Steciana

2016, Vol. 20(3): 173–182
doi:10.12657/steciana.020.018

www.up.poznan.pl/steciana

ISSN 1689-653X

SEM STUDIES ON SPORANGIA AND POLLEN MORPHOLOGY OF *GINKGO BILOBA* CULTIVARS

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(Received: July 20, 2016. Accepted: August 25, 2016)

Abstract. The present study was conducted to illustrate the micromorphology of mature sporangia and pollen grains of four *Ginkgo biloba* cultivars: 'Horizontalis', 'Lasiocarpa', 'Pragensis' and 'Tubifolia' and is a continuation of earlier investigations on this subject. The sporophylls bear typically two pendulous sporangia, whereas three were found in one cultivar. The sporangia varied in shape from oval, elliptical elongate to boat-shaped and dehisced along a longitudinal slit. The sporangial walls are multilayered. The sculpture of the sporangium is rugose covered with wax crystals (tubules) and the endothecium shows clearly visible fibrous thickening on the wall. The pollen grains are monosulcate, the non-apertural surface is irregularly ridged (striate) or rugulate, with perforations and the germinal aperture (furrow) is covered with verrucae. The current study did not differentiate significantly four cultivars based on sporangia and pollen morphology. The micromorphological traits may be useful for identification and classification of fragments of fossil sporangia and pollen grains.

Key words: *Ginkgo biloba*, male cone, sporangial wall, pollen grains, LM, SEM

INTRODUCTION

Ginkgo biloba L., one of the most primitive extant gymnosperms, is the only living species of the ancient lineage of Ginkgophyta (Zhou & Zheng 2003). It medicinal properties have been known and its decorative value has been appreciated since antiquity. Leaves of an unusual shape and venation as well as edible seeds used for medicinal purposes probably contributed to the popularisation of this species in China and Korea (Li 1956). In China *G. biloba* used to be planted mainly around Buddhist and Taoist temples (Del Tredici 2000). In the 12th century this species was introduced in western Japan (Tsumura et al. 1992) and from there it reached Europe six centuries later. The first *G. biloba* tree in the Old Continent was planted around 1730 in Holland, in the Utrecht Botanical Garden. Fascination with this unusual species resulted in its rapid spread throughout Europe and its introduction in North America in 1784 (Del Tredici 1991).

Over the last decades more than 200 *G. biloba* cultivars have been selected and bred (Santamour et al. 1983, Li et al. 2013, our own information – S.K.), differing in habit or shape and size of their leaf blades. Curently, the cultivar systems of *G. biloba* are confused.

At present *G. biloba* is commonly planted as an ornamental tree in parks and along streets, while it is grown as a medicinal plant mainly in China, Korea, France, Germany and the United States (Singh et al. 2008). In the opinion of Sinclair et al. (1987), Honda (1997) and Singh et al. (2008) resistance of *G. biloba* to pests and pathogens as well as air pollution, including ozone and sulphur dioxide, makes it a perfect species for urban areas. The trees are dioecious. The male trees are more desirable for planting, because female plants produce foul-smelling seeds (Singh et al. 2008). The pollen-bearing organs are catkin-like and originate singly in the leaf axis of short shoots. Each sporangiophore consists of a stalk and a sterile extension and each bears two (rarely more) pendant pollen sacs, which dehisce with a longitudinal slit. The sporangial walls are multilayerd (Page 1990). The pollen grains of *G. biloba* possess one simple aperture that extends from one extremity of the pol-

len grain to the other. The terms used to describe the aperture of these pollen grains vary from monocolpate to one sulcate (Erdtman 1952, Yamazaki & Takeoka 1962, Nakamura 1980, Liu et al. 2006). The dry and acetolised pollen grains are boat-shaped with a single longitudinal furrow. However, fully swollen grains are also observed, being spherical with a rounded or oval germinal aperture, bordered by a rim from the proximal hemisphere and differing in sculpture (Sahashi & Ueno 1986).

The aims of our study were to examine and describe sporangia and pollen morphology of four, previously not studied *Ginkgo biloba* cultivars and to determinate their characters for systematic importance.

