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# Study of male sterility in *Taiwania cryptomerioides* Hayata (Taxodiaceae)

**S.-H. Chen**[1,2,*], **N.-J. Chung**[3], **Y.-N. Wang**[3,4], **C.-L. Lee**[1], **Y.-L. Lee**[1], and **P.-F. Tsai**[1]

[1] Department of Life Science, National Taiwan University, Taipei
[2] Institute of Ecology and Evolutionary Biology, National Taiwan University, Taipei
[3] Experimental Forest, National Taiwan University, Nantou
[4] Department of Forest and Resource Conservation, National Taiwan University, Taipei

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**Summary.** A study of male sterility over a period of three consecutive years on a conifer species endemic to Taiwan, *Taiwania cryptomerioides* Hayata (Taxodiaceae), was done for this article. With the aids of fluorescence and electron microscopic observations, the ontogenic processes in the fertile and sterile microsporangia are compared, using samples collected from Chitou Experimental Forest and Yeou-Shoei-Keng Clonal Orchard of the National Taiwan University, Nantou, Taiwan. The development of male strobili occurred from August to the end of March. Microsporogenesis starts with the formation of the archesporium and ends with the maturation of 2-celled pollen grains within the dehiscing microsporangium. Before meiosis, there was no significant difference in ultrastructure between the fertile and sterile microsporangia. Asynchronous pollen development with various tetrad forms may occur in the same microsporangium of either fertile or sterile strobili. However, a callose wall was observable in the fertile dyad and tetrad, but not in the sterile one. After dissolution of the callose wall, the fertile microspores were released into the locule, while some sterile microspores still retained as tetrads or dyads with intertwining of exine walls in the proximal faces. As a result, there was no well developed lamellated endexine and no granulate ectexine or intine in the sterile microspores. Eventually, the intracellular structures in sterile microspores were dramatically collapsed before anthesis. The present study shows that the abortion in pollen development is possibly attributed to the absence of the callose wall. The importance of this structure to the male sterility of *T. cryptomerioides* is discussed.

**Keywords:** *Taiwania cryptomerioides*; Male sterility; Microsporogenesis; Pollen development; Callose wall.

## Introduction

*Taiwania cryptomerioides* is an endemic, rare, and extant species to Taiwan. It was discovered by Konishi in 1904 at an altitude of about 2000 m in Nantou County, Taiwan, and was nomenclatured by Hayata as a new genus and species in 1906 (Hayata 1906). Later, it also was found in southern China, Burma, and northern Vietnam, but in a sparsely distributed manner (Wang and Yang 2002). The fossils have been found in Northeastern China (Koidzumi 1942) and Japan (Miki 1954). These indicate that this plant is disjunctive in distribution. Nowadays, *T. cryptomerioides* is identified as one of the classic Tertiary relic gymnosperms, along with *Sequoiadendron*, *Metasequoia*, and *Ginkgo* species.

In Taiwan, the majority of *T. cryptomerioides* are distributed in the central ranges of the island, usually occurring between broadleaved subtropical forest and temperate cloud forest at an elevation ranging from 600 m to 2800 m. It usually appears in association with other endemic conifers, such as *Chamaecyparis formosensis* and *C. obutsa* var. *formosana*. (Huang 1994, Wang and Yang 2002). Although *T. cryptomerioides* plants have been found at several localities in Taiwan, their abundance at each locality is very low. A conjunction of unfavourable factors resulting from environmental perturbations and specific biological features are thought to promote extinction vulnerability (Terborgh 1974).

*Taiwania cryptomerioides* belongs to the family Taxodiaceae. Embryological observations in this species were first made by Sugihara (1941). There have been studies on the morphology of male strobili (Kung and Kiang 1969), development of strobili (Wang et al. 1969), pollen germination (Kung et al. 1969), pollen morphology (Huang 1972), ultrastructure of pollen (Roscher 1975, Xi and Wang 1989), and the life cycle (Liu and Su 1983) of this plant.

