UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE BIOCIÊNCIAS - DEPARTAMENTO DE BOTÂNICA PROGRAMA

DE PÓS-GRADUAÇÃO EM BOTÂNICA

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DESENVOLVIMENTO DO GRÃO DE PÓLEN EM CYPERACEAE: ASPECTOS MORFOLÓGICOS, CITOLÓGICOS E MOLECULARES

Porto Alegre

2019

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Tese apresentada ao Programa de Pós-Graduação em Botânica da Universidade Federal do Rio Grande do Sul, como parte dos requisitos para a obtenção do Título de Doutor em Botânica

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Porto Alegre

2019

Agradecimentos

À minha família, pelas décadas de apoio,

Ao meu orientador Jorge Ernesto Araújo Mariath e meu co-orientador André Luís Laforga Vanzela, pela confiança depositada em mim e pelos ensinamentos ao longo da minha formação,

Aos membros da banca examinadora, Marccus Vinícius Alves, Janete Palma Fett e Alexandra Antunes Mastroberti, pela disponibilidade em avaliar esse trabalho,

A todos envolvidos no Programa de Pós-Graduação em Botânica da Universidade Federal do Rio Grande do Sul, pelo trabalho envolvido em tornar a minha formação e a de tantos outros possível,

Às agências de fomento que financiam a grande maioria da pesquisa cientifica no Brasil, em especial a CNPq pela bolsa concedida. Sem essa bolsa, a realização dos trabalhos contidos neste doutorado e a minha formação profissional não teriam sido possíveis.

Aos colegas de trabalho do Laboratório de Anatomia Vegetal da UFRGS e do Laboratório de Citogenética e Diversidade Vegetal da UEL, pela ajuda durante a execução deste trabalho, e aos colegas de trabalho do Departamento de Biologia Estrutural, Molecular e Genética da Universidade Estadual de Ponta Grossa, pela ajuda e compreensão durante o meu período como professor substituto,

À minha segunda família, principalmente: Fernanda Nogueira, Raquel Bastos, Lucas Johan, Fátima Arcanjo, Mariana Pires, Eloá Castro, e especialmente, Leandro Fazzano, por tudo.

Resumo

A família Cyperaceae é conhecida por apresentar indivíduos com características citológicas peculiares, como a meiose assimétrica que resulta em uma estrutura chamada pseudomônade durante a microsporogênese. Nessa estrutura, três micrósporos são abortados, enquanto um, o funcional, dá origem ao grão de pólen. Esta tese abordou vários dos aspectos desse processo, utilizando técnicas de microscopia de luz e eletrônica, bioinformática e biologia molecular. A análise dos diferentes estádios de desenvolvimento da pseudomônade a partir do final da microsporogênese mostrou que a microgametogênese também possui aspectos peculiares em Cyperaceae, como a vacuolação tardia do microscópio funcional que ocorre concomitantemente com a morte celular dos degenerativos. Durante esse processo, as linhagens degenerativa, generativa e vegetativa são isoladas por paredes de calose. O grão de pólen formado acumula reservas diferentes dependendo do ambiente, e possivelmente é mais longevo e de rápida germinação em relação a outros polens similares. Ele apresenta ainda um sistema de endomembranas contendo retículo endoplasmático cortical e concêntrico, além de secreção não-convencional. Apesar dessas características aparecerem em um pólen originado a partir de uma microgametogênese peculiar, há registro na literatura de ocorrências similares em outras angiospermas. As análises de bioinformática e biologia molecular revelaram que alguns genes relacionados a morte celular programada estão presentes em células mães de micrósporo e inflorescências de *Rhynchospora*. Um deles é o gene de uma proteína de choque térmico que, segundo a literatura, está relacionada ao acúmulo de espécies reativas de oxigênio e resposta hipersensitiva.

Abstract

Representatives of the Cyperaceae family are known for presenting peculiar cytological features such as an asymmetric meiosis which results in a structure called pseudomonad during microsporogenesis. In this structure, three microspores are aborted while the functional one gives rise to the pollen grain. This thesis addressed several aspects of this process, using light and electron microscopy, bioinformatics and molecular tools. The analysis of different stages of pseudomonad development from the end of microsporogenesis to the mature pollen revealed that microgametogenesis also presents peculiar aspects in Cyperaceae, such as the late vacuolation of the functional microspore that occurs concomitantly to cell death of degenerative microspores. During this process, callose walls isolate the degenerative and functional cellular lineages in pseudomonads. The pollen grain accumulates different reserves depending on the environment, and probably is more longevous and rapid germinating in relation to similar pollen. It presents a endomembrane system containing cortical and concentric endoplasmic reticulum networks, as well as unconventional secretion. Although these features are present in a group with particular microsporogenesis and microgametogenesis, there is record in the literature for similar occurrences in other angiosperms. Bioinformatics and molecular analysis revealed some genes related to cell death occurring in microspore mother cells and inflorescences of *Rhynchospora*. One of them is a heat shock protein gene that, according to the literature, is related to the accumulation of reactive oxygen species and hypersensitive response.

Súmario

Prefácio

A presente tese, que teve sua execução entre os anos de 2015 e 2019, teve como objetivo contribuir para o entendimento de uma estrutura peculiar formada durante o desenvolvimento do grão de pólen em Cyperaceae, a pseudomônade. Essa tese está organizada na forma de capítulos, formatados conforme as normas da revista Botanical Journal of the Linnean Society.

O primeiro capítulo abrange um referencial teórico, com informações a respeito do ciclo de vida das plantas e os processos que atuam na formação de micrósporos e grãos de pólen em angiospermas em geral, na microsporogênese e na microgametogênese, respectivamente. Em seguida, os mesmos processos são explicados na família Cyperaceae. Ainda nesse capítulo são mostrados os principais objetivos e hipóteses que guiaram a execução dessa tese.

O segundo capítulo é um artigo publicado em 2018, onde o desenvolvimento da pseudomônade foi sistematizado em cinco estádios de desenvolvimento, contemplando principalmente os eventos após a microsporogênese. Nesse trabalho também foram realizados estudos com citoquímica a fim de elucidar aspectos relacionados a biologia celular dessa estrutura, bem como aspectos preliminares da ultraestrutura do grão de pólen.