MATERIAL AND METHODS

Sporangia of *G. biloba* were collected by the authors in May, 2015 from four cultivated plants: 'Horizontalis', 'Lasiocarpa', 'Pragensis' and 'Tubifolia'. They were selected from around 60 varieties in the collection of the Department of Dendrology, Pomology and Nursery, Poznań University of Life Sciences, Poland. Each specimen was represented by 15 pollen cones. The pollen grains were studied by light microscopy (LM) and SEM to obtain comprehensive information concerning the general morphology and exine sculpture. The SEM examinations were made on pollen grains which were air-dried, while for LM examinations pollen was treated with 10% KOH (Frederiksen 1978, Korszun & Klimko 2014) and without chemical treatment. The length of the polar axis (P) and equatorial axis (E) were measured in 100 pollen grains per specimen and the P/E ratio was calculated. The arithmetical mean and standard deviation were calculated for each mentioned trait (Table 1). The biometric data were analysed statistically. For each pollen grain feature, one-way analysis of variance (ANOVA) was used to examine differences in means between the cultivars studied. If significant differences were observed, multiple comparisons were carried out based on Tukey's test for equal sample sizes. Statistical analyses were performed using Statistica 12.5 (StatSoft, Poland). Micrographs were taken with a SEM type EVO 40 microscope (Carl Zeiss, Jena, Germany) at an accelerating voltage of 15 kV, at the Confocal and Electron Microscopy Laboratory, the Faculty of Biology, the Adam Mickiewicz University, Pozna, Poland. Prior to the observations the prepared material was sputtered with gold (for 15 s) using an SCB 050 ion sputter (Balzers AG, Liechtenstein). The study was documented with photographs taken during the observations, primarily at a magnification ×150 and surface from ×2000 to ×20000 for sporangia, for pollen grains ×8000 and from ×20000 to ×35000 for exine sculpture. Micromorphological traits of sporangia were observed on the dorsal and exine surfaces in the proximal and distal view. With regard to the sporangial surface, mainly the terminology of Barthlott (1981) was applied. The pollen terminology was adopted from Fægri & Iversen (1964), while the shape classification followed that of Erdtman (1952) based on the P/E ratio (Table 1).

RESULTS

DESCRIPTION OF POLLEN CONE

The sporophylls bear mainly two pendulous sporangia, whereas three were observed in cv. Tubifolia (Fig. 1e). The mean length ranged from 1.32 to 1.50 mm, width from 0.75 to 0.86 mm, while the length/width ratio from 1.61 to 2.03 (Table 1). The coefficient of variation for length ranged from 9.1% ('Horizontalis') to 21.6% ('Tubifolia'), for width from 3.3% ('Tubifolia') to 23.2% ('Horizontalis') and for the length/width ratio it ranged from 15.5% ('Lasiocarpa') to 21.1% ('Horizontalis') (Table 1).

The sporangia in the examined cultivars were oval (Fig. 1a, e) or elongate-elliptical (Fig. 1c) in all taxa and dehisced along a longitudinal slit (Fig. 1c). The surface was rugose and cell shape was not visible in SEM (Fig. 1b, d, f). The dorsal and ventral surfaces were convex. The cuticular layer covering the sporangial wall was broken into irregularly shaped (Fig. 1b, d, f) and covered densely with wax crystals (tubules) (Fig. 1g, h). The sporangia were eusporangiate and consisted of the epidermis, endothecium and the tapetum. The endothecium cells in LM were polygonal or elongated at the dehiscence of the sporangium with a clearly visible fibrous thickening on the wall (Fig. 2a-d).

DESCRIPTION OF POLLEN GRAINS

General morphology of pollen grains was similar, as all were distally monosulcate. The mean length of pollen grains (without chemical treatment) was from 35.82 μm ('Horizontalis') to 41.66 μm ('Tubifolia'), while width ranged from 18.92 μm ('Lasiocarpa') to 24.65 μm ('Tubifolia') (Table 1). In cv. Horizontalis 5.88% pollen grains were subprolate and 94.4% prolate. In 'Lasiocarpa' 31.82% pollen grains were prolate and 68.16% perprolate. In 'Pragensis' 77.27% pollen grains were prolate and 22.72% perprolate, while in 'Tubifolia' 89.47% pollen grains were prolate and 10.52% perprolate. Prolate pollen was predominant in three cultivars, i.e. 'Horizontalis', 'Pragensis' and 'Tubifolia'. Perprolate pollen was found in 'Lasiocarpa' with the P/E ratio of 2.13. Values of P, E and P/E showed low variability: for P it ranged from 7.8% ('Lasiocarpa') to 13.0% ('Tubifolia'), for E it was from 9.6% ('Tubifolia') to 18.9 ('Horizontalis'), whereas for the P/E ratio it was from 13.1% ('Lasiocarpa') to 14.8% ('Tubifolia'). Pollen grains were medium-sized according to Erdtman's classification

Table 1. Mean values (±SE) and minimum – maximum ranges of morphological traits of *Ginkgo biloba* sporangia and pollen grains (without and with chemical treatment)