Due to its very good wood quality, relatively rapid growth rate, and high resistance to insect infection, *T. cryptomerioides* has been selected as an important species for silvicul-

* Correspondence and reprints: Department of Life Science, National Taiwan University, 106 Taipei, Taiwan.
E-mail: suchen@ntu.edu.tw

ture in middle altitude mountain areas of Taiwan (Kung 1974). For this purpose, a clonal orchard at Yeoi-Shoei-Keng, the Experimental Forest of the National Taiwan University, was established with grafting in 1966 to obtain proper seed sources for reforestation (Kung and Gong 1966). However, there was no filled seed (Chung et al. 1998) or fertile pollen (Hsu et al. 2002) obtained in this clonal orchard. In contrast to this, fertile pollen grains have been obtained in an artificial forest of *T. cryptomerioides* in the Chitou Experimental Forest of the National Taiwan University at an altitude 300 m higher than that of the Yeoi-Shoei-Keng clonal orchard (Liu and Su 1983). Lin and Kuo (2004) assumed that the reason of the failure of seed production was due to the abortion of male strobili in the orchard which might be related to a higher temperature in winter (3 °C higher) during strobilus development.

Some investigations of conifers indicate that pollination is the most important prezygotic factor controlling reproductive success (Owens et al. 1990a, b; Owens and Morris 1998). Indeed, in some species, the presence of viable and functional pollen grains in the ovule is necessary for the development of female reproductive structures (Owens et al. 1990b, Wilson and Owens 1999). Therefore, the understanding of abortion of male strobili of *T. cryptomerioides* becomes important for silviculture in Taiwan.

Male sterility has been extensively reported in higher plants, including herbaceous or tree angiosperms (Rutledge 1998). It may be caused by an alteration during microsporogenesis, such as meiotic defects (Pichot and Maâtaoui 2000, Maâtaoui and Pichot 2001), alteration in mitochondrial genome (Abad et al. 1995), tapetal abnormality (Bino 1985a, b), absence of callose wall and fault timing of callase activity (Tsuchiya et al. 1995), and/or alteration of quantity and quality of protein and enzyme (Kaul 1988). However, only little is known about the viability and sterility of gymnosperm pollen (Andersson 1947, Orr-Ewing 1978, Taira 2000, Pichot and Maâtaoui 2000, Maâtaoui and Pichot 2001, Parantainen and Pulkkinen 2002, Wilson and Owens 2003). For *T. cryptomerioides*, the reason for male sterility is still unknown. In the present study, we examine the ultrastructural changes of microspores during microsporogenesis of this plant in order to compare the differences between fertile and sterile ones. For this purpose, the study was done from autumn 2001 to spring 2003, employing both fluorescence and transmission electron microscopy.

## Material and methods

The fertile and sterile male strobili of *Taiwania cryptomerioides* were collected weekly from Chitou Experimental Forest (23°40′N, 12°47′E, 1050 m alt.) and Yeou-Shoei-Keng Clonal Orchard (23°42′N, 12°47′E, 750 m alt.), National Taiwan University, Nantou County, respectively, over the period from September of 2001 to April of 2003. The samples were fixed immediately in 1% glutaraldehyde solution after collection in the field. During fixation, samples were degassed occasionally, each time for 15 min, until the samples were totally immersed in fixing solution. They were then stored at 4 °C for further study.

For sectioning, samples were transferred and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) at 4 °C for 2 days, then rinsed in 0.1 M phosphate buffer and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 2 days. Subsequently, samples were dehydrated through an alcohol series and embedded in Spurr resin (Spurr 1969) and hardened at 60 °C for 24 h. Semithin sections (0.5 μm) were stained with toluidine Blue O in 1% borate buffer (Roland and Vian 1991). Ultrathin sections (90 nm) were sectioned on an ultramicrotome (Reichert Ultracut E), and subsequently were stained with 2% ethanolic uranyl acetate for 25 min and 0.2% aqueous lead citrate for 6 min (Reynolds 1963). The stained specimens were examined either with a Hitachi H-600 (75 kV) or with a Hitachi H-7100 (75 kV) transmission electron microscope (TEM).