O terceiro capítulo é um artigo a ser submetido, onde a ultraestrutura do grão de pólen de quatro espécies de Cyperaceae foi estudada. Nele, podemos perceber que assim como a microsporogênese, o grão de pólen em Cyperaceae também apresenta alguns aspectos peculiares, principalmente relacionados ao sistema de endomembranas, mais especificamente o retículo endoplasmático, aparelho de Golgi e outras organelas.

O quarto capítulo traz um estudo preliminar sobre a expressão gênica de pseudomonades em Cyperaceae, onde foi possível perceber que um gene de proteína de choque térmico é amplamente encontrado no transcriptoma sequenciado e também é expresso em maior quantidade em relação ao gene padrão utilizado, actina. Esse gene, assim como alguns outros, estão relacionados a um tipo de morte celular chamado resposta hipersensível que envolve a formação de espécies reativas de oxigênio, e por sua vez podem estar atuando nos processos de desenvolvimento e morte celular que ocorrem nas pseudomônades.

Por último, são discutidas algumas das principais conclusões finais que puderam ser obtidas durante a execução dos três trabalhos, bem como as perspectivas futuras para o estudo do desenvolvimento do grão de pólen em Cyperaceae.

CAPÍTULO I

Referencial teórico

Reprodução sexual em plantas

O ciclo de vida das plantas é marcado pela alternância entre gerações: cada geração é representada por um indivíduo multicelular diploide e um indivíduo multicelular haploide. O indivíduo multicelular diploide é chamado esporófito, já que este produz, assexuadamente, esporos por meiose. Ao germinarem, os esporos se desenvolvem em indivíduos multicelulares haploides, chamados gametófitos, já que se reproduzem sexuadamente produzindo gametas por mitose. A união dos gametas produzidos pelos gametófitos irá gerar um zigoto diploide, que, por sua vez, dará origem a um indivíduo multicelular diploide, completando assim o ciclo de vida. Os esporófitos compõem, portanto, a geração esporofítica, enquanto que os gametófitos compõem a geração gametofítica. Essas gerações alternam entre si durante o ciclo de vida das plantas, sendo que em alguns grupos uma dessas gerações pode ser mais ou menos reduzida (Mariath *et al.*, 2012).

As briófitas, musgos e liquens são grupos cedo-divergente de plantas. Nelas, a geração gametofítica é a geração dominante, com rizoides, caulídios e filídios. Na maioria dos exemplares desses grupos, existem dois tipos de gametófitos, um masculino e o outro feminino. Cada um deles produz um tipo específico de gametas, masculino e feminino. O gameta masculino é flagelado e chamado anterozoide. Ele é produzido em estruturas presentes nos gametófitos masculinos chamados anterídios. Quando há água no ambiente, os anterídios podem se deslocar para o gametófito feminino, onde são produzidos os gametas femininos, também chamados de oosferas, em estruturas chamadas arquegônios. Quando um anterozoide fecunda a oosfera, é formado um zigoto diploide que germina no próprio gametófito feminino, dando origem ao esporófito. O esporófito, por sua vez, é efêmero e dará origem aos esporos que irão se propagar no meio. Existem dois tipos de

esporos que porem ser gerados no esporófito: os micrósporos (ou andrósporos) e os megásporos (ou ginósporos). Os micrósporos, ao germinarem, darão origem aos gametófitos masculinos, o microgametófito ou androgametófitos, enquanto que os megásporos darão origem aos gametófitos femininos, o megagametófito ou ginogametófito (Raven, Evert & Eichhorn, 2001a; Mariath, Santos & Bittencurt, 2006; Maciel-Silva & Pôrto, 2014).

As plantas vasculares sem sementes, as pteridófitas, também possuem alternância de gerações. Entretanto, diferente do que acontece nas briófitas, nas pteridófitas a geração esporofítica é a geração dominante, compondo o corpo vegetativo da planta, com caules, raízes e folhas. No esporófito também existem estruturas de reprodução assexual, que podem ser soros ou estróbilos. Em algumas pteridófitas, como as samambaias, são produzidos nessa estrutura um único tipo de esporo, que, ao germinar, dará origem a um gametófito bissexual efêmero de vida livre. Esse gametófito possui tanto anterídios quanto arquegônios, produzindo, portanto, ambos os gametas masculino e feminino, os anterozoides e as oosferas, respectivamente. Na presença de água, os anterozoides podem se deslocar até os arquegônios, fecundando a oosfera e gerando um zigoto diploide. O zigoto dará origem a um novo esporófito, completando assim o ciclo de vida. Em outras pteridófitas, como *Selaginella*, o esporófito pode produzir tanto micrósporos quanto megásporos, que darão origem aos microgametófito e megagametófito, respectivamente, que são ambos efêmeros. No meio ambiente, os microgametófitos irão produzir os anterozoides, que podem se deslocar para os arquegônios do megagametófito, produzindo assim um zigoto diploide (Raven, Evert & Eichhorn, 2001b; Mariath, Santos & Bittencurt, 2006; Jian-Guo 2014).

Nas plantas com sementes, as gimnospermas e angiospermas, a geração gametofítica é ainda mais reduzida, sendo que os microgametófito e megagametófito agora correspondem a indivíduos microscópicos compostos de apenas algumas células. Apesar disso, o ciclo de vida ocorre com alternância de gerações, da mesma maneira que ocorre nas demais plantas: Os esporófitos produzem micrósporos e megásporos por meiose, que, ao germinarem, produzem microgametófito e megagametófito haploides. Esses, por sua vez, produzem os gametas masculinos e femininos, que, ao unir-se, dão origem a um zigoto diploide (Mariath *et al.* 2012).