Cultivars of Ginkgo biloba	Sporangia trait			Pollen trait					
				polar length (P) (μm)		equatorial width (E) (μm)		P/E ratio	
	length (mm)	width (mm)	lenght/width ratio	without	with	without	with	without	with
				chemical treatment		chemical treatment		chemical treatment	
'Horizontalis'	1.32 ± 0.12 a 1.10–1.50	0.86 ± 0.20 a 0.50–1.40	1.61 ± 0.34 a 1.00–2.40	35.82 ± 4.25 a 29.22–42.09	28.57 ± 2.48 a 24.01–35.15	22.93 ± 4.34 bc 15.39–31.36	26.48 ± 3.10 ab 21.89–31.07	1.59 ± 0.21 a 1.24–1.97	1.09 ± 0.12 a 0.93–1.24
'Lasiocarpa'	1.45 ± 0.22 a 0.95–1.80	0.81 ± 0.07 a 0.70–1.00	1.80 ± 0.28 ab 1.18–2.25	39.70 ± 3.12 bc 31.20–44.80	31.40 ± 2.07 b 27.45–36.48	18.92 ± 2.88 a 13.88–26.70	28.95 ± 2.24 c 22.78–34.04	2.13 ± 0.28 c 1.73–2.57	1.09 ± 0.05 a 1.00–1.25
'Pragensis'	1.50 ± 0.17 a 1.10–1.80	0.75 ± 0.10 a 0.60–0.90	2.03 ± 0.34 b 1.37–2.83	38.95 ± 3.41 abc 32.14–48.27	30.72 ± 3.22 b 25.64–41.19	20.63 ± 3.05 ab 15.76–29.06	27.64 ± 2.97 bc 23.44–37.83	1.92 ± 0.28 b 1.69–2.38	1.12 ± 0.12 a 0.92–1.25
'Tubifolia'	1.48 ± 0.32 a 1.00–2.20	0.85 ± 0.12 a 0.65–1.10	1.76 ± 0.36 ab 1.11–2.44	41.66 ± 5.43 c 29.05–53.82	29.45 ± 3.68 ab 20.86–35.09	29.45 ± 3.68 ab 20.86–35.09	26.21 ± 3.03 ab 18.39–32.80	1.69 ± 0.25 a 1.40–2.14	1.12 ± 0.07 a 1.01–1.23
ANOVA	F = 2.919 p < 0.05	F = 3.019 p < 0.05	F = 4.633 p < 0.05	F = 5.926 p < 0.01	F = 6.94 p < 0.01	F = 11.449 p < 0.01	F = 7.62 p < 0.01	F = 19.587 p < 0.01	F = 1.11 p = 0.3537

ANOVAs were performer separately for each pollen features to determine the differences among cultivars studied. Same letters indicate a lack of statistically significant differences between analysed cultivars according to Tukey's a posteriori test (P < 0.05).

