. Tetrasporangiate anther found in *R. serpentina* is also found in most of the Apocynaceae. However, Sud (1984) confirmed bisporangiate anther in *Trachelospermum fragrans*. The development of anther wall in *R. serpentina* is obviously dicotyledonous type (Lakshminarayana, 1988) and prior to maturity it is multilayered, a single middle layer later divides to two. Precisely same pattern has been seen in *Holarrhena antidysenterica* (Lattoo, 1974), *Vallaris heynei* (Rau, 1940) and 2-3 middle layers reported in *T. fragrans* (Sud, 1984). This is contrary to Maheshwari (1971) who reported a 10-14 middle layers in *V. foetida*. Some cells of the tapetum also divide, and at places it becomes two-layered. Such a condition has also been reported by Lattoo (1974) in *H. antidysenterica* and by Maheswari (1964) in species of subfamily Asclepiadoideae (formerly known as a separate family Asclepiadeae) for example *Cynanchum callialata*, *Pergularia daemia* and *Tylophora indica*. The most important characteristic in *R. serpentina* found in this study was both successive and simultaneous cytokinesis during the meiosis of pollen mother cells. Similar characteristics can be seen in other Apocynaceae members like, *Catharanthus pusillus* (Bhasin, 1971) and *R. canescence* (Meyer, 1938). But
previous studies in *R. serpentina* (Maheshwari, 1970; Lamba, 1976) and *R. sumatrana* (Lakshminarayana, 1988) described only simultaneous cytokinesis which should be revised at this time. Only simultaneous cytokinesis in microspore mother cells has been reported in *T. fragrans* (Sud, 1984) whereas only successive cytokinesis has been found in *H. antidisponentica* (Lattoo, 1974). Apart from Apocynaceae both successive and simultaneous cytokinesis in a same species has been observed in *Vernonia cinerea* (Asteraceae) by Dakshini and Dadlani (1978) and in one cultivar of *Codiaeum variegatum var. pictum* (Euphorbiaceae) by Albert et al. (2009). It is rare for a species to have both types of cytokinesis and specially highly unusual for eudicots species, since in higher eudicots, cytokinesis is uniformly described as simultaneous (Longly and Waterkeyn, 1979; Blackmore and Barnes, 1988; Otegui and Staehelin, 2000; Ressayre et al., 2000) with few exceptions e.g. Porteaceae (Blackmore and Barnes, 1995) *Rafflesia* (Rafflesiaceae) (Furness and Rudall, 2004) and Podostemaceae (Furness and Rudall, 2004; Jager-Zurn et al., 2006). In contrast to eudicots, cytokinesis is highly labile in basal angiosperms and monocots (Furness and Rudall, 1999; Furness et al., 2002). *R. sumatrana* pollen grains are shed at the three-celled stage like as in most other Apocynaceae. However, anthesis at two-celled stage has been reported by Bhasin (1971) in *C. pusillus* and by Lamba (1976) in *R. serpentina*. Present study results confirm Lamba’s (1976) study on same species contradict in two important embryological features, that is, cytokinesis of microspores and number of cells in mature pollen grains. Three-celled pollen grains feature had already been reported in *R. serpentina* (Maheshwari, 1970) which is confirmed in *R. sumatrana* (Lakshminarayana, 1988) and *R. serpentina* (this study). In general, the mode of cytokinesis, shape of tetrad and number of cells in mature pollen are considered as the most consistent characters in generic level, in some cases within the family level as well (Tobe, 1989). Within monocotyledon microsporogenesis is a significant character at the ordinal level and is usually (but not always) consistent within a family (Furness and Rudall, 1999; 2000). But surprisingly, earlier reports on these characters in genus *Rauvolfia* vary with each other (Maheshwari, 1970; Lamba, 1976; Lakshminarayana, 1988) even within same species *R. serpentina* (Maheshwari, 1970; Lamba, 1976). However, from this study we are clear about these two features in *R. serpentina* that: cytokinesis followed by both types which has also been reported for another species *R. canescence* by Meyer (1938) and pollen grain released at three celled stage also reported by Maheshwari (1970) for *R. serpentina* and by Lakshminarayana (1988) for the *R. sumatrana*.

In conclusion our result is more consistent and clear about the most of anther characteristics of *R. serpentina*. *R. serpentine* undoubtedly follows both type of cytokinesis, even though simultaneous is predominant and pollen grain shed at three celled stage. Through this study we clarified all these controversial information on microsporogenesis and pollen grain concerning to genus *Rauvolfia*. As we might articulate from previous information and this study microsporogenesis is highly diverse in family Apocynaceae. However, further studies are required on other genera of Apocynaceae before to make any comment on definite evolutionary significance and diversity of microsporogenesis.

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