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Original Paper

Meiotic behavior and diploidy in *Schinus terebinthifolia* (Anacardiaceae)

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Abstract

The genus *Schinus* (Anacardiaceae) comprises 30 species found in Peru, Bolivia, Chile, Paraguay, Uruguay, Argentina and Brazil. *Schinus terebinthifolia* (Brazilian pepper tree) is socioeconomically important in the Brazilian state of Espírito Santo. Dried fruit is used as a pepper-like spice and natural populations are exploited. Studies on the meiotic behavior and pollen stainability from an ecological and evolutionary point of view are essential, since they can promote future sustainable management strategies for this species. The objective was to evaluate meiotic behavior and characteristics of pollen grains of individuals from a natural population of *S. terebinthifolia* localized in the municipality of Vitória, Espírito Santo state, Brazil. Young anthers were squashed in 1% acetocarmine dye to obtain the pollen mother cells. The pollen stainability with 1% acetocarmine, Lugol's iodine solution and Sudan IV and pollen shape were used to assess the pollen potential viability, cytochemistry and morphology. All individuals had a gametic number of n = 7, a regular meiotic behavior and so the somatic number is 2n = 2x = 14 chromosomes. Median pollen stainability was 94%. Pollen grains are dispersed as monads, 3 - colporate and subspheroidal. The exine has striate-reticulate ornamentation. Pollen grains are starch positive and lipid negative. This is the first report of the occurrence of diploidy for the species. The high pollen stainability is the result of the occurrence of regular meiosis. The presence of starch as pollen reserve can increase the resistance of pollen grains to hostile environments in this species.

Key words: Brazilian pepper, diploidy, meiosis, pollen.

Resumo

O gênero *Schinus* (Anacardiaceae) compreende aproximadamente 30 espécies sul americanas distribuídas no Peru, Bolívia, Chile, Paraguai, Brasil, Argentina e Uruguai. *S. terebinthifolia* (pimenta vermelha brasileira) é socioeconomicamente importante no estado do Espírito Santo, Brasil. Populações naturais vêm sendo exploradas devido à coleta não sustentável de frutos e sementes que são utilizados na indústria alimentícia. Estudos sobre o comportamento meiótico e a viabilidade polínica dessa espécie são essenciais sob o ponto de vista ecológico e evolutivo, pois estão relacionadas com o seu sucesso reprodutivo, promovendo estratégias de manejo sustentável. Objetivou-se avaliar o comportamento meiótico e as características polínicas de indivíduos de uma população natural de *S. terebinthifolia* localizada no município de Vitória-ES (Brasil). Anteras jovens foram esmagadas em carmim acético 1% para a obtenção das células-mãe dos grãos de pólen. A coloração com carmim acético 1%, solução de Lugol e Sudan IV e a forma dos grãos de pólen foram utilizadas para avaliar a viabilidade potencial, a citoquímica e a morfologia polínicas. Todos os indivíduos apresentam um número gamético de n=7 e um comportamento meiótico regular, sugerindo um número cromossômico somático de 2n=2x=14 cromossomos. Viabilidades polínicas de 94%, em média, foram observadas. Os grãos de pólen apresentaram-se dispersos como mônades, tricolporados e subesferoidais com ornamentação estriada-reticulada sendo amido positivos e lipídeo negativos. Esse é o primeiro relato da ocorrência de diploidia para a espécie. As elevadas viabilidades polínicas são resultantes da ocorrência de meiose regular. A presença de amido pode aumentar a resistência dos grãos-de-pólen dessa espécie a ambientes hostis.

Palavras-chave: aroeira vermelha, diploidia, meiose, pólen.

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Introduction

Schinus terebinthifolia Raddi (Anacardiaceae), also called Brazilian pepper- along with many regional names- is a pioneer species with a tropical and subtropical distribution (Lorenzi & Matos 2008). It is found in Argentina, Paraguay, Uruguay and in Brazil (Sabbi *et al.* 2010) it occurs from the southern state of Rio Grande do Sul to the northeastern state of Pernambuco. As a pioneer species it can be found in a variety of environmental conditions and can rapidly appear in almost any perturbed location, becoming an invasive species in many places (Correa 1926). Introduced into the United States as an ornamental it quickly became established and spread in the state of Florida (Dawkins & Esiobu 2016). Ecologically, the Brazilian pepper is found in almost all soils type and rainfall regimes (Falkenberg 1999).