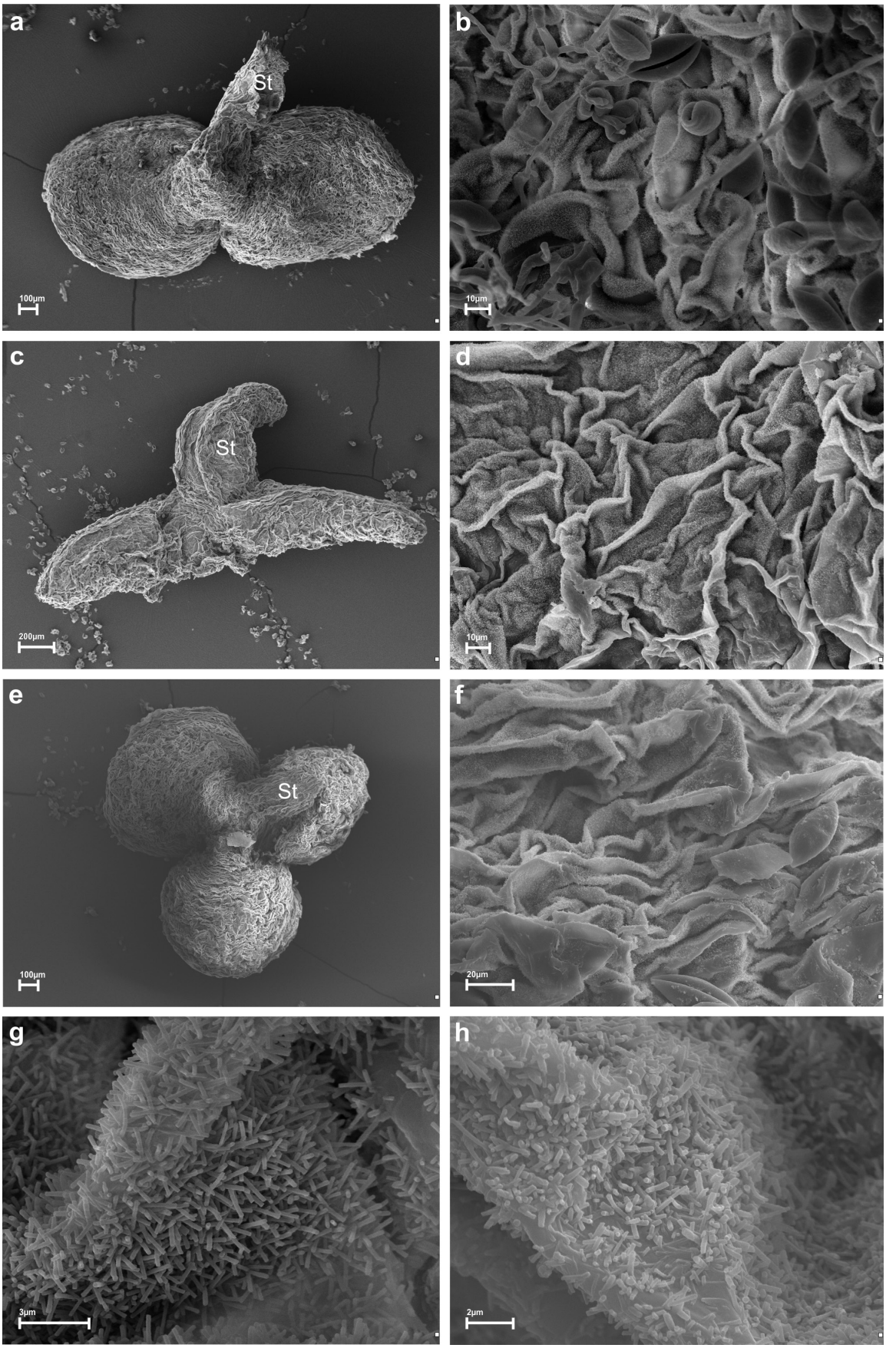

Fig. 1. SEM micrographs of sporangia of *Ginkgo biloba*: (a, b) 'Horizontalis' and (c, d) 'Pragensis', sporophyll bearing two sporangia, (e, f) 'Tubifolia' sporophyll bearing three sporangia. Crystaloid wax (tubules) on sporangia, (g) 'Horizontalis', (h) 'Tubifolia'; *St* – stalk

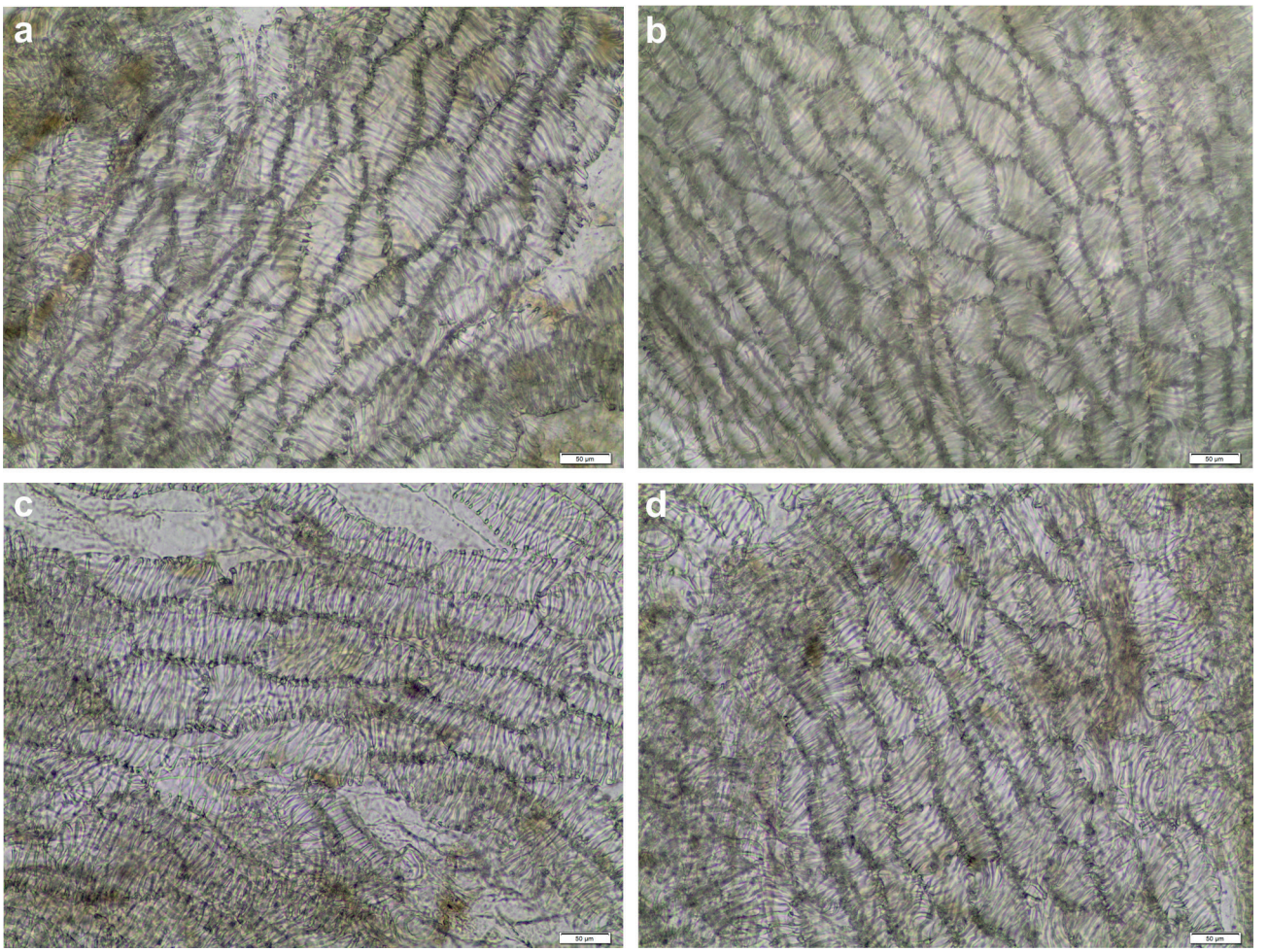

Fig. 2. LM. Fibrous thickening on endothecium walls of *Ginkgo biloba*: (a) 'Horizontalis', (b) 'Lasiocarpa', (c) 'Pragensis', (d) 'Tubifolia'