Microsporogênese e microgametogênese em angiospermas

O processo de formação de micrósporos ocorre na microsporogênese. Os esporófitos em angiospermas produzem as flores, onde encontram-se as anteras. As anteras são os microesporófilos, e possuem tecidos estéreis e o tecido esporogênico. Os tecidos estéreis compõem a parede da antera, e organizam-se em epiderme, endotécio, uma ou algumas camadas médias e tapete. O tecido mais interno da antera é o tecido esporogênico, onde estará ocorrendo a microsporogênese. Nesse processo, as células mães de micrósporo irão se reproduzir por meiose, originando os micrósporos haploides (Bhandari, 1984). A microsporogênese pode ser do tipo sucessiva, em que ocorre a formação de parede celular após ambas as divisões meióticas, ou simultânea, em que não ocorre a formação de parede na meiose I, e os micrósporos são individualizados por parede simultaneamente após a meiose II. A microsporogênese simultânea é considerada um caractere derivado, e é comum em monocotiledôneas (Furness & Rudall, 1999). De qualquer maneira, na maioria das angiospermas, são produzidos quatro micrósporos por célula mãe, formando uma tétrade de micrósporos. Os quatro micrósporos inicialmente encontram-se unidos e revestidos cada um por uma espessa camada de calose, um

carboidrato polimérico formado por moléculas de glicose ligadas entre si por ligações 1,3-β. Estudos apontam que a parede de calose é importante no desenvolvimento dos micrósporos, garantindo que eles possam expressar seu genoma haploide sem interferência dos tecidos esporofíticos adjacentes que compõem a parede da antera (Bhandari, 1984). A parede de calose eventualmente é digerida pela ação da enzima calase, o que libera os quatro micrósporos da tétrade. Uma vez livres, cada micrósporo irá formar um microgametófito no processo de microgametogênese (Knox, 1984; Bhandari, 1984).

A microgametogênese na maioria das angiospermas inicia com o crescimento e vacuolização do micrósporo. O núcleo do micrósporo é deslocado para a periferia da célula, e logo acontece a primeira divisão da mitose, a mitose polínica I. Essa mitose ocorre de maneira assimétrica, gerando duas células desiguais: a célula generativa menor e a célula vegetativa maior. A célula generativa logo é englobada dentro da célula vegetativa, que, por sua vez, acumula reservas energéticas na forma de carboidratos e/ou lipídeos (Knox, 1984). A estrutura resultante é chamada microgametófito ou grão de pólen e, em aproximadamente 70% das angiospermas, ele é liberado da antera na forma bicelular, contendo uma célula generativa e outra vegetativa. Nesses casos, a célula generativa irá se dividir novamente por mitose durante a formação do tubo polínico, na mitose polínica II, gerando os gametas masculinos. Em outras angiospermas, a mitose polínica II ocorre ainda quando o grão de pólen está na antera (Brewbaker, 1967). Já em alguns casos, como em *Annona* (Annonaceae), podem ser liberados grãos de pólen tanto na forma bicelular quanto na forma tricelular (Lora, Herrero & Hormaza, 2009).

Os processos de microsporogênese e microgametogênese descritos acima são característicos da maioria das angiospermas, e nesses casos, são liberados ao meio grãos

de pólen individuais na forma de mônades. Entretanto, em alguns grupos, ocorre a agregação do grão de pólen em estruturas maiores (Harder & Johnson, 2008). Em alguns casos, como por exemplo na família Juncaceae, ocorre a formação de parede após a meiose II, individualizando os quatro micrósporos. Entretanto, após a dissolução da calose, os micrósporos permanecem unidos na tétrade. Cada micrósporo dará origem a um grão de pólen, que será liberado como uma tétrade de grãos de pólen (Munro & Linder, 1997; Furness & Rudall, 1999). Em outros casos, grãos de pólen podem formar agregados ainda maiores, como as polínias presentes em indivíduos da família Orchidaceae (Johnson & Edwards, 2000). Acredita-se que a agregação do grão de pólen em tétrades ou agrupamentos maiores aumente as chances de ocorrer fecundação, já que a maioria dos grãos de pólen falha na sua função principal que é encontrar o estigma e germinar. Em agregados de pólen, todos os grãos de pólen do conjunto são viáveis e podem germinar (Harder & Johnson, 2008). Em outros casos ainda mais peculiares, também não ocorre a liberação da tétrade de micrósporos. Porém, três dos micrósporos formados degeneram, enquanto o micrósporo funcional dá origem ao grão de pólen. Nesses casos, o grão de pólen é disperso na forma de mônade, mesmo essa única mônade tendo sido originada a partir de uma tétrade de micrósporos. Por isso, diz-se que esses grãos de pólen são dispersos na forma de pseudomônade (falsa mônade). Pseudomonades aparecem em alguns gêneros da família Ericaceae e em todos os indivíduos da família Cyperaceae estudados até então (Furness & Rudall, 2011).

A família Cyperaceae

A família Cyperaceae possui distribuição cosmopolita e é uma das maiores entre as monocotiledôneas (Goetghebeur, 1998), sendo esse um dos grupos mais diversos entre as angiospermas (Escudeiro *et al.*, 2012). Representantes dessa família são caracterizados

por serem herbáceas perenes ou anuais, com colmos geralmente triangulares e folhas alternas trísticas com corpos de sílica (Goetghebeur, 1998). Dados moleculares e morfológicos sugerem a divisão de Cyperaceae em duas grandes subfamílias: A cedo divergente e menor Mapanoide e a tarde divergente e maior Cyperoide (Muasya *et al.*, 2009). Nessas duas subfamílias encontram-se mais de 100 gêneros divididos em várias tribos. Na subfamília Cyperoide, vários gêneros apresentam um grande número de espécies, como *Carex:* 2000 espécies, *Cyperus:* cerca de 550 espécies, *Eleocharis:* 200 espécies e *Rhynchospora:* cerca de 250 espécies (Goetghebeur, 1998) A família Cyperaceae está incluída na ordem Poales e é irmã da família Juncaceae e, juntas, formam um clado irmão à família Thurniaceae (Bouchenak-Khelladi, Muasya & Linder, 2014). Os representantes dessa família possuem valor comercial significativo em algumas economias locais (Goetghebeur, 1998), bem como valor histórico: os papiros do antigo Egito eram feitos a partir de *Cyperus papyros*, um exemplar de Cyperaceae (Marota *et al.*, 2002). Entretanto, a família se destaca pela presença de algumas características peculiares em relação às demais plantas com flores em geral. Uma delas é a presença de cromossomos holocêntricos ou holocinéticos, que, em contraste aos monocêntricos ou monocinéticos, apresentam ausência de constrição primária e cinetócoro difuso ao longo de toda sua extensão (Heilborn, 1924; Vanzela *et. al.*, 1998; Cabral *et al.*, 2014). Graças a essa característica, os cromossomos desses indivíduos podem se fissionar sem que ocorra perda de DNA, já que os fragmentos cromossômicos gerados pela fissão possuirão cinetócoro e poderão se segregar na divisão celular normalmente. Graças a isso, é observado um grande espectro de números cromossômicos para a família, sendo que são descritas espécies de apenas dois cromossomos e até espécies com mais de 100 cromossomos, em células haploides. Essa variação é, inclusive, encontrada até mesmo entre espécies do mesmo gênero (Roalson, 2008). Também graças a essa característica

podem ser observados também indivíduos de uma mesma espécie com diferentes números cromossômicos (Luceño & Castroviejo, 1991). Nessa família também se observa a ocorrência de meiose pós-reducional, em que a primeira divisão meiótica ocorre de maneira equacional com a divisão das cromátides irmãs, enquanto que a segunda divisão meiótica ocorre de maneira reducional, com a separação dos cromossomos homólogos (Cabral *et al.*, 2014). Outra característica peculiar encontrada na família é a formação de pseudomônades durante a microsporogênese e a microgametogênese (Hakansson, 1954).