The Brazilian pepper has medicinal and phytochemical value and so may also be economically important (Guerra *et al.* 2000; Amorim & Santos 2003). The essential oil extracts have medicinal uses including fungicidal, insecticidal, antibacterial and may help wounds heal (Degáspari *et al.* 2005; Ribas *et al.* 2006; Lorenzi & Matos 2008; Santos *et al.* 2009; Gundidza *et al.* 2009; Santos *et al.* 2010; Silva *et al.* 2010). The species is also important in reforestation efforts where their wide environmental tolerances allow them to be used to reforest riparian areas and stabilize sand dunes (Ferreti *et al.* 1995; Kageyama & Gandarra 2000). Brazilian pepper ecology-wide tolerance, high fecundity, competitive ability and pioneer species- also favors its use in areas with strong anthropic effects where soils have been damaged (Carvalho 1994).

Dried Brazilian pepper fruits are used as a food condiment locally called pink pepper with a slightly sweet, but peppery flavor and which has found an international market (Lenzi & Orth 2004; Bertoldi 2006). Most production of the Brazilian pepper is in the state of Espírito Santo (Mazza *et al.* 2011), typically in regions that were formerly mangroves and costal plains (*restinga*). The species has been constantly exploited economically through unsustainable extraction of its fruits and seeds that are commercialized for processing industries. Extractivism has been occurring without a standard procedure in the management of the species (Jesus & Gomes 2012).

Cytogenetic approaches, which regard the chromosome number contribute to the understanding of evolutionary processes in plants (Shan *et al.* 2003). Moreover, the analysis of meiotic behavior has been widely used in the study of plants as they provide relevant information about the viability of a species (Lavinscky *et al.* 2007). Palynology can provide insights into biodiversity because pollen grains vary in form. This variation is heritable and shows a high level of consistency within a táxon, although, interestingly, a wide range forms can sometimes be encountered at low levels of abundance within a single species (Blackmore 2007).

Thus, cytogenetics and palynology are important tools for understanding intra- and inter-populational genetic variability. While the Brazilian pepper is common, few studies have attempted to examine cytogenetics and palynology of the genus *Schinus.* From the cytogenetic point of view, all reports in the literature show the occurrence of polyploidy for *S. terebinthifolia.* Chromosome count of 2n = 60 was demonstrated by Sarkar *et al.* (1973), and by Moore (1977) and 2n = 4x = 28 by Pedrosa *et al.* (1999), Franco-Cairo *et al.* (2009), Luz *et al.* (2015) and Bitencourt (2016). Only one reference about meiotic characteristics for this species is reported. Bitencourt (2016) described the occurrence of high meiotic index (95%) of tetraploid plants from Santa Catarina state- Brazil.

Pollen characteristics of *S. terebinthifolia* were described by Cruz-Barros & Granito (1997), Barros *et al.* (1999), Takeda *et al.* (2000), Willard *et al.* (2004) and Bitencourt (2016). These studies show that pollen intraspecific variations are common. Some differences in the pollen shape (prolate spheroidal, prolate, subprolate and oblate spheroidal) and ornamentation pattern (reticulate, striate, finely reticulate or reticulate-striate) were described. Information about pollen viability and cytochemistry for *S. terebinthifolia* are reported by Bitencourt (2016) that observed 68% of pollen stainability and starch and lipid positive pollen grains in tetraploid specimens. However, data about meiotic behavior for *S. terebinthifolia* not are existent in the literature. Meiosis is a mechanism highly conserved among sexually reproduction species and according to the studies revised by Jing *et al.* (2019) is an essential cell-division process for ensuring genetic diversity across generations. Moreover, the analysis of meiotic process associated with palynology is important for the evaluation of plant fertility and consequently, for the knowledge of its reproductive success.

The knowledge of reproductive aspects of individuals present in natural populations is important for the development of adequate management strategies associated with maintaining the balance of ecosystems, because meiotic instability caused by irregularities can affect the fertility of the plant and can lead to the decadence of a plant variety (Pagliarini 2001) and an inadequate quantity or quality of pollen can reduce plant reproductive success- seed quantity or quality. (Ashman *et al.* 2004). Thus, considering the economic potential of *S. terebinthifolia,* its possible sustainable use and the scarcity of this studies about specimens from Espírito Santo, Brazil we will describe the meiotic behavior, the pollen potential viability and some morphological and cytochemistry descriptions of pollen grains from individuals from a natural population.