(1952). Exine sculpturing was observed in SEM on the proximal (Figs 3a-c, d, g, h; 4a-c, f- h) and distal views of boat-shaped pollen (air-dried). The proximal surface has the same sculpturing as the distal surface (Figs 3f, i, j; 4d, e i, j), but variance in exine sculpturing was observed between the non-apertural and apertural areas (Figs 3f, j; 4e, j). Ornamentation on the proximal face was irregularly ridged (striate) in *G. biloba* 'Horizontalis' (Fig. 3a-d) and in 'Lasiocarpa' (Fig. 4a, b, c); rugulate and irregularly ridged in 'Pragensis' (Fig. 4h) and rugulate in 'Tubifolia' (Fig. 3g, h). The enlarged proximal surfaces are shown in Figures 3c, d; 4b, c, h. It had very clearly visible perforations (irregular pores, holes) and small granules. The ornamentation observed on the outer face of the germinal aperture (furrow) of *Ginkgo* pollen grains under SEM was similar and covered with verrucae (Figs 3f, j; 4e, j).

The mean length of pollen grains (with chemical treatment) was from 28.57 μm ('Horizontalis') to 31.40 μm ('Lasiocarpa') and width from 26.21 μm ('Tubifolia') to 28.95 μm ('Lasiocarpa'). Prolate-spheroidal pollen was predominant in all the cultivars with a P/E ratio from 1.09 to 1.12 (Table 1). However, pollen grains were oblate-spheroidal, prolate-spheroidal and subprolate. In 'Lasiocarpa' 3.84%, 'Pragensis' 10.12% and 'Horizontalis' 31.6% pollen grains were oblate-spheroidal. In 'Horizontalis', 'Pragensis' and 'Lasiocarpa' 52.03%, 70.08% and 88.5% pollen grains were prolate-spheroidal. A total of 7.7% 'Lasiocarpa', 10.5% 'Horizontalis', 22.6% 'Pragensis' and 29.2% 'Tubifolia' pollen grains were subprolate. Values of P, E and P/E showed low variability: for P it was from 3.5% ('Horizontalis') to 12.5% ('Tubifolia'); for E from 7.7% ('Lasiocarpa') to 11.7% ('Horizontalis') and P/E from 4.6% ('Lasiocarpa') to 11.1% ('Horizontalis'). Exine sculpturing in our study was difficult to describe on LM images (Fig. 5).

DISCUSSION

The application of a greater magnification in SEM showed that the wax covering the sporangial wall was crystals (tubules), not platelets (Korszun & Klimko 2014). The type of wax is a good diagnostic feature for *Ginkgo*, because it is found on generative and vegetative organs (Riederer 1989, Majumder et