To verify the deposition of callose wall, the tetrads from fertile and sterile microsporangia were squeezed and stained with aniline blue (Jensen 1962) before viewing by fluorescence microscopy.

## Results

Under observation with TEM, there was no significant difference in ultrastructures between fertile and sterile microsporangia before the occurrence of meiosis. A remarkable difference between them was first observed after this stage. By staining with aniline blue for the callose wall, all of the tetrads in the fertile microsporangia exhibited stark fluorescence, showing a positive staining reaction (Fig. 1A–C), while only 42% of those in the sterile microsporangia gave a positive reaction (Fig. 1D). In addition, the intensity of fluorescence for those in the sterile microsporangia was very weak, suggesting a reduction in the amount of callose wall (Fig. 1E, F).

In fertile microsporangia, a callose wall was observed between the plasmalemma and the original cellulosic wall of the microsporocyte. Until the end of meiosis II, the callose wall expanded centripetally to four separate microspores (Fig. 2A). Simultaneous cytokinesis proceeded along the planes defined by the intersection of vesicles at the microspore interfaces (Fig. 2A). Those tetrad-microspores were characterised by a richness in the cytoplasm containing prominent amyloplasts and a nucleus with a clear nuclear membrane (Fig. 2A). In contrast to this, the majority of tetrads in the sterile microsporangia were enclosed by a very thin callose wall, or none at all, with a moderate amount of vesicles and a reduced cytoplasm with many electron-dense oil particles (Fig. 2B).

Different types of tetrads were observed in both the fertile and sterile microsporangia, including tetrahedral, tetragonal, linear, and T-shape tetrads. The tetrahedral type occurred

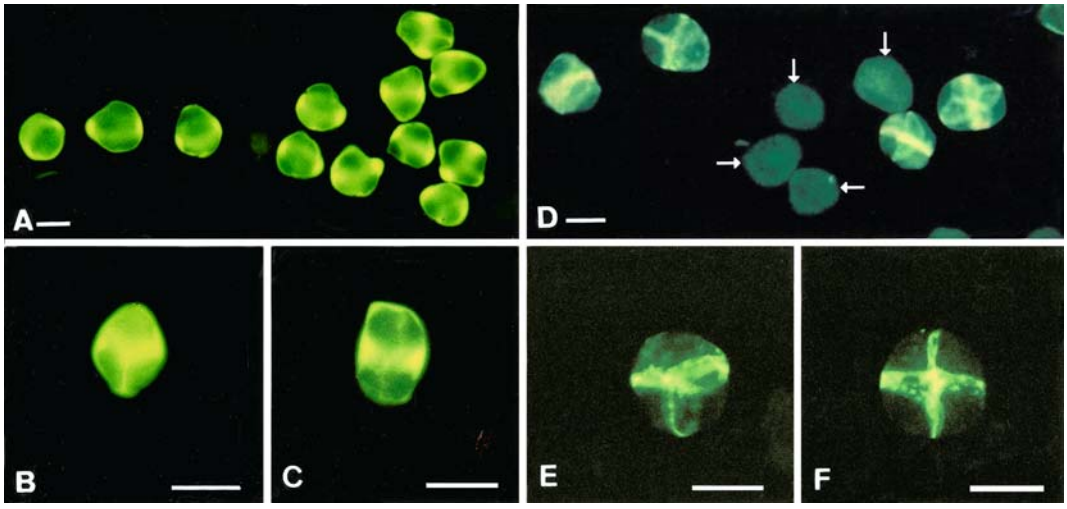


**Fig. 1.** Callose at the tetrad stage stained with aniline blue for fluorescence microscopy in fertile (**A–C**) and sterile (**D–F**) microsporangia of *Taiwania cryptomerioides*. **A** In fertile microsporangia, all different types of tetrads demonstrate stark fluorescence of the callose wall. **B** A tetrahedral tetrad with thick and homogeneous callose wall. **C** A tetragonal tetrad with thick and homogeneous callose wall. **D** In sterile microsporangia, some tetrads demonstrate fluorescence, while the others show weak or no fluorescence (arrows). **E** A tetrahedral tetrad with tenuous and heterogeneous callose wall. **F** A tetragonal tetrad with tenuous and heterogeneous callose. Bars: A and D, 20 μm; B, C, E, and F, 5 μm

most frequently, with the least frequently occurring type being the T-shape tetrad (data not shown). The sterile tetrads did not differ significantly from the fertile ones in the relative amount of these tetrad structures.