Microsporogênese e microgametogênese em Cyperaceaae

Ao contrário do observado nas demais angiospermas, em que a meiose da célula mãe de micrósporos origina uma tétrade de micrósporos livres, na família Cyperaceae uma tétrade de núcleos é formada após a meiose em uma célula cenocítica (Simpson *et al.*, 2003). Após o estabelecimento dessa estrutura, três núcleos são deslocados para uma das extremidades da célula e separados por parede em pequenos compartimentos citoplasmáticos, formando as células ou micrósporos degenerativos, enquanto um permanece ao centro, compondo a célula ou micrósporo funcional (Brown & Lemmon 2000; Coan *et al.,* 2010; Furness & Rudall 2011; Mariath *et al.,* 2012). A estrutura resultante é chamada pseudomônade (Håkansson, 1954; Strandhede, 1965), de aspecto piriforme com duas regiões distintas: uma maior voltada para o tecido do tapete chamada abaxial; e outra menor voltada para o lóculo da antera chamada adaxial (Ranganath & Nagashree, 2000; San Martin *et al.*, 2013). As células degenerativas podem aparecer tanto na região adaxial quanto na região abaxial, dependendo do gênero em que são observadas (Tanaka 1941), e permanecem viáveis por um longo período no desenvolvimento, com citoplasma e nucléolos funcionais (Rocha *et al.*, 2016). Entretanto, seus núcleos tornam-

se sucessivamente menores, até que, em algum momento, essas células são abortadas por morte celular programada, PCD (*Programmed Cell Death*, San Martin *et al.*, 2013; Rocha *et al.* 2016). A célula funcional, por sua vez, segue o que provavelmente seria o desenvolvimento típico encontrado nas demais angiospermas, passando por uma mitose polínica assimétrica (PM I) e dando origem às células generativa e vegetativa (Ranganath & Nagashree 2000, Brown & Lemmon 2000). Antes mesmo da liberação do grão de pólen do saco polínico, a célula generativa passa por uma segunda divisão mitótica, dessa vez simétrica, chamada mitose polínica II (PM II), dando origem aos gametas do grão de pólen (San Martin *et al*., 2013). Entretanto, tal processo de microgametogênese não é descrito em detalhe na literatura.

Esse tipo de desenvolvimento, em que a meiose da célula mãe de micrósporos origina uma pseudomônade, não é exclusivo da família Cyperaceae, aparecendo estruturas similares em Ericaceae (Furness & Rudall, 2011). Porém, apenas em Cyperaceae pode ser considerado um caractere autapomórfico (Kirpes *et al.*, 1996; Furness & Rundall, 2011). Apesar disso, pouco se sabe sobre quais seriam os fatores envolvidos no estabelecimento das pseudomonades, visto que a perda de três dos quatro produtos meióticos poderia gerar a redução da quantidade de grãos de pólen e, com isso, perda de variabilidade.

Em diversas plantas modelos, como *Arabidopsis thaliana* e *Oryza sativa*, muitos genes envolvidos no desenvolvimento do grão de pólen já foram caracterizados. Alguns deles, como *SIDECAR POLLEN* e *GEMINI POLLEN1* foram identificados como sendo fundamentais no estabelecimento da assimetria na PM I (Chen & McCormick 1996; Park, Howden & Twell, 1998). Outros, como o *TAPETUM DEGENERATION RETARDATION*, são necessários para a degeneração do tecido do tapete e

desenvolvimento da antera (Li *et al.,* 2006). Mais genes já foram identificados, assim como os prováveis papéis de vários deles no desenvolvimento e na morfologia já foram descritos (Honys & Twell 2003; Becker *et al.,* 2003; Huang *et al.,* 2009, Huang *et al.,* 2011). Adicionalmente, alguns genes já foram descritos atuando em diversos processos de morte celular em plantas, como por exemplo na resposta hipersensitiva, que ocorre em reação a um determinado patógeno (Lam, Kato & Lawton, 2001). Dentre esses genes, certas proteínas de choque térmico são conhecidas por poderem participar no processo (Kim & Hwang, 2015). Contudo, apesar de envolver a formação de micrósporos, grãos de pólen e morte celular programada, não há relato algum na literatura que indique um possível gene ou fator envolvido no estabelecimento de pseudomônades em Cyperaceae.

Justificativa

Até o presente momento, muitos dos trabalhos envolvidos no estudo de pseudomônades na família Cyperaceae tiveram como objetivo caracterizar os aspectos citológicos, morfológicos e ultraestruturais da microsporogênese (Hakansson, 1954; Strandhede, 1973; Kirpes *et al.,* 1996; Furness & Rudall, 1999; Brown & Lemmon, 2000; Coan *et al.,* 2010; Mariath *et al.,* 2012; San Martin *et al.,* 2013). Esses estudos mostraram que o posicionamento de núcleos varia entre gêneros da família, apesar do processo de formação e desenvolvimento parecer o mesmo. Na maioria dos gêneros estudados, como *Carex*, *Eleocharis*, *Hellmuntia*, *Hypolytrum*, os núcleos degenerativos encontram-se voltados para a região adaxial da pseudomônade (Brown & Lemmon 2000; Coan *et al.,* 2010; Furness & Rudall 2011; Mariath *et al.,* 2012). Esse posicionamento levou a hipótese de que a distância entre os núcleos degenerativos do tecido do tapete estaria envolvida no seu abortamento por PCD (Håkanson, 1954; Kirpes *et al.,* 1996). Entretanto, no gênero *Rhynchospora*, núcleos degenerativos encontram-se voltados para a região

abaxial, próximos ao tecido do tapete (Coan *et al.*, 2010; Mariath *et al.*, 2012; San Martin *et al.*, 2013).