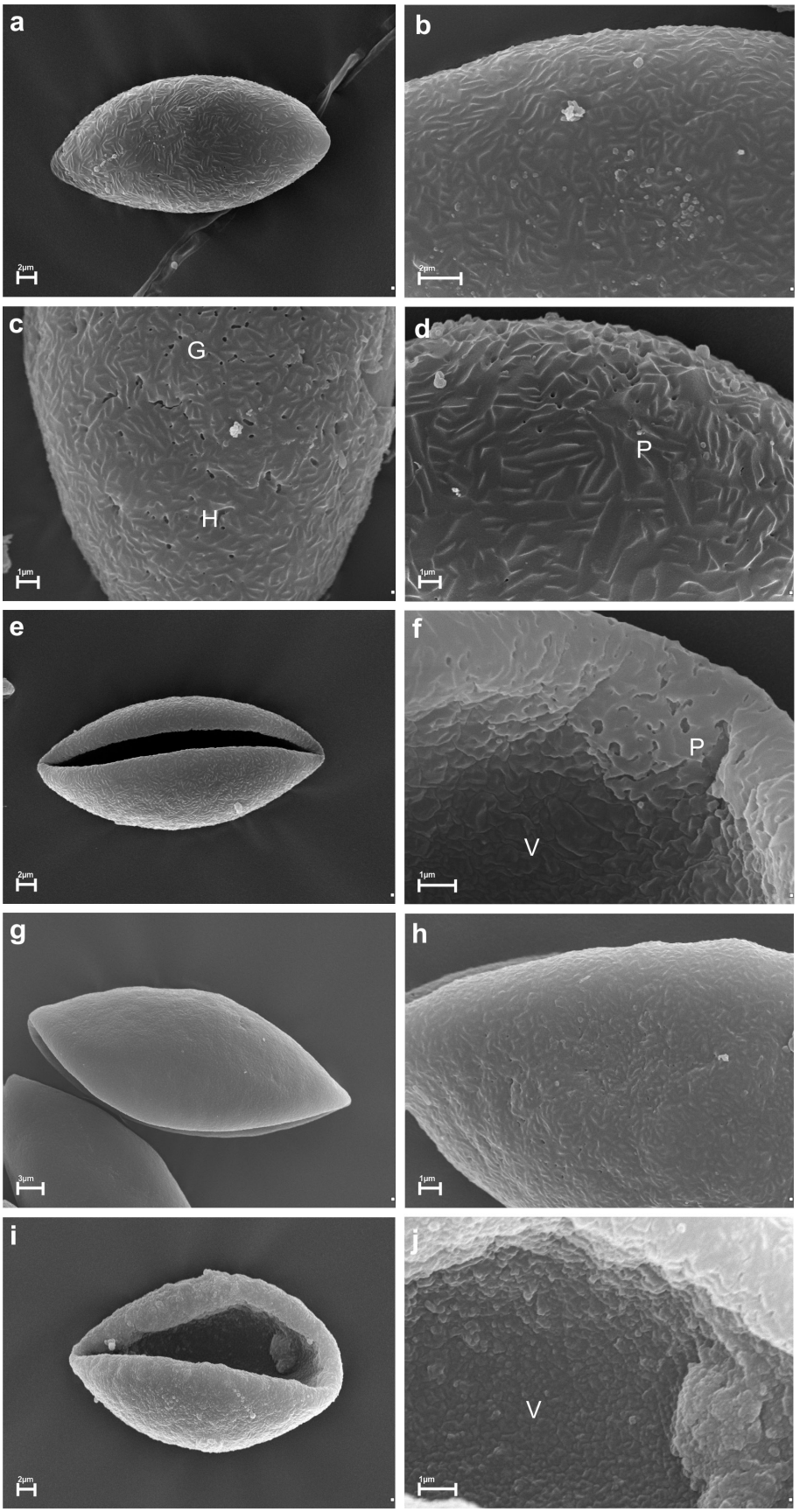

Fig. 3. SEM micrographs of pollen grain of *Ginkgo biloba*: (a–f) 'Horizontalis', (a) proximal view, (b, c), proximal surface, enlargement of (a), (d) proximal surface, (e) distal view, (f) distal face of germinal aperture surface, (g–j) 'Tubifolia', (g) proximal view, (h) enlargement of (g), (i) distal view, (j) distal face of germinal aperture surface; *G* – grana, *H* – holes, *P* – puncta, *V* – verrucae

