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# Reproductive biology of the "Brazilian pine" (*Araucaria angustifolia* – Araucariaceae): Development of microspores and microgametophytes

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ABSTRACT

*Araucaria angustifolia* Bert. (O. Ktze), also known as the "Brazilian pine", is native to the South of Brazil. This species has a long reproductive cycle, taking about 29–34 months. Its cones begin to develop in early January and remain dormant from March to July. In August, they become active again and microsporogenesis occurs, which proceeds until September. From September to October, microgametogenesis is established and pollination occurs from October to November. Meiosis is asynchronous, with simultaneous cytokinesis, and the tetrads are of the tetrahedral and isobilateral type. During gametogenesis, microgametophytes gradually develop an axial row of cells that are isolated by internal callose and undergo four mitotic cycles until pollen dispersal. In mature pollen grains, the vegetative cells do not possess a cell wall, but maintain strong internal polarization. The pollen of *A. angustifolia* is suboblate, without apertures or air sacs. Histochemical analysis of the sporoderm was also performed and when compared to other conifer families, showed the most simplified intine structure among the group. Embryological characteristics analyzed during the phenological phases of this species showed certain peculiarities, knowledge about which may be helpful contributing to the management and conservation of *A. angustifolia*.



## Introduction

The family Araucariaceae comprises three genera: *Araucaria* Juss., *Agathis* Salisbury and *Wollemia* Jones, Hill & Allen. All species of the family are native to the Southern Hemisphere. Only two species occur in South America: *Araucaria araucana* (Mol.) K. Koch, also known as the Chilean pine, which grows in the South of Chile and in the Argentine Province of Neuquén (Marchiori, 1996) and *Araucaria angustifolia* (Bert.) O. Ktze, known as the "Brazilian pine". *Araucaria angustifolia* occurs in the South of Brazil and forms dense clusters of individuals, especially in the eastern and central part of the Brazilian plateau (Hueck, 1972). This formation, named the 'Araucaria Forest' is a particular type of the Atlantic Forest biome.

*Araucaria angustifolia* is dioecious and rarely monoecious (Stefenon and Caprestano, 2009) and according to Anselmini and Zanette (2008), the reproductive cycle is concluded in about 29–34 months, from the time of cone formation until seed maturity.

Currently, *A. angustifolia* is classified as vulnerable regarding the risk of extinction (Brazilian Decree N° 42,099, 2002). The species suffered from heavy devastation at the end of the nineteenth century, due to the high value of its timber (Machado and Siqueira, 1980) and the lack of environmental management against exploitation (Lacerda et al., 2012). This risk is even greater due to the long and delicate reproductive mechanism of the species, making its natural regeneration difficult. According to current estimates, less than 2% of the original Araucaria Forest remains, placing it as the most threatened formation in the Atlantic Rainforest (Koch and Corrêa, 2002). As a dominant tree, this situation is even more far-reaching, because populations of adult individuals allow shade-tolerant plant species of other taxa to grow and develop. Moreover, the seeds, which have a high energy value, feed the wild fauna, including many mammals and birds, which are the main *Araucaria* seed dispersers (Koch and Corrêa, 2002).

Studies on conifer embryology are very scarce compared to those performed on angiosperms. This situation can be explained by the long reproductive cycle of conifers and also by the great size reached by many of them. For *A. angustifolia*, studies performed so far help to understanding of certain aspects of its reproduction. However, studies by Burlingame (1913, 1915a,b), Alencar (1941)

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and Shimoya (1958, 1962) lack improved techniques for a better quality of analysis.

This article therefore, describes the microsporogenesis and microgametogenesis with respect to the detailed histochemistry of the pollen structures and the spatial arrangement of the mature gametophyte, supplying both embryological and phenological data on *Araucaria angustifolia*. This will contribute important knowledge concerning the relations among the conifers, can supply information on the reproductive biology of this species, and will thereby increase chances for a successful management of the natural environment of this species, aiming to recover its presently rather reduced and in part degraded stands.

## Materials and methods

Cones of *Araucaria angustifolia* were obtained from individuals in two different areas of the city of Nova Petropolis, Rio Grande do Sul, Brazil. Material was collected from four microsporangiate individuals. The collections were performed weekly during January and February, daily between August and beginning of September, and twice weekly between September and October in 2010 and 2011. For the rest of the year, the monthly collections were done. Voucher specimens were deposited in the ICN herbarium of the Federal University of Rio Grande do Sul, under numbers 171957 and 171958.

Microsporangia were fixed in 1% glutaraldehyde and 4% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.2 (McDowell and Trump, 1976). Following Gabriel (1982) they were then washed in sodium phosphate buffer (0.1 M, pH 7.2), dehydrated in an ethanol series (10–100%) and embedded in (2-hydroxyethyl)-methacrylate (Gerrits and Smid, 1983). Sections were cut 2–4 μm thick, using a Zeiss rotating microtome and stained with Toluidine Blue 0.05%, pH 4.4 (O'Brien and McCully, 1981).

The microsporangia were dissected and stained with 2% Acetic Carmine (Guerra and Souza, 2002) to characterize the reproductive stage of the material. Chemical composition was detected using different histochemical tests: IKI (Lugol's solution; Johansen, 1940) to detect starch, Coomassie Brilliant Blue (Southworth, 1973) for total proteins, Ruthenium Red (Johansen, 1940) for polysaccharide acids and pectic acids, Alcian Blue 8GX (Lillie, 1965) for mucopolysaccharides, Basic Fuchsin (Faegri and Iversen, 1964) to identify ectexine and endexine, Calcofluor White (Hughes and McCully, 1975) for cellulose, Aniline Blue (Martin, 1959) for callose, Auramine O 0.01% (Nepi and Franchi, 2000) for lipidic compounds and DAPI (Kuroiwa and Suzuki, 1980) to identify DNA. Pectinase (20%) and hemicellulase (2%) enzymes were used (Guerra and Souza, 2002), to confirm the type of the pectic compound present in pollen grains, with a subsequent reaction with 1% Astra Blue in aqueous solution (Gerlach, 1984), Alcian Blue 8GX (Lillie, 1965), and Ruthenium Red (Johansen, 1940).

Acetolysis was performed to analyze pollen morphology (Erdtman, 1960) and the arithmetic means of the polar and distal axis measurements were calculated from a sample of 30 pollen grains.

Observations and image capture were performed under bright-field light microscopy or epifluorescence (340–380 nm and 450–490 nm), using a Leica DM R HC microscope with a Leica DFC 500 digital camera and LAS Leica Application Suite v. 4.1.

For scanning electron microscopy (SEM), the samples were dehydrated in an acetone series and dried using the critical-point method (Gerstberger and Leins, 1978), with BAL-TEC, CPD 030 equipment. The samples were then mounted onto stubs and coated with gold using a BAL-TEC SCD 050 sputtering device. Observations and micrographs were performed with a JEOL JSM 6060 microscope under 30 kV.

For the three-dimensional reconstruction of pollen, serial 2 μm-thick sections were stained with Toluidine Blue O and were digitized, aligned in the Adobe Photoshop CS5.1 program and imported to the Rhinoceros 4.0 program with a scale reference. These images were applied on parallel planes to outline the structure contours (vectorization) and their three-dimensional surfaces were generated.

Environmental data for the city of Nova Petrópolis during the sampling period were obtained from the meteorological database of the National Institute for Meteorology (INMET; Estation 83942, Caxias do Sul, RS). Data were originally recorded on a daily basis and monthly mean, maximum, and minimum temperatures (Centigrade degrees), as well as air humidity (percent), and rainfall (millimeters) were subsequently computed.

## Results

### *Microsporogenesis*

The cones of *A. angustifolia* begin to develop during the summer, between January and February. The reproductive meristems appear in the dorsal region of the microsporophyll and are initially apparent as a set of meristematic cells in which anticlinal and periclinal divisions are observed in the dermal and hypodermal cell layers. During February, the number of parietal strata of the microsporangium increases and in March, during the beginning of autumn, these are completely differentiated. The microsporangiate cones of *A. angustifolia* do not show morphometric variations, and the microsporangium remains unchanged until July.

Meiosis in the sporogenic cells in the same microsporangium is asynchronous and shows a basipetal progress of maturation, which occurs during the beginning of spring, in August and September. The microspore mother cells separate from each other (Fig. 1a), their sizes increase and their chromosomes condense, entering prophase 1 (Fig. 1b). The histochemical analysis of these cells shows the existence of a large amount of starch in their cytoplasm (Fig. 1c), the composition of cell walls to be cellulosic (Fig. 1d), mucopolysaccharidic (Fig. 1e) and proteinic (Fig. 1f), and callose being absent. The tapetum cells also show major modifications in this stage, with endomitosis, starch in their cytoplasm (Fig. 1c) and large vacuoles.

Anaphase I, telophase I, metaphase II and telophase II were demonstrated by different techniques (Fig. 1g–k). The histochemical tests performed in each phase indicate that callose is present on the wall of the late microspore mother cell only in telophase I, and that the starch grains remain in the cytoplasm during cell division. In telophase II, callosic walls are laid down centripetally, separating each of the microspores (Fig. 1l). Thus, the meiosis of *A. angustifolia* is characterized as simultaneous, because cytokinesis does not occur until the second meiotic cycle.

The tetrads formed are of the tetrahedral and isobilateral type, with the tetrahedral form occurring more frequently (Fig. 2a–b). The mature tetrads have callosic walls (Fig. 2c) and starch grains in the cytoplasm that are distributed around the nuclei (Fig. 2d). During the tetrad phase, the first primexine displays a mucopolysaccharide (Fig. 2e) and proteinic (Fig. 2f) composition. During the same stage, the first signs of degeneration of the tapetum cells occur, which is identified by cytoplasmic projections into the locular cavity and cell walls with a weak reaction for cellulose. However, the nuclei still have a parietal position and active nucleolei (Fig. 2a and g).

The microspores exhibit a proximal pole with three flattened faces and the distal pole is rounded, with the nucleus in a central position (Fig. 2g and h). The starch grains are still in the cytoplasm (Fig. 2i) and only the outer layer of the exine, the ectexine, is