A observação do comportamento de elementos do citoesqueleto e sistema de endomembranas nas pseudomônades mostra um deslocamento de um fragmoplasto organizado tardiamente, o que leva a compartimentalização de núcleos degenerativos em pequenos volumes citoplasmáticos (Brown & Lemmon 2000; San Martin *et al.*, 2013). Sugere-se também que essa compartimentalização poderia ser responsável pela diferenciação dos micrósporos degenerativos e funcional (Håkanson 1954; Strandhede 1973). Entretanto, após a PM I, o núcleo generativo é de modo similar compartimentalizado em um pequeno volume citoplasmático formando a célula generativa que, apesar de adjacente às células degenerativas, é funcional e forma os gametas por mitose (Ranganath & Nagashree, 2000).

Esses dados indicam que o processo de diferenciação de células degenerativas e funcional (vegetativa e generativa) seria provavelmente um evento controlado e mediado pela expressão de diferentes genes. Porém, ainda não é caracterizado sequer um único gene envolvido nesse processo que parece ter poucos paralelos entre as angiospermas. É comum a degeneração de três dos quatro megásporos após a meiose durante o desenvolvimento do rudimento seminal ou óvulo (Papini *et al.*, 2010). Entretanto, esses são formados por uma meiose regular, com células de tamanho e composição citoplasmática similares (Bajon *et al.*, 1999). No caso da formação de micrósporos na família Cyperaceae, os esporos da tétrade apresentam uma organização citológica interna altamente discrepante, sendo que a célula funcional apresenta uma quantidade maior de área citoplasmática e organelas (Ranganath & Nagashree, 2000). O próprio processo de PCD também parece não possuir relação com outros encontrados em angiospermas. No

caso das células degenerativas, apesar de possuírem núcleos sucessivamente menores, esses permanecem funcionais mesmo após a PM I (Rocha *et al.*, 2016).

O processo de formação de pseudomônades e estabelecimento do grão de pólen em Cyperaceae é um caso ainda não compreendido e muito peculiar de desenvolvimento, envolvendo assimetria celular e PCD. Entretanto, esse processo ocorre em um órgão em que esses eventos são comumente observados, porém em estádios diferentes do desenvolvimento ou em células diferentes, como por exemplo, a PM I e células do tecido do tapete (Tanaka 1997, Papini *et al.*, 1999). É de se esperar, portanto, que os genes envolvidos nesses casos possam ser os mesmos envolvidos no desenvolvimento de pseudomônades. Sendo assim, um estudo que relacione a expressão gênica envolvida no seu desenvolvimento com suas respectivas alterações morfológicas poderia elucidar as vias de diferenciação e de PCD encontradas durante o desenvolvimento das pseudomônades na família Cyperaceae. O entendimento dessas vias poderia esclarecer como uma estrutura que aparentemente leva a perda de variabilidade pôde ser fixada nessa família. Mais ainda, a descrição completa de um caso tão peculiar de desenvolvimento do grão de pólen certamente contribuiria com o conhecimento de como é formada essa estrutura em angiospermas em geral. Pseudomônades ainda apresentam um caso fascinante de diferenciação celular, em que várias linhagens celulares completamente distintas se desenvolvem confinadas no pequeno ambiente do grão de pólen. A compreensão de como essas linhagens podem se estabelecer nessa estrutura a partir de uma única célula poderia ainda esclarecer vias e mecanismos de diferenciação celular e aborto celular em plantas em geral.

Hipóteses

Em relação ao desenvolvimento do grão de pólen em Cyperaceae, foram levantadas as seguintes hipóteses:

-O perfil de desenvolvimento de pseudomônades é similar nos diferentes indivíduos da família Cyperaceae, exceto pela posição dos núcleos degenerativos;

-A microgametogênese em Cyperaceae provavelmente segue os padrões vistos nas demais angiospermas.

-Genes já descritos que atuam em outros eventos do desenvolvimento da antera ou de outros tecidos das plantas estariam relacionados com as alterações morfológicas encontradas em pseudomônades.

Objetivos

Geral

Descrever as alterações morfológicas e ultraestruturais nas pseudomonades de Cyperaceae e buscar uma relação entre essas alterações e a expressão gênica ocorrendo nesses tecidos.

Específicos

- Obter o perfil de desenvolvimento de anteras de indivíduos dos gêneros *Rhynchospora* e *Eleocharis* em pelo menos cinco fases distintas: célula mãe de micrósporo, meiose, pseudomônade, mitose polínica I e mitose polínica II (grão de pólen imaturo).

- Para cada um dos estádios previamente descritos, proceder a analise morfológica, citoquímica e ultraestrutural dos tecidos da antera, bem como dos micrósporos/pseudomonades/tétrades/grãos de pólen;

-Localizar, a partir do transcriptoma sequenciado de *Rhynchospora pubera*, genes de interesse, relacionados ao desenvolvimento dos esporos e do grão de pólen;

-Isolar o RNA das inflorescências de *Rhynchospora* e converter RNA extraído de anteras em cDNA para que possa ser feita a amplificação dos genes selecionados;

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CAPÍTULO II

Comparative study of microgametogenesis in members of Cyperaceae and Juncaceae: a shift from permanent pollen tetrads to pseudomonads

Artigo publicado na revista *Botanical Journal of The Linnean Society*, 2018, **188:** 59-73.

Title: Comparative microgametogenesis study between Cyperaceae members and Juncaceae: a shift from permanent pollen tetrads to pseudomonads

Running title: Comparative microgametogenesis study between Cyperaceae and Juncaceae

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Abstract

Cyperaceae is the third largest monocot family and sister to Juncaceae. During microsporogenesis in sedges, an asymmetric tetrad of microspores is formed, establishing a pseudomonad. Three microspores undergo programmed cell death, while the functional one goes though microgametogenesis. The comprehension of this process remains elusive, but understanding its stages, cell death meaning and coexistence of multiple cell lineages occurring in such restricted spaces is of great cytological interest. Therefore, a comparative study was made in Cyperaceae and Juncaceae to characterize pollen features and development using light and electron microscopy, as well as cytochemical tests. Evidences from sporopollenin suggest pseudomonads are derived from pollen tetrads like

those in Juncaceae, and data collected allowed the establishment of five pseudomonad development stages. Summarizing, the late meiosis cytokinesis and precocious pollen mitosis I seem to be associated with pseudomonad formation. Vacuolation happens afterwards, with continuous autophagy of degenerative microspores, suggesting cell death in benefit of pollen fitness. Reserves are accumulated as starch, depending on environmental conditions. During microgametogenesis, different cellular lineages appear isolated by callose, giving each cell metabolic autonomy. This process of pseudomonad establishment allows the formation of a longevous, rapidly germinating tricellular pollen, which could be key to Cyperaceae great adaptative success.