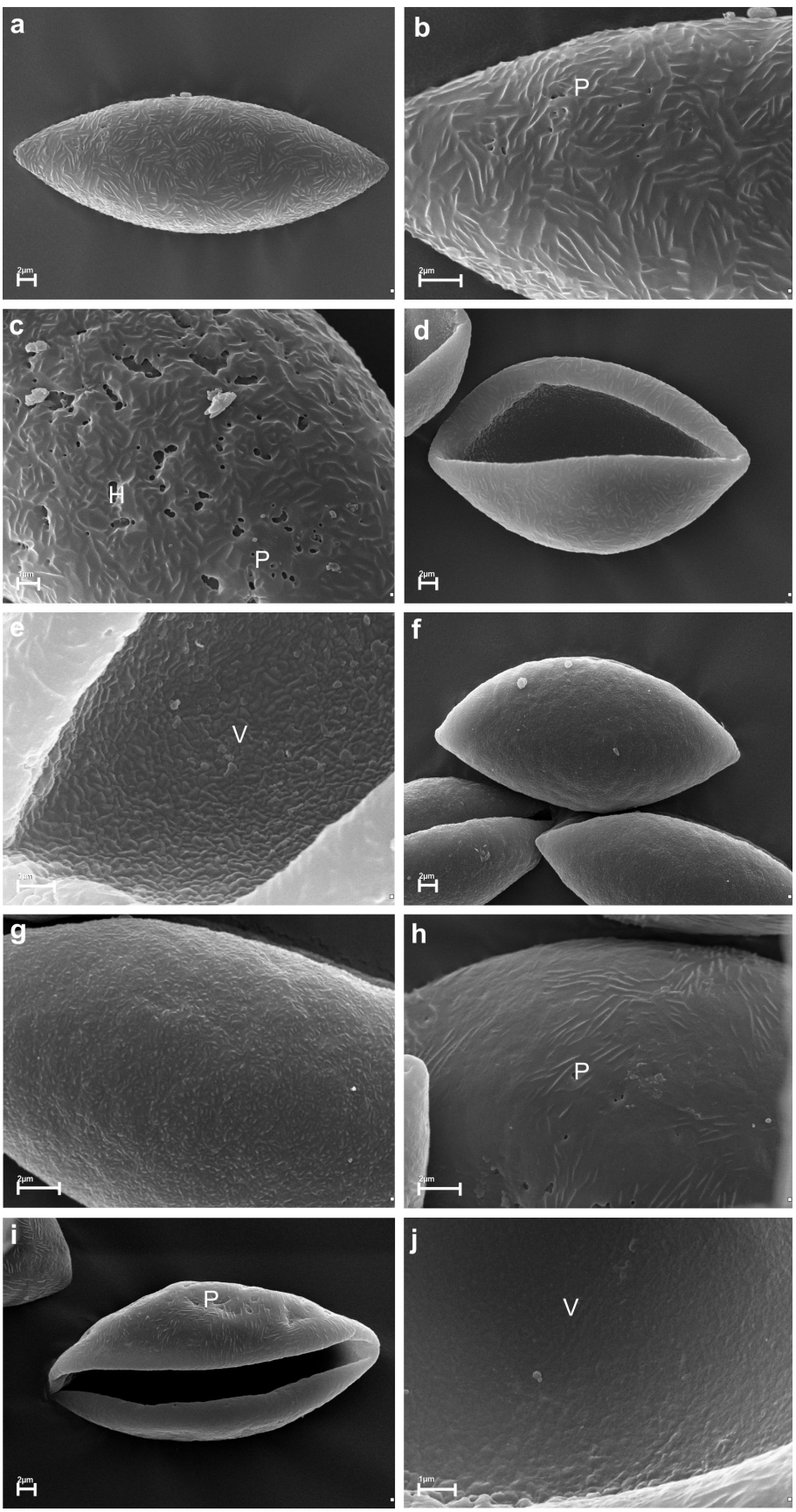

Fig. 4. SEM micrographs of pollen grain of *Ginkgo biloba*: (a–e) 'Lasiocarpa', (a) proximal view, (b) proximal surface, enlargement of (a), (c) proximal surface, (d) distal view, (e) distal face of germinal aperture surface, (f–j) 'Pragensis', (f) proximal view, (g, h) proximal surface, (i) distal view, (j) distal face of germinal aperture surface; *H* – holes, *P* – puncta, *V* – verrucae

al. 2013, Tomaszewski & Zieliński 2014, Klimko et al. 2015, 2016). In other gymnosperms wax layers were studied on vegetative organs e.g. in *Cryptomeria japonica* D. Don, *Taxodium distichum* (L.) Rich., *Taxus baccata* L., *T. cuspidate* Siebold et Zucc., and *Sequoia sempervirens* (D. Don) End., and wax tubules and in *G. biloba* platelets (Neinhuis & Barthlott 1997). The sporangia were eusporangiate and consisted of the epidermis, endothecium and the tapetum (Lu et al. 2011). Lu et al. (2011) reported that in early April a fibrous thickening was detected in the outer endothecium wall. Our description is consistent with earlier findings by Liu et al. (2006), Lu et al. (2011) and Korszun & Klimko (2014). However, this study showed that *G. biloba* cultivars had smaller sporangia than it had been reported previously (Liu et al. 2006, Lu et al. 2011, Korszun & Klimko 2014). In cultivars described by Korszun & Klimko (2014) the mean size of sporangia was 1.3–3.25 × 0.37–1.5 mm, while in our study it was 1.32–1.5 × 0.75–0.86 mm. In turn, they were very similar in shape, i.e. most typically oval or elongate-elliptical. Greater differences were observed in pollen grain size. Pollen grain preparation for analyses was significant. In the cultivars examined by Korszun & Klimko (2014) chemically treated pollen grains were on average 36.72–39.33 μm in length (P) and 15.03–16.02 μm in width (E). In turn, in our study pollen grains not subjected to chemical treatment were larger and had 35.82–41.66 μm in length (P) and 18.92–24.65 μm in width (E). Those chemically treated were much smaller, with 28.57–31.40 μm in length (P) and 26.21–28.95 μm in width (E). Moreover, described pollen grains differed in shape. In the cultivars analysed by Korszun & Klimko (2014) the P/E ratio was 2.3–2.49, while in those used in this study it was 1.59–2.13 (pollen grains not treated chemically) or 1.09–1.12 (pollen grains treated chemically).

The proximal surfaces of pollen grains have very clearly visible perforations (irregular pores, holes) and small granules. In contrast, Sahashi (1997) reported that perforations were almost invisible on the surface without chemical treatment. The ornamentation on the outer face of the germinal aperture (furrow) of *Ginkgo* pollen grains under SEM was similar and covered with verrucae. According to Sahashi & Ueno (1986), the inner face of the exine of the germinal aperture in *Ginkgo* pollen grains, which can be