Material and Methods

Plant material

Schinus terebinthifolia Raddi is dioecious with pentameric, actinomorphic flowers. The male flower has 10 paired, stamen in two concentric rows, with yellow anthers. We collected inflorescences from five randomly chosen male plants in an area of mangroves on the Goiabeiras Campus of the Federal University of Espírito Santo, municipality of Vitória, Espírito Santo state, eastern Brazil (20º16'38"S, 40º18'27"W). Exsiccates for each specimens were deposited in the Herbaria VIES: *K. Bernardi* (VIES 025704), *LBS. Calazans* (VIES 48744, VIES 48745, VIES 48746 and VIES 48747).

Climate in the study region is tropical seasonal with a minimum temperature during the coldest month (July) of 12–18ºC, and the highest temperature during the warmest month (February) is 31–34ºC. Rainfall is greatest from October to January (average maximum rainfall in November at 230 mm) is when most rainfall occurs, while June has the least (60 mm) (Incaper 1999).

Meiotic behavior

To obtain pollen mother cells (PMCs), male young floral buds from five individuals were fixed for 24 h in absolute ethanol: glacial acetic acid 3:1. For slide preparation, anthers from five flowers were squashed in 1% acetocarmine dye (Belling 1921). Chromosome number was determined at the diakinesis stage of prophase I. Were examined 100 cells per slide undergoing meiosis to describe the stages and 10 slides per individual. Cells were photomicrographed from slides using Olympus BX-51 microscope with Digital Image System.

Pollen stainability

Pollen stainability and pollen shape were used as an indicative of pollen viability. Male inflorescences were collected prior to anthesis and immediately fixed in absolute ethanol: glacial acetic acid 3:1. Squash preparations were stained with 1% acetocarmine according to procedures described by Kearns & Inouye (1993). Stained pollen grains with well-preserved shapes were considered potentially viable. Grains that do not stain or are empty were considered unviable. Samples of 1000 pollen grains were analyzed from each individual and analyzed five slides per individual. We compared pollen viability among plants to examine how viability varied among plants, using Analysis of Variance. Statistical tests were carried out in R Development Core Team (2019).

Pollen morphology and cytochemistry

Pollen grains were obtained from mature anthers in pre-anthesis of five individuals. The polliniferous material was processed according to the acetolysis method established by Erdtman (1960) with modifications in the time and temperature of incubation in acetic anhydride: sulfuric acid solution. Fixed anthers were separated and placed in microtubes with glacial acetic acid for 10 min, followed by macerations and separation with a needle. The resultant material was centrifuged at 2,500 rpm for 10 min. The supernatant was collected and discarded, followed by addition of acetic anhydride: sulfuric acid 9:1 solution. After stirring, the material was washed in 85 ºC water for 2 min and then followed again by centrifugation at 2,500 rpm for 10 min. The supernatant was discarded, distilled water and two drops of ethyl alcohol were added. Stirring followed by centrifugation was repeated, and again the supernatant was discarded, followed by the addition of glycerine and distilled water 1:1 (v/v). Once again, stirred and centrifuged, discarding the supernatant leaving the pollen grains. Slides were mounted in glycerinated gelatin. The polar and equatorial diameter and exine thickness were measured in equatorial and polar view, respectively. Twenty-five measurement were taken at random from each individual. Terminology for pollen shape, exine ornamentation and other characteristics follows Erdtman (1952), Punt *et al.* (2007) and Willard *et al.* (2004).

For cytochemistry analysis, five slides per plant were stained using Lugol's iodine solution and Sudan IV for starch and lipids (Baker & Baker 1979). Dark- brown and red stained pollen grains were considered starch and lipid positives, respectively. Samples of 1000 pollen grains were analyzed from each individual.

Pollen was photographed using an Olympus CX41 microscope with digital camera. Image J software (Abramoff *et al.* 2004) was used for pollen grains measurements. All analyses were carried out in the Botanical Sector of the Federal University of Espírito Santo in Vitória, Espírito Santo, Brazil.