KEYWORDS: Asymmetry ● Autophagy ● Callose ● Eleocharis ● Juncus ● Monocotyledons ● Rhynchospora ● Sporoderm ● Starch ● Vacuole.

Introduction

Cyperaceae, the sedge family, is the third largest among monocotyledons, occurring in a variety of ecosystems worldwide (Goetghebeur, 1998) and is established to be sister to rush family Juncaceae (Bouchenak-Khelladi, Muasya & Linder, 2014). Sedges are of high cytological interest due to the presence of some unusual features, such as holocentric chromosomes and possible inverted meiosis (Vanzela & Guerra, 2000; Melters *et al.*, 2012; Cabral *et al.*, 2014). Members of this family also present an atypical microsporogenesis in which an asymmetrical tetrad is formed (Furness & Rudall, 1999).

Anthers present the typical layers found in angiosperms (Rocha *et al.*, 2016), however, after meiosis, four nuclei are formed in wedge shaped coenocytic cell. Three of them are moved either to the broader basal region of the pseudomonad (sometimes called the abaxial region, facing away from the locule axis), or the narrower apical region of the pseudomonad (the adaxial region, near the locule axis). Meanwhile one remains in the

center (Tanaka, 1941; Brown & Lemmon, 2000; Ranganath & Nagashree, 2000; Simpson *et al.*, 2003; Coan, Alves & Scatena, 2010). The resulting structure is referred generically as pseudomonad (Håkansson, 1954) meaning that pollen is released in monads arising from tetrads. The four meiotic products are later cut off by cell walls, forming two cellular domains (Brown & Lemmon, 2000; San Martin *et al.*, 2013). One, the degenerative domain, is composed by three degenerative cells, which are the microspores that will degenerate. These microspores remain functional for some time, but they become successively smaller until they are finally aborted in a peculiar form of programmed cell death (PCD) associated with DNA fragmentation and vacuolation (Ranganath & Nagashree, 2000; Rocha *et al.*, 2016). The other domain is called functional, and it is composed of a functional cell or functional microspore. Concomitantly to the degenerative microspores development and PCD, the functional microspore gives rise to a generative cell inside a vegetative cell. The generative cell further divides forming two sperm cells (Brown & Lemmon, 2000; San Martin *et al.*, 2013).

Pseudomonads have been well documented (Tanaka 1941; Furness & Rudall, 1999; Coan *et al.*, 2010). However, some peculiarities, like the different positioning of degenerative microspores in species of *Rhynchospora* (Tanaka, 1941; Coan *et al.*, 2010; San Martin *et al.*, 2013), are reported. Yet, little is known about these and development is only partially described. It is assumed that the pollen grain is formed by regular microgametogenesis (Brown & Lemmon, 2000; San Martin *et al.*, 2013). However, literature is scarce on the matter, probably because embryology studies usually focus on the peculiar sporogenesis (Tanaka, 1941; Nijalingappa, 1976). Pseudomonad development involves themes such as cell asymmetry and PCD happening in the restricted space of a pollen grain and understanding how it happens would be of great cytological interest.

In this paper, we investigate morphological and developmental aspects of the different cellular lineages during microgametogenesis process in Cyperaceae. Ten representatives of *Rhynchospora* and the two external groups *Eleocharis sellowiana* (Cyperaceae) and *Juncus tenuis* (Juncaceae) were studied comparatively. The questionings that guidelined this investigation were: i) What are the stages of pseudomonad development, from tetrad to mature pollen grain, and what are their typical features? ii) Does the positioning of degenerative microspores affect in any way the pseudomonad development? iii) Is microgametogenesis in Cyperaceae occurring in the same way it does in angiosperms in general? iv) Are the cytochemical properties of pollen grains common to all species? v) How can multiple cellular lineages develop within the small space of a pseudomonad? In this paper, we attempt to provide data and insights for these and other questions.

Material and methods

Plant material

Eleocharis sellowiana Kunth, *Juncus tenuis* Willd., *Rhynchospora albobracteata* A.C. Araújo, *Rhynchospora breviuscula* H.Pfeiff., *Rhynchospora ciliata* (Vahl) Kük., *Rhynchospora globosa* Roem. & Schult., *Rhynchospora holoschoenoides* (Rich.) Herter, *Rhynchospora nervosa* Boeckeler, *Rhynchospora pilosa* Boeckeler, *Rhynchospora pubera* Boeckeler, *Rhynchospora setígera* Boeckeler and *Rhynchospora tenuis* Link were collected in field and maintained in the greenhouse of the Laboratory of Cytogenetics and Plant Diversity at the State University of Londrina. Vouchers were deposited in FUEL herbarium (See table 1 for detailed information).

Preparation for light and electron microscopy

For light microscopy, inflorescences were collected and fixed in 2% glutaraldehyde in 0.05M phosphate buffer, pH = 6.8. Material was immersed in this solution for 24 hours and washed in 0.05M phosphate buffer, pH = 6.8. Samples were then dehydrated in a graded ethanol series (10-100%), processed through etanol:chloroform series (1:3, 1:1 and 3:1, v:v) and embedded in hydroxyethylmethacrylate (Gerrits & Smid, 1983). Thin sections (2 µm) were obtained, mounted on a glass slide and stained with 0.05% toluidine blue O, pH 4.4 (Feder & O'Brien, 1968). After staining, slides were mounted using Entellan (Merck). Photomicrographs were acquired under bright field using a Leica DMR HC microscope, with a Leica DFC 500 digital camera and the Leica Application Suite (LAS software, version 4.1). For transmission electron microscopy (TEM), anthers were fixed in 2% glutaraldehyde in 0.05M phosphate buffer, pH = 6.8. Afterwards, anthers were washed in the same buffer and later post-fixed in 1% osmium tetroxide for 2 hours. After washing again in buffer, the material was dehydrated in a graded ethanol series (70, 80, 90 and 100%), processed through propylene oxide and embedded in Araldite® resin. Ultrathin sections (70 nm) were collected in a copper grid and stained with 9% uranyl acetate and Reynold's lead citrate solution (Reynolds, 1963). Sections were analyzed using a FEI Tecnai 12 transmission electron microscope at 70 kV. Images were acquired with Analysis FEI software.