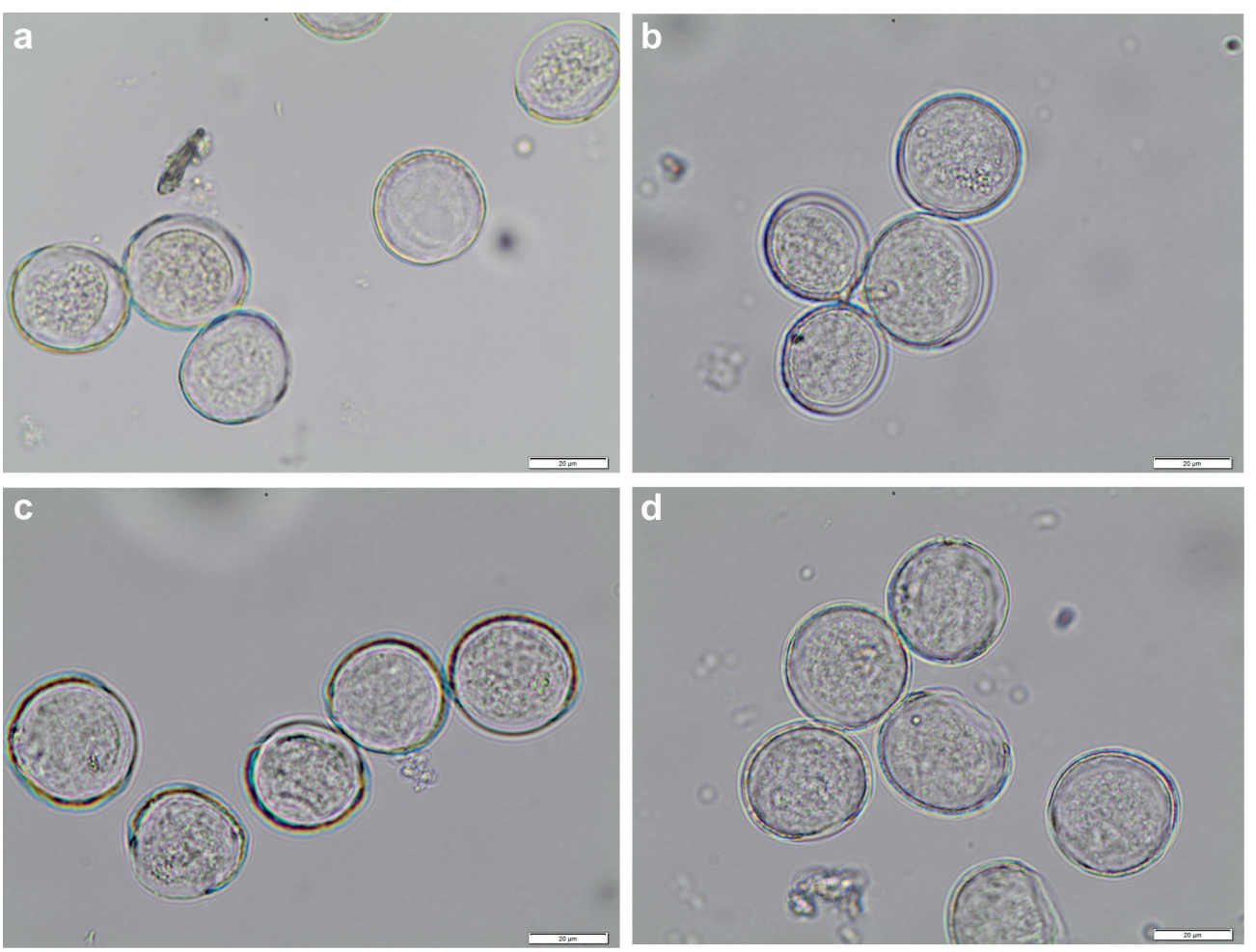

Fig. 5. LM. Groups of pollen grains (with chemical treatment) of *Ginkgo biloba*: (a) 'Horizontalis', (b) 'Lasiocarpa', (c) 'Pragensis', (d) 'Tubifolia'

seen in sections, has quite remarkable reticulum-like sculpturing.

Ornamentation seen on the proximal face (without chemical treatment) of pollen grains in SEM was described by Sahashi (1997) as randomly arranged linear ridges with some granules, as covered by arranged ridges, with minute verrucoid details between the ridges by Yamazaki & Takeoka (1962), spinulous by Ueno (1960) or reticulate with short and slightly elevated crests by Rohr (1977), and covered with rugules by Audran & Masure (1978), Zavialova et al. (2011). The pollen grains do not have one strict pattern even within the same tree (Korszun & Klimko 2014, this study). Ornamentation seen on chemically treated pollen grains in LM was described as minutely roughened by Wodehouse (1935), subreticulate or with a spiny wave by Yamazaki & Takeoka (1962), as granulate by Shimakura (1973) and as verrucate by Nakamura (1980). Sahashi (1997), Tekleva et al. (2007) and Zavialova et al. (2011, 2014) published photos of pollen grain with a concave aperture, with swollen apertural areas, an apertural area with a rim and a rugulate sculpture on the proximal surface in SEM images (materials were dehydrated and treated by critical point drying). After acetolysis the ornamentation was foveolate, perforated with tiny holes (Frederiksen 1978) or with clear linear ridges without granules (Sahashi 1997). As it was shown by analyses conducted to date, the size, shape and ornamentation of pollen grain exine are influenced by the method of specimen preparation.

Our results showed that *Ginkgo biloba* cultivars cannot be separated based on sporangia and pollen grain micromorphology. Statistical analysis showed no significant differences between cultivars in the means of sporangia and pollen grains. Ornamentation of air-dried pollen grains does not have one strict pattern even within the same tree, e.g. *G. biloba* 'Pragensis'. This conclusion correlates to the findings reported previously. The micromorphological traits may be useful for identification and classification of fragments of fossil sporangia and pollen grains.

ACKNOWLEDGEMENTS

We are grateful to Wojciech Klimko for his assistance with computer data recording. The authors would like to thank two anonymous reviewers for their suggestions and comments made on an earlier version of the manuscript. The study was partly supported by the Department of Botany and the Department of Dendrology, Pomology and Nursery, the Poznań University of Life Sciences.

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For citation: Klimko M., Bykowska J., Korszun S. (2016): SEM studies on sporangia and pollen morphology of *Ginkgo biloba* cultivars. Steciana 20(3): 173–182. doi: 10.12657/steciana.020.018