Results

Meiotic behavior and pollen stainability

Meiocytes in all meiotic stages were clearly observed. Meiosis in *S. terebinthifolia* is typical and no irregularities were seen (Fig. 1). We identified meiotic divisions during zygotene (Fig. 1a), diakinesis (Fig. 1b), metaphase I (Fig. 1c), telophase I (Fig 1d), beside microspores tetrad (Fig. 1e). During diakinesis seven bivalents were noted (Fig. 1a) which allows us to infer that the somatic number of chromosomes is 2n = 2x = 14. Daughter nuclei in telophase I have n = 7 chromosomes (Fig. 1d). Viable (colored pollen grains, Fig. 1f) and empty pollen grains are, in fact, unviable grains were identified through staining.

Pollen stainability varied among plants ($F_{4,120} = 39.0$, $r^2 = 0.80$, $P < 0.001$. Plant viability varied from the low average of 75% in plant 4, to the high average of 97% in plant 5. Within plant with pollen potentially viable varied from 35% to 90% in plant 4 and from 94% to 100% in plant 5. Plant 4 had the lowest pollen stainability which was lower and different from all the rest, followed by plants 1, 2 and 3, which were all similar (with stainability from 90–95%) and plant 5 with the greatest viability. Overall, median pollen stainability was high, at 94%, and so typical pollen stainability is high, and even in the least potential viable plant, the median pollen stainability was 79%.

Pollen grain morphology and cytochemistry

Pollen in *S. terebinthifolia* are monads, subcircular, sub-spheroidal (prolate-spheroidal) and tricolporate, (Fig. 2a-d). The colpus tend to be relatively long and almost reach the poles when viewed from the pole (Fig. 2c). The exine is striate-reticulate (Figs. 2b-d). Pollen grains features of *S. terebinthifolia* are described in Table 1. Pollen is positive for starch since staining with Lugol's iodine results in a dark brown color (Fig. 3a). They are lipid negative considering the test performed (Fig. 3b).

Discussion

Meiotic behavior, diploidy and pollen stainability

Meiocytes obtained in the present study enabled the observation of meiotic behavior and the determination of the chromosome number. In some cases, meiotic counts can be more accurate due to the reduced number of chromosome units (bivalents), natural spreading of bivalents in diakinesis, and large cell size of meiocytes (Guerra 2008) as was observed for *S. terebinthifolia*. Surprisingly, we found that the plants analyzed are diploid (2n = 2x = 14). This chromosome count is very different from the chromosome numbers described in previous studies. A chromosome count of 2n = 60 was demonstrated by Sarkar *et al.* (1973), and by Moore (1977) and 2n = 4x = 28 by Pedrosa *et al.* (1999), Franco-Cairo *et al.* (2009), Luz *et al.* (2015) and Bitencourt (2016) for *S. terebinthifolia.*

Polyploidy has been very important for speciation and evolution in plants (Otto & Whitton 2000). Severe environmental fluactuations and/or stress may bring about unreduced gamete formation in germline or mitotic errors in somatic tissues, enabling the establishment of new genomic states. However, fixation of any such variation should involve merchanisms that produce reproductively isolated lineages. This could be realized either through polyploidy that may lead to stable genomic states owing to their tolerance to post-polyploid genetic modification (Lavania 2020).

The family Anacardiaceae is known for polyploidy in a variety of species and its evolution was at the tetraploid level. Moreover, the family has been little examined cytologically. Raven (1975) describes the basic chromosome number for the family as x = 7, with the majority of species being tetraploid (2n = 4x = 28). For example, other species in the genus *Schinus* in Argentina all were 2n = 4x = 28 (*S. areira, S. fasciculatus, S. johnstoni, S. longifolius, S. o'donelli, S. patagonicus, S. praecox, S. roigii*e, *Schinus* sp., Peñas *et al.* 2006). Cytogenetic analyses of 22 accession samples of *S. terebinthifolia* in Rio Grande do Sul were