Cytochemistry

Inflorescences were processed the same way described above for light microscopy. To detect pollen grain chemical composition, the following cytochemical tests were made: i) Periodic acid–Schiff staining to detect total polysaccharides except

cellulose and callose (O'Brien & Mccully, 1981), ii) IKI solution to detect starch (Lugol solution, Johansen, 1940), iii) 0,05% Basic Fuchsin (C.I. 425000; Roeser, 1972; Luque, Sousa & Kraus, 1996), which has a general affinity for structures with different compositions, but mainly suberin, cutin, lignin, and other phenolic substances (Kraus *et al.*, 1998), iv) 1% Astra blue (Sigma; Roeser, 1972; Luque *et al.*, 1996) to detect polysaccharides of primary cellular walls such as pectins (Kraus *et al.*, 1998), v) Calcofluor White (Sigma) reaction followed by excitation in ultraviolet light (filter 340-380 nm) to detect cellulose (O'Brien & Mccully, 1981), vi) Auramine O (C.I. 41000) reaction followed by excitation in ultraviolet light (filter 450-490 nm) to detect sporopollenin and lipid components (Nepi & Franchi, 2000) and vii) Detection of callose using aniline blue (Riedel-de-haën) followed by excitation in ultraviolet light (filter 340-380 nm; Martin, 1959).

Results

Pseudomonad development

The pseudomonad development progress was analyzed from the end of meiosis (end of sporogenesis) to the mature, pre-anthesis pollen grain (end of gametogenesis) in several species (Figures 1-3, Supplementary figure 1). For didactical purposes, development was divided in five stages based on a group of distinguishable features, although some progressive variations within each one can be noticed.

Stage I. The tetrad recognized after meiosis. Simultaneous microsporogenesis without cytokinesis at first resulted in a transformed coenocytic megaspore mother cell (MMC), with four similar nuclei at first (Figure 1a, three out of four are shown). As development progressed, three nuclei first occupied one region of the transformed MMC (Figure 2b), and then became more condensed, originating an asymmetrical tetrad of

nuclei (Figures 1b and 1c; Figures 2a and 2c-f). The three are the degenerative nuclei, while the remaining central nucleus is the functional nucleus. While in most cases the functional nucleus is clearly distinguishable by its uncondensed aspect, in other cases it was just as condensed as the degenerative nuclei (Figure 1b, Figure 2f). In all *Rhynchospora* species, degenerative nuclei occupied the basal region of the transformed MMC. Due to sectioning, it was common that only two out of the three degenerative nuclei were seen in *Rhynchospora*, probably because they occupied the broader region of the pseudomonad (Figure 1b; Figure 2). In *Eleocharis sellowiana*, the degenerative nuclei occupied the apical region (Figure 1c). After nuclear migration and differentiation, cell walls were laid forming two domains: the degenerative domain, composed by three degenerative cells or microspores, and the functional domain, composed by a functional cell or microspore. When cell walls were clearly visible, functional nuclei were beginning prophase I of pollen mitosis I, PM I (Figure 2e).

Stage II. In this stage, PM I occurred regularly in the functional nucleus, which gave rise to the generative and vegetative cells, as observed in *R. pubera* (Figure 3). In prometaphase, chromosomes were condensed, and the nuclear envelope was not visible. Degenerative microspores also entered cell division. Chromosomes in the degenerative microspores appeared to be smaller than those in the functional cell (Figure 3a). In anaphase, two sets of chromosomes are seen in the functional microspore: one in the basal region and one in the apical region. Degenerative microspores presented chromosomes but without distinguishable sets (Figure 3b). During telophase, generative and vegetative nuclei could be observed. Both presented very uncondensed chromatin, however, generative nucleus appeared smaller than the vegetative one. Degenerative microspores nuclei presented condensed chromatin (Figure 3c). After generative nucleus differentiation, cytokinesis occurred, forming the generative cell (Figure3d). Generative

cell was always cut off next to the degenerative microspores, regardless of their location, and presented approximately the same size and chromatin condensation in *R. pubera* (Figure 1d), while in *R. albobracteata* and *E. sellowiana*, generative cells nuclei were condensed but distinct from degenerative microspores (Figures 1e and 1f). By this stage, degenerative microspores exhibited no PCD features. The vegetative nucleus was large and uncondensed (Figure 1d-f; Supplementary figures 1a, 1e and 1i).

Stage III. This stage is recognized by the appearance of large vacuoles that take up most of the vegetative cell cytoplasm. Vacuoles were observed in every species analyzed in this stage. They ranged from numerous medium sized vacuoles (*R. globosa*, Supplementary figure 1b), to a single large one (*R. albobracteata*, Figure 1h). The generative cell and vegetative nucleus remained similar to previous stage (Figures 1g-i; Supplementary figures 1b, 1f and 1j). During this stage, degenerative microspores initiated PCD. They were observed presenting a large range of cell death features: from pyknotic nuclei in *R. pubera* (Figure 1g) to cellular debris in *E. sellowiana* (Figure 1i), *R. breviuscula* and *R. ciliata* (Supplementary figures 1f and 1j). In some pseudomonads, degenerative microspores were not observed anymore, due to degeneration or sectioning (Figure 1h, Supplementary figure 1b).

Stage IV. During this stage, generative cells were observed in all phases of pollen mitosis II: prophase (Figure 1l), metaphase (Figures 1k; Supplementary figure 1c), anaphase (Supplementary figure 1g) and telophase (Figures 1j; Supplementary figure 1k). Vegetative nucleus remained uncondensed. The middle and big sized vacuoles formed in the previous stage appeared fragmented into smaller ones at different ranges. In most cases, only small vacuoles were observed, such as in *R. pubera, R. breviuscula, R. ciliata*,

R. globosa and *E. sellowiana* (Figures 1j and 1l; Supplementary figures 1c, 1g and 1k). In *R. albobracteata*, large vacuoles were still present (Figure 1k).