After the dissolution of the callose wall in the microsporangia, the fertile microspores were released into the locules. At that time, the fertile microspores were still rich in cytoplasm with prominent granules of amyloplast and only a few vacuoles in the cytoplasm (Fig. 2C). The microspores at this stage exhibited a well developed, lamellated endexine, granulate ectexine, and a clear germinal zone (Fig. 2C). The sterile microspores, in contrast, were characterised by a rudimentary ectexine and endexine, and remarkably reduced cytoplasm with numerous swollen and degenerating organelles, and some electron-dense lipid bodies (Fig. 2D). In addition, double nuclei in sterile microspores occurred occasionally. Numerous fragments of cellular constituents were observed in certain nuclei, possibly resulting from abnormal meiosis. In the locule of the sterile microsporangia, numerous visible debris were observed, which may have been the remains of the collapsed cytoplasmic structures of abnormal microspores (Fig. 2D).

At the subsequent stage, the fertile microspores were rounded up due to vacuolation and exhibited visible accumulation of the intine on the wall (Fig. 3A). The granules of amyloplast were still visible. In sterile microsporangia, on the contrary, some microspores demonstrated empty contents, some were with reduced cytoplasm and abnormally condensed chromatins (Fig. 3B). No intine was accumulated in most of the sterile microspores. In addition, certain sterile microspores still remained as tetrads with intertwining of parts of the exine walls in the proximal faces (Fig. 3B).

Like in many other members of the Taxodiaceae (Moitra and Bhatnagar 1982), no prothallial cells are formed in *T. cryptomerioides*. The microspore nucleus directly functions as antheridial initial and further divides into a small antheridial cell and a large tube cell (Fig. 3C) with an intine thicker than that at the previous stage. In the sterile microsporangia, the majority of pollen exhibited neither a well developed exine nor a regularly accumulated intine (Fig. 3D). Eventually, the intracellular structures were dramatically collapsed. As a result, numerous debris were revealed in the sterile microsporangia.

## Discussion

Male sterility in angiosperms has been well studied, such as in *Arabidopsis thaliana* (Regan and Moffatt 1990, Zhang et al. 2002), tobacco (Worrall et al. 1992), tomato (Tsuchiya et al. 1995), maize (Mariani et al. 1992), *Brassica napus* (Grant et al. 1986, Shukla and Sawhney 1994), *Petunia hybrida* (Izhar and Frankel 1971; Bino 1985a, b; Hanson 1991), and *Beta vulgaris* (Majewska-Sawka et al. 1993), and results from genetic, morphological, or biochemical abnormality have been reported (Kaul 1988, Raghavan 2000, Shivanna 2003). Natural male sterility has been reported in a few conifer species, including *Picea abies* (Andersson

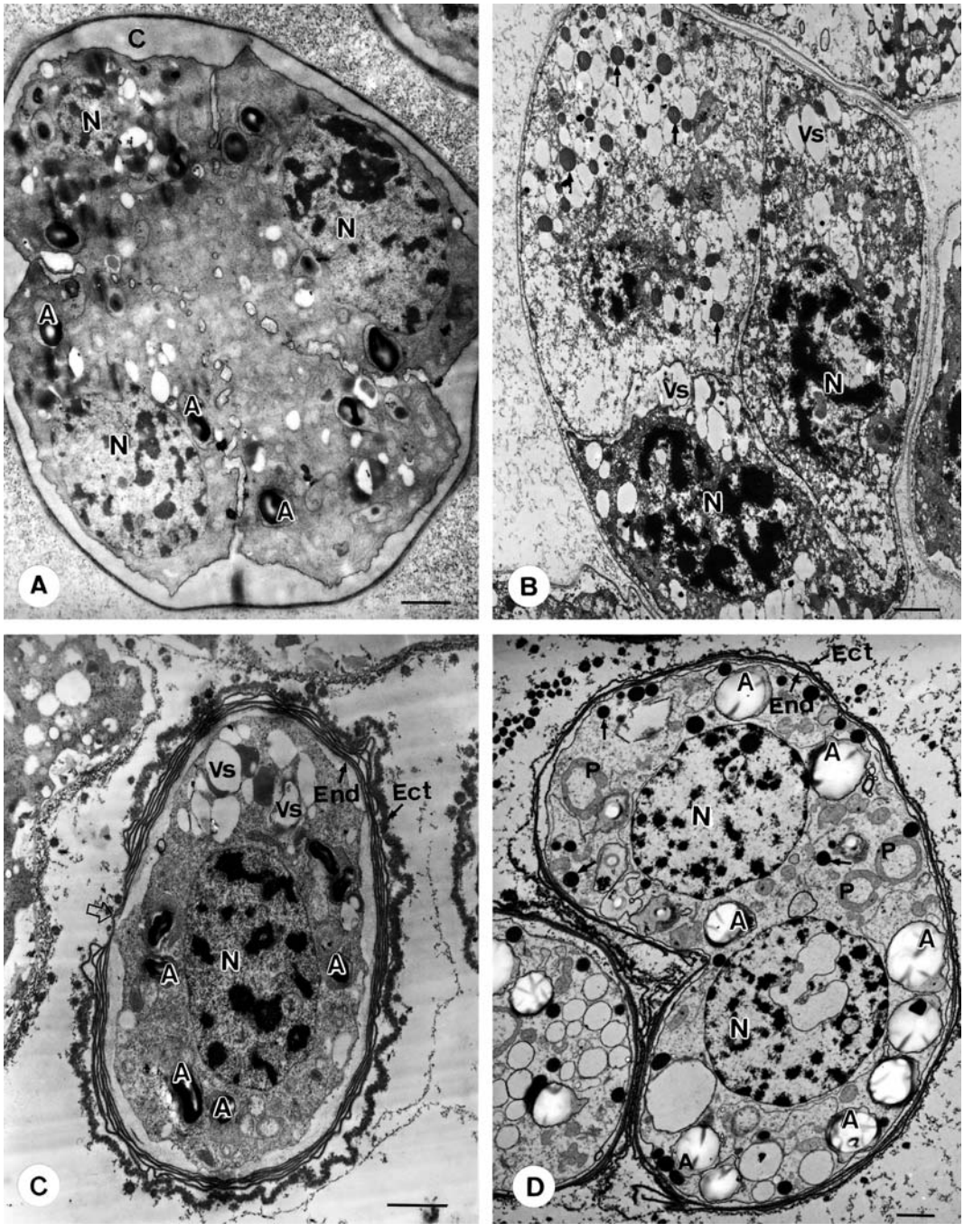


**Fig. 2.** TEM sections of fertile (**A** and **C**) and sterile (**B** and **D**) tetrads and free microspores of *Taiwania cryptomerioides*. **A** A fertile, tetragonal tetrad showing callose wall between plasmalemma and wall of microsporocyte. Many vesicles intersecting at the microspore interfaces, amyloplasts present. **B** A sterile, tetrahedral tetrad showing very thin or no callose wall, reduced cytoplasm with oil particles (arrows). **C** A fertile free microspore with lamellated endexine and granulate ectexine, a germinal zone (open arrow) shown on left side. **D** A sterile binucleate free microspore with reduced cytoplasm and underdeveloped endexine and ectexine. This free microspore is full of oil particles (arrows). *A* Amyloplast, *C* callose wall, *Ect* ectexine, *End* endexine, *N* nucleus, *P* plastid, *Vs* vesicle. Bars: A–C, 3 μm; D, 2.5 μm

1947), *Pseudotsuga menziesii* (Orr-Ewing 1978), *Cupressus dupreziana* (Pichot and Maâtaoui 2000, Maâtaoui and Pichot 2001), *Cryptomeria japonica* (Taira 2000), and *Pinus monticola* (Wilson and Owens 2003), but only the latter two studies described anatomical details. Taira (2000) reported that male sterility of *Cryptomeria japonica* in Japan occurred because the microspores collapsed after they separated from the tetrads. Wilson and Owens (2003) reported that male

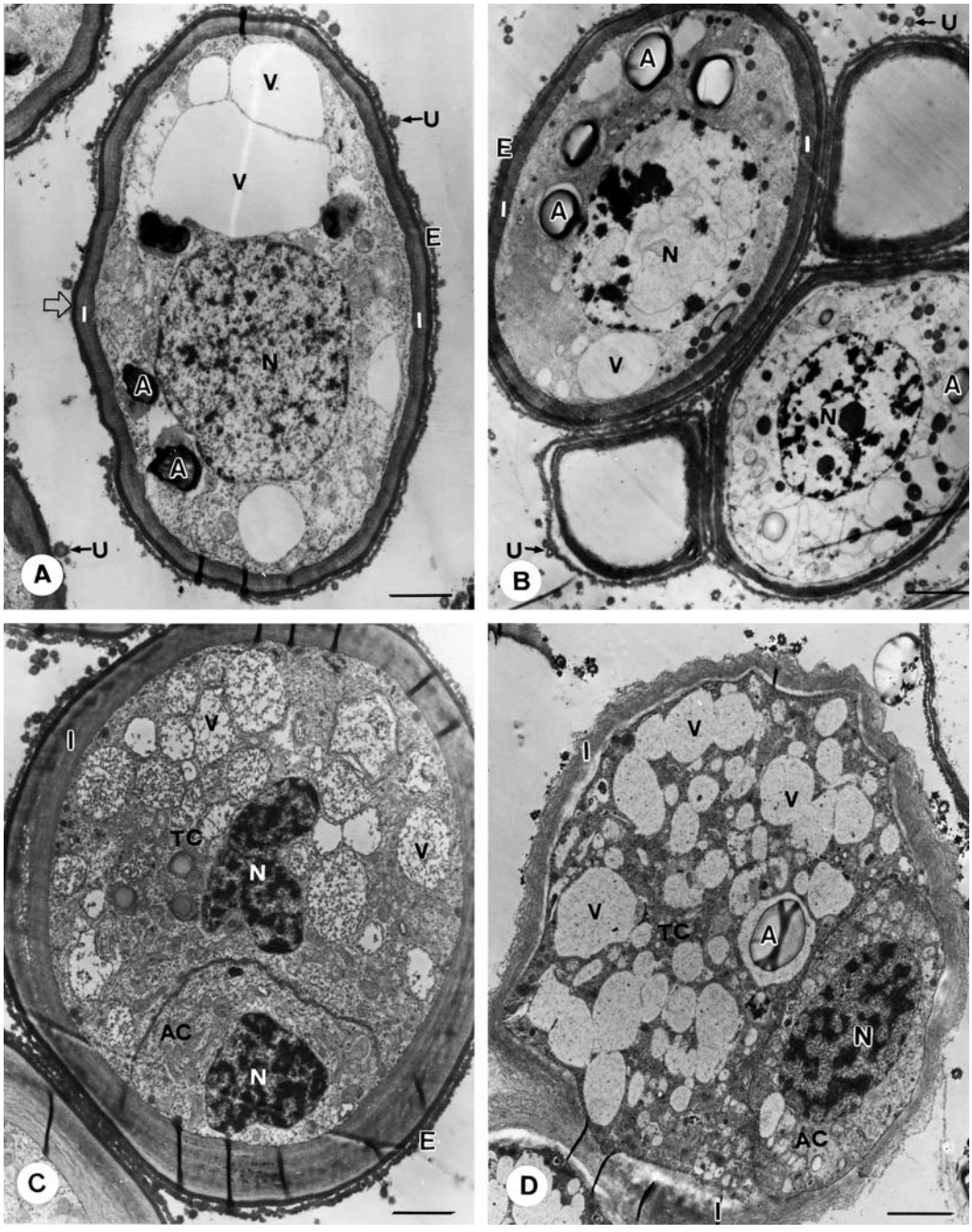


**Fig. 3.** TEM sections of fertile (**A** and **C**) and sterile (**B** and **D**) vacuolate microspores and bicellular pollen grains of *Taiwania cryptomerioides*. **A** A fertile vacuolate microspore with thick intine and thin exine, a germinal zone (open arrow) shown at left side, amyloplasts present. **B** Four sterile vacuolate microspores remain as tetrad, one microspore with well developed exine and thick intine (upper left), while the other three have only intine. **C** A fertile bicellular pollen grain with a small antheridial cell and a large tube cell, this grain has well developed exine and thick intine. **D** A sterile bicellular pollen grain with irregularly thickened intine but no exine. *A* Amyloplast, *AC* antheridial cell, *E* exine, *I* intine, *N* nucleus, *TC* tube cell, *V* vacuole, *U* Ubisch body. Bars: A, 3 μm; B–D, 3.5 μm

sterility of *Pinus monticola* in Canada occurred because of the reduction or absence of starch granules in sterile microspores during mitosis and maturation, reduced cytoplasm in the uninucleate microspore, irregular and thin exine deposition in the sacci, and persistent, abnormal tapetal cell activity. A Mediterranean endangered species, *Cupressus dupreziana*, yielded unreduced and abortive pollen due to meiotic defects which might lead to a natural extinction

(Pichot and Maâtaoui 2000, Maâtaoui and Pichot 2001). In the present study, similar abortion of pollen was observed for *T. cryptomerioides*. It is presumed that the rareness of this species in Taiwan might be attributed to male sterility.

Plants of *T. cryptomerioides* grafted in the Yeoi-Shoei-Keng clonal orchard did not produce mature pollen or seed cones (Lin and Kuo 2004). The present study shows for the first time that the male sterility of *T. cryptomerioides* might be related to the abnormal microsporogenesis under the natural environment in Taiwan. The microsporogenesis in the sterile strobili of *T. cryptomerioides* did not display any noticeable difference from the fertile strobili before the occurrence of meiosis. This is similar to those observed in a number of angiosperms (Heslop-Harrison 1971, Dickinson and Heslop-Harrison 1977, Regan and Moffatt 1990, Zhang et al. 2002). After meiosis, a number of disorders occurred in sterile microsporangia, such as the absence or reduction of the callose wall in tetrads, reduced and disorganised cytoplasm and abnormal mitosis in microspores, failure in development of exine and intine, and persistence as tetrads after pollen wall deposition. Eventually, all of these disordered microspores were collapsed in sterile microsporangia. This is not comparable to other conifers.

Although certain plant species such as *Pandanus odoratissimus* (Periasamy and Amalathas 1991) and lettuce (Curtis et al. 1996) do not require callose wall for pollen development, callose formation around the meiocytes seems to be a basic requirement for normal meiosis of the majority of plants (Reznickova and Bogdanov 1972). The role of the callose wall in microsporogenesis has been reviewed by Shivanna and Johri (1985), Shivanna (2003), and Scott et al. (2004). It might provide an isolation of meiocytes from other sporophytic tissue and production of individual microspores, and a protection for meiocytes from dehydration under water stress. In addition, the callose wall is essential for the orderly deposition of the pollen exine. After breakdown, the callose wall might also serve as a source of soluble carbohydrates for further development of pollen. In fact, lack of callose deposition (Tsuchiya et al. 1995), its early breakdown (Worrall et al. 1992), or abnormal deposition (Abad et al. 1995) would result in pollen sterility. In the present study, an absence or reduction of the callose wall was associated with the sterility of *T. cryptomerioides*. Apparently, the callose wall should have played an important role in microsporogenesis for this plant.

Blackmore and Crane (1988) proposed that the fusion of the exine in some naturally produced permanent tetrads (such as Juncaceae, Ericaceae, and Oenotheraceae) is related to the extent and timing of callose wall deposition. The absence of callose in the tetrad cross walls may result in permanent tetrads. Similarly to this, our present study of *T. cryptomerioides* shows that some of the sterile microspores remained as tetrads with intertwining of parts of the exine walls in the proximal faces. Possibly, such an abnormality results from a lack of structural support of the callose wall during development.

If a mutation is expressed sporophytically, it is expected that the numbers of normal and affected pollen resulting from meiosis would vary in each tetrad (McCormick 2004). However, if a mutation is gametophytic, the ratio of normal to affected pollen resulting from meiosis should be 2 : 2 (Johnson and McCormick 2001). In the present study, a different viability or different maturation timing occurred in the tetrad of sterile microsporangia of *T. cryptomerioides*. Thus, it is likely that the male sterility of this plant results from a sporophytic mutation. Microsporogenesis is completed either through successive or simultaneous cytokinesis (Scott et al. 2004). As a consequence, the former case leads to the formation of a tetragonal tetrad as revealed in the majority of monocots, while the latter leads to the formation of a tetrahedral tetrad as revealed in most dicots. In non-saccate conifers, the symmetry of the tetrad varies among genera. For example, the microspore tetrads of *Cryptomeria japonica* (Uehara and Sahashi 2000), *Cunninghamia lanceolata* (Kurmann 1990), *Tsuga canadensis* (Kurmann 1989), and *Chamaecyparis lawsoniana* (Lugardon 1995) are tetrahedral, while those of *Taxus baccata* (Pennell and Bell 1986) and *Taxus brevifolia* (Anderson and Owens 2000) are tetragonal. In *T. cryptomerioides*, various forms of tetrads, including tetrahedral, tetragonal, linear, and T-shaped, are present in the same microsporangium. Though *T. cryptomerioides* is a member of the conifers (Taxodiaceae), in pollen development it differs apparently from other members of this division. Thus it would be interesting to further study the phylogenetic position of *T. cryptomerioides* among conifers.

Temperature is an environmental factor that might influence microsporogenesis. Under stress conditions, pollen development might be affected, giving rise to cytoplasmic male sterility (CMS). High temperature rendered many CMS lines sterile, such as corn (Duvick 1965), *Petunia hybrida* (Izhar 1975), and *Brassica napus* (Banga 1992). In the present study, the temperature at the Yeou-Shoei-Keng clonal orchard was usually mild and in winter even slightly higher than at other localities of *T. cryptomerioides*. Whether such a temperature is already a stress for the development of pollen has not been studied. To elucidate the effect of temperature, further study is necessary.

It has been known that the first visible cytological deviations in many CMS species are related to the tapetum (Laser

and Lersten 1972, Raghavan 2000) and the callose wall (Worrall et al. 1992, Tsuchiya et al. 1995, Abad et al. 1995). In *Petunia hybrida*, the faulty timing or absence of callase, which was released from metabolic abnormalities in the tapetum, is related to the failure of callose wall dissolution and may be an indirect effect of male sterility (Izhar and Frankel 1971, 1976; Bino 1985a). A comparison of ultrastructure in microsporogenesis between fertile and CMS *Petunia hybrida* indicates that sterility occurred because of vacuolation in the sterile tapetum and meiocytes accompanied by the condensation of cytoplasmic organelles (Bino 1985b). In oilseed rape (*Brassica napus*), degeneration of microsporocytes and proliferation of the tapetum occur simultaneously and lead to CMS (Grant et al. 1986). In sugar beet (*Beta vulgaris*), a reduction in mitochondrial size during microsporogenesis was accompanied by a reduction in ribosome and a failure to produce Ubisch bodies from the tapetum which led eventually to CMS (Majewska-Sawka et al. 1993). Previously, we have discussed the relationship between the callose wall and male sterility. The present study documents the existence of male sterility in *T. cryptomerioides* and some abnormality in the development of sterile microsporangia and its possible relationship with the absence of the callose wall. However, we do not present the development of the tapetum and/or chromosomal aberrance during microsporogenesis that might be closely correlated with the development of the callose wall. In order to elucidate the reason of male sterility of *T. cryptomerioides*, apparently a further study is necessary.

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