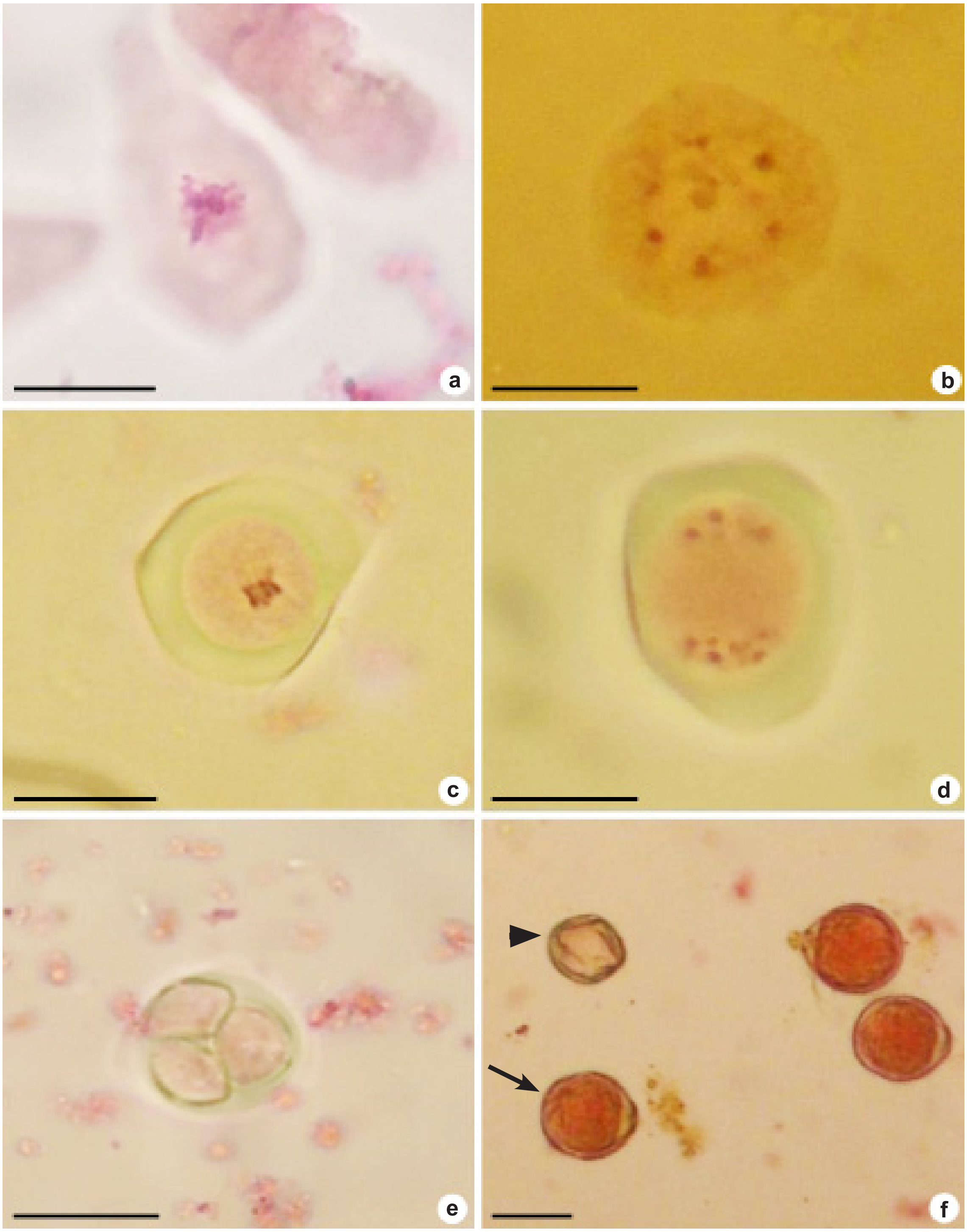

Figure 1 – a-f. Meiotic behavior and pollen viability in *Schinus terebinthifolia* – a. zygotene; b. diakinesis with seven bivalents; c. metaphase I; d. telophase I; e. tetrad; f. viable (colored, indicated by the arrow) and unviable (translucent grain, indicated by the arrowhead). Bars: a-e = 10µm; f = 20µm.

performed by Luz *et al.* (2015). These authors determined a chromosome number of 2n = 4x = 48 indicating there is no intraspecific variability in *S. terbinthifolia.* The regularity of meiotic process that we found here, and the consistent count of 2n = 2x = 14 is surprising.

A general rule to the direction of events is that tetraploids or higher ploidy levels are always derived from diploids or low ploidy levels (Guerra 2008). However, according this author, the most intriguing question is the frequent observation of high chromosome numbers or high ploidy levels in clades characterized by basal traits while those with more derived characters are diploids or low polyploids, in an apparent contradiction of the diploid-derived-polyploid rule. This trend has been reported in some families as Rutaceae (Stace *et al.* 1993) and Passifloraceae (Melo *et al.* 2001). Therefore, further studies on the origin and evolution of polyploidy in *S. terebinthifolia* should be performed. Chromosome variations such as polyploidy, hybridization or dysploid

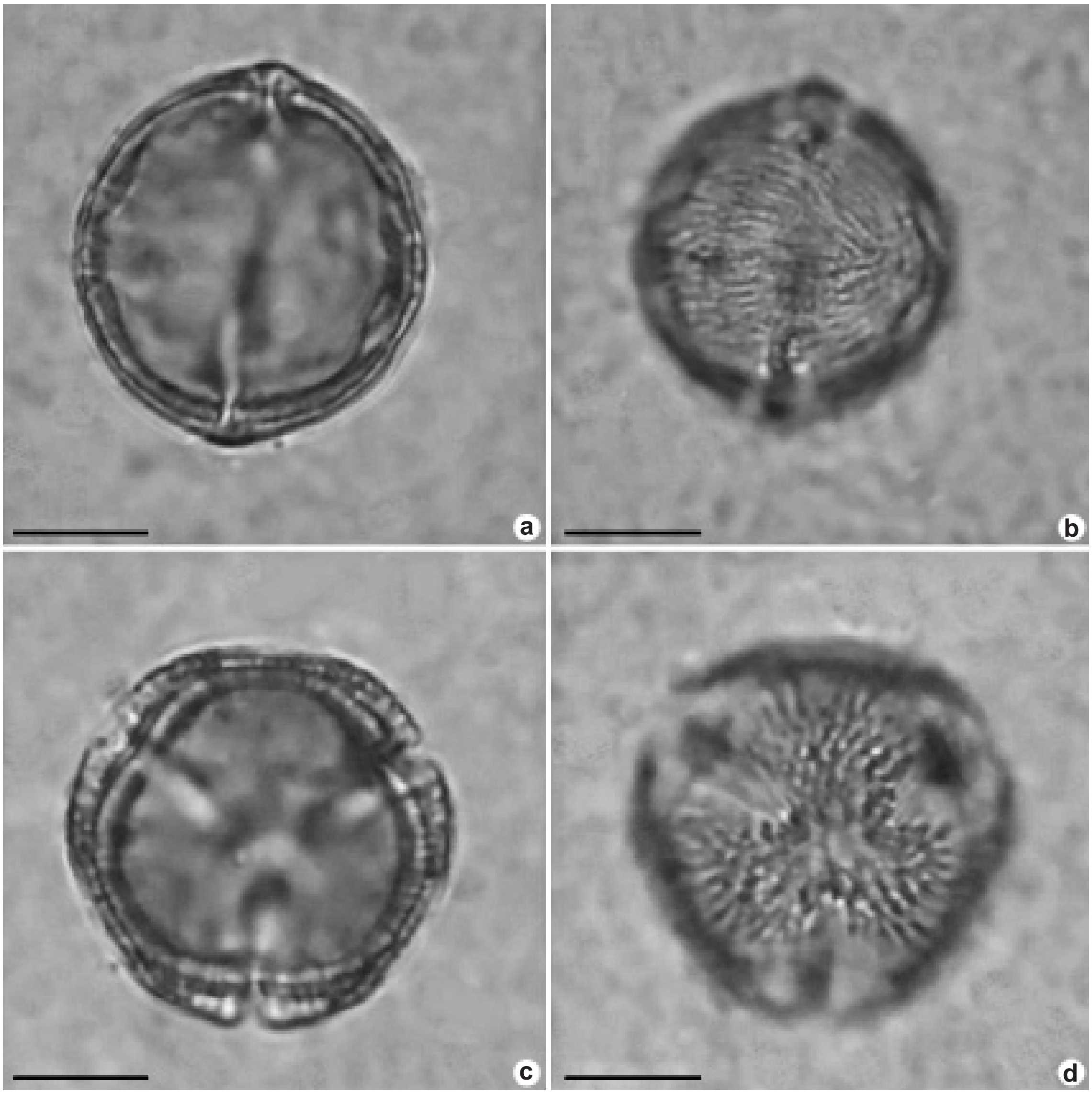

Figure 2 – a-d. Photomicrographs of pollen grains of *Schinus terebinthifolia* – a. equatorial view indicating the exine and two apertures; b. equatorial view illustrating striate-reticulate ornamentation; c. polar view illustrating triangular shape with three, near polar, apertures; d. polar view indicating striate-reticulate ornamentation. Bars: 10 µm.

Table 1 – Measurements of polar and equatorial diameters and exine thickness, P/E, shape, amb, apertures and exine ornamentation of *Schinus terebinthifolia*.

Pollen features	
Measurements (µm)	
Polar Diameter (P)	24 ± 1,42
Equatorial Diameter (E)	22 ± 1,30
Exine thickness	1,3 ± 0,37
P/E	1,09
Shape	Subesferoidal (prolate sferoidal)
Ambit	Subcircular
Apertures	Tricolporate
Exine ornamentation	Striate-reticulate

change may provide the cytological basis for ecological differentiation, adaptation and isolation (Winterfield *et al.* 2020) and the identification of changes in the ploidy level is useful to know the evolutionary history of a species.

In plants, irregular meiosis is often responsible for unviability of the pollen grains due to the formation of unbalanced microspores (Souza *et al.* 2006). Thus, pollen viability is a direct consequence of regular and consistent meiosis (Pagliarini 2002). Here, with this first examination of meiosis and pollen potential viability in the state of Espírito Santo, our results are surprising and different from those chromosome reports carried out elsewhere. Also, here we observed several phases of meiosis in contrast to other studies that only observed tetrads at the final stage of meiosis (Bitencourt 2016).

Pollen stainability was extremely high in almost all individuals analyzed, typically greater than 90%. Determination of pollen viability can be done using cytological parameters, such staining techniques (Belhadj *et al.* 2007), *in vitro* and *in vivo* germination (Perez *et al.* 2019), measures of respiration of chemical conductivity, content of proline and capacity to effect seed set (Dafni & Firmage 2000). Although the histochemical test is quick, easy and inexpensive, it should not be

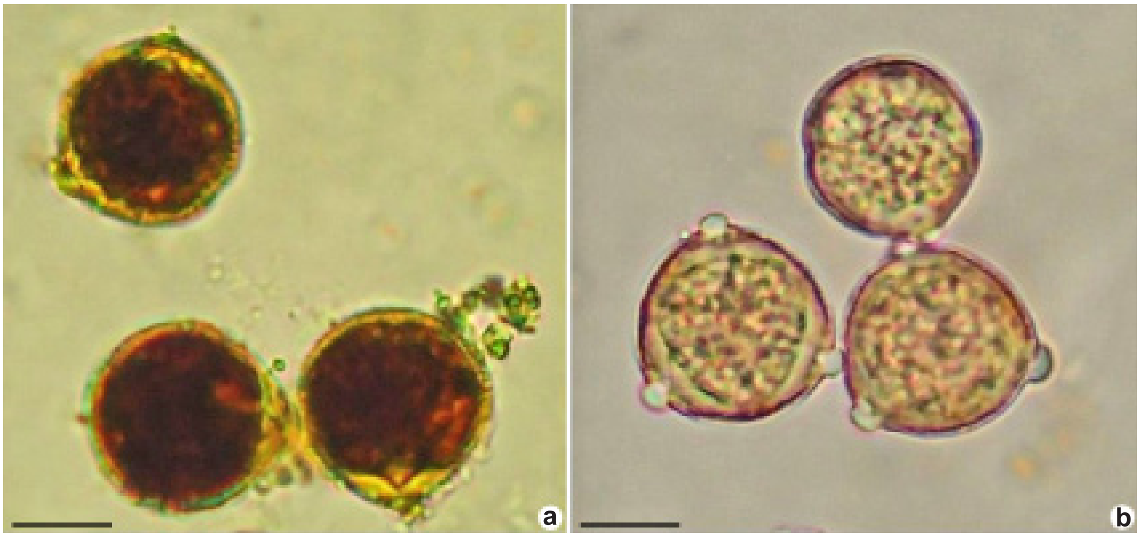

Figure 3 – a-b. Photomicrographs of pollen grains of *Schinus terebinthifolia* – a. pollen stained with Lugol indicating starch positive; b. pollen stained with Sudan IV indicating lipid negative. Bars: 10 µm.

the only method used to estimate pollen viability, since it does not supply information on the germinative capacity (Soares *et al.* 2016). Thus, it is recommended that several tests would be used simultaneously to reflect several componentes of pollen performance (Dafni & Firmage 2000). Although plants may have shown high pollen stainability will require further study.

Pollen morphology and cytochemistry

Pollen morphology and cytochemistry are useful taxonomic and ecological tools (Takahashi 1997). Both are strongly heritable features and very consistent within taxa (Santiago *et al.* 2004; Blackmore 2007), but intraspecific variations can be observed.

In this work, pollen grains for all individuals showed uniformity in shape, exine sculpturing and aperture number. However, *S. terebinthifolia* pollen shape and ornamentation is variable when compared among different studies. Cruz-Barros & Granito (1997) for example, describe pollen of *S. terebinthifolia* var. *acutifolia* as prolate spheroidal (subspheroidal) with reticulate ornamentation. Others authors describe pollen of *S. terebinthifolia* Raddi as prolate, striate reticulate (Barros *et al.* 1999); subprolate and reticulate (Takeda *et al.* 2000), subprolate with sculpture finely reticulate (Willard *et al.* 2004) and oblate-spheroidal with striate-reticulate ornamentation (Bitencourt 2016). Intraspecific variability is actually expected at every scale in evolutionary ecology, but this raw material of natural selection can also be due to non-adaptative processes, e.g. genetic drift between small and isolated populations. Detecting intraspecific variability is usually the first step before discussing the adaptiveness of a given trait and linking ecological constraints and evolutionary changes (Bonhomme *et al.* 2013).

In some studies, polyploidy has been shown to influence pollen morphology (Yan *et al.* 1997; Ortiz 1997; Cavalcante *et al.* 2000). In the plant family Malvaceae, chromosome number is associated with the number of apertures in the pollen grains (*Modiolastrum* spp., n = 5, with 3 to 5 apertures, *Modiola* spp., n = 9, with 6 apertures, *Tropidococcus* spp. n = 12, with 12 apertures; Cuadrado 2003). Shape, size and number of apertures also varied in *Lippia alba* (Verbenaceae) depending on polyploidy level. Diploid plants (2n = 2x = 30) had triangular grains with three apertures, while tetraploid plants (2n = 4x = 60) had triangular and quadrangular grains with three and four apertures (Pierre 2008). Other species in the genus *Lippia* also had a relationship between chromosome number and pollen morphology (Sousa *et al.* 2013). With *S. terebinthifolia*, similarity in number of apertures was despite polyploidy (in all studies were observed tricolporate pollen grains) and variations in pollen shape and exine ornamentation were observed.

Pollen in the Phanerogamae (seed-producing plants) can be divided into having starch or lipids as their energy reserves. We found that in *S. terebinthifolia*, pollen stored energy as starch. This was in contrast to studies in which both starch and lipids were used for storage in this species (Bitencourt 2016). Cytochemistry techniques can be used to obtain information about cytological localization of the substances (Nepi & Fanchi 2000). However, these are qualitative techniques that can be influenced of the type of fixation on the preservation cellular compounds (Nepi & Franchi 2000), stain time and stain concentration (Konyar & Dane 2013). Thus, these results can be considered as technical limitations of the method. Typically, pollen with starch reserves are dispersed by the wind or animals that do not consume the pollen and these grains are relatively large (Zona 2001). The Brazilian pepper tends to be pollinated by flies, bees and wasps that collect and consume pollen grains (Lenzi & Orth 2004). Starch in pollen can also have other functions, such as protecting the grain against dessication (Franchi *et al.* 1996). Thus, plants that are quickly pollinated following anthesis should not store starch because they are not likely to become dessicated. On the other hand, wind-pollinated plants or when pollination is not rapid after anthesis, starch offers a source of sacarose and other oligosaccharides for protection against drying. In *S. terebinthifolia*, pollination is not necessarily rapid and can occur hours after anthesis (Lenzi & Orth 2004).

The meiotic behavior was described for the first time for *S. terebinthifolia*. Moreover, the Brazilian pepper is surprising in that plants in Espírito Santo analyzed in the present study were all diploid, while elsewhere the species was found to be polyploid. The specimens presented regular meiotic behavior and pollen stainability was very high, being potential indicators of fertility for this species.

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