Stage V. The mature pre-anthesis tricellular pollen grain stage with two sperm cells, which were rounded (Figure 1n, Supplementary figure 1d) or falciform (Figures 1m and 1o; Supplementary figures 1h and 1l). Later, as observed in *E. sellowiana* (Figure 1o) and *R. ciliata* (Supplementary figure 1l), they were associated to the vegetative nucleus forming the male germ unit. The vegetative cytoplasm presented smaller vacuoles in relation to the stage IV (Figures 1m and 1o; Supplementary figures 1d, 1h and 3l). *R. albobracteata* presented a few medium sized vacuoles (Figure 1n). Overall, pollen in Cyperaceae presented the same basic morphological features as described above, independently of genera or species sampled (Figures 1m-o; Supplementary figures 1d, 1h and 1l).

Polysaccharide accumulation in pollen gains cytoplasm

Polysaccharide accumulation was detected in Cyperaceae and Juncaceae pollen grains cytoplasm using Periodic acid–Schiff (PAS) and IKI solution (lugol) staining. Unlike Cyperaceae, Juncaceae pollen grains were dispersed in permanent tetrahedral tetrads, therefore, pollen tetrads were observed in *J. tenuis.* Due to sectioning, two or three individual pollen grains from the tetrad appeared enveloped by a thick wall. (Figure 4a-c). PAS staining revealed that some pollen grains cytoplasm presented some polysaccharide granules, as observed in *J. tenuis, R. pubera, R. ciliata*, and *E. sellowiana* (Figures 4a, 4d, 4g and 4p, respectively). In *R. nervosa* few small granules were detected (Figure 4j), and in *R. setigera*, no granules were clearly observed (Figures 4m). Lugol staining revealed that these granules are composed by starch and confirmed that *R. pubera, R. ciliata, E. sellowiana, R. globosa* and *R. holoschoenoides* pollen were rich in

this carbohydrate (Figures 4f, 4i and 4r; Figures 5b and 5c), while *R. nervosa, R. setigera* and *R. albobracteata* pollen were relatively starchless (Figures 4l and 4o; Figures 5a). *Juncus tenuis* pollen cytoplasm also presented starch granules (Figure 4c), however, these were fewer and smaller in relation to other starch containing Cyperaceae pollen.

Detection of cell wall components in pseudomonads and pollen grains

Callose was detected in stage II pseudomonads of *R. pubera* and *E. sellowiana* using aniline blue. It was revealed that both degenerative microspores and generative cells were completely embraced by callose walls (Figures 6a and 6b). Sporoderm was uniform and no clear apertures could be noticed during this study. Its constituents were investigated using PAS, astra blue, auramine O and calcofluor. PAS and astra blue staining revealed the intine is rich in polysaccharides of primary cellular walls (Figures 4a, 4b, 4d, 4e, 4g, 4h, 4j, 4k, 4m, 4n ,4p and 4q), and cellulose was also found in intine, as evidenced by calcofluor (Figure 6c-f). In Juncaceae pollen, the conspicuous exine was heavily stained by basic fuchsin, while in Cyperaceae, the exine was weakly stained or non-stained (Figures 4b, 4e, 4h, 4k, 4n and 4q). Sporopollenin present in the pollen wall could be detected in both Juncaceae pollen tetrads and Cyperaceae pollen by Auramine O (Figures 6c-f). Pollen tetrads in Juncaceae are internally divided by intine and enveloped by a thick exine (Figures 6c).

Pollen ultrastructure

In the stage V, vegetative cells of *R. pubera* pollen grains exhibited cytoplasm rich in amyloplasts, lipid droplets and small vacuoles (Figures 7a and 7b). In both *R. pubera* and *R. ciliata,* pollen wall was easily distinguished between tectate exine and intine (Figure 7c-d). Still at this stage, debris from degenerative microspores could be seen. In light microscopy, these debris were seen in *R. ciliata* as a small heavily stained

structure in the basal region (Figure 4h), and using electron microscopy, these debris are seen as heterogenous material. The degenerative microspore debris is located between the pollen exine and the vegetative cell intine. A small vacuole in the vegetative cell cytoplasm can be seen adjacent to these cellular debris (Figure 7d).

Discussion

Pseudomonad development

Pseudomonads appear to be a synapomorphy for the Cyperaceae family (Furness & Rudall, 2011). However, uncertainties remain regarding pollen in the early diverging Mapanioideae subfamily, which closely reassembles pollen from regular monads (Simpson *et al.* 2003). Evidence of pseudomonad formation in *Hypolytrum* (Coan *et al.* 2010) might be indicative of pseudomonads appearing early in the family evolution. Still, more data needs to be produced to support this statement. In sedges, alterations from the standard development seen in angiosperms are noticeable when an asymmetric simultaneous microsporogenesis takes place in MMCs. After nuclear migration and differentiation, a late cytokinesis takes place, forming three degenerative cells and a functional one (Brown & Lemmon, 2000). These cells correspond to the microspores of regular monads, except they are not released as individuals and three of them degenerate. In *Eleocharis* and other genera studied, degenerative cells or microspores are in the apical region, but in *Rhynchospora*, they occur in the basal region (Tanaka, 1941; Padhye, 1968; Nijalingappa, 1976; Kirpes, Clark & Lersten, 1996; Furness & Rudall, 1999; Brown & Lemmon, 2000; Coan *et al.,* 2010), suggesting a synapomorphy for *Rhynchospora*. However, it is important to check this status in the close related genus *Pleurostachys*. Regardless of the positioning, degenerative nuclei fail to divide, presenting lower C values in comparison to the functional microspore nucleus (Rocha *et al.* 2016), which

undergoes regular PM I after meiosis cytokinesis, without post meiotic stages often described in microspores (Knox 1984; Pacini, Jacquard & Clément, 2011). Afterwards, vacuoles take most of the vegetative cell cytoplasm, making microgametogenesis in Cyperaceae different than in most angiosperms, in which one or two peaks of vacuolation happen after tetrad stage and before PM I (Pacini *et al.*, 2011). However, similar features are observed in seagrasses (Knox 1984).

Asymmetry establishment for PM I in angiosperms is assumed to be initiated as early as in tetrad stage. Its mechanisms are not entirely known, but it could be based on cytoplasmic gradients ensuring that different cells are created with uneven differentiation factors (Twell, Park & Lelanne, 1998). In many ways, asymmetry establishment during PM I reassembles asymmetry establishment during pseudomonad formation in Cyperaceae. Orientation is variable in genera and species (Twell *et al.*, 1998), as it is seen during pseudomonad development in different genera of sedges. Asymmetric meiosis and PM I also happen in subsequent stages of development and have the same orientation, as shown here and in other genera (Nijalingappa, 1976; Brown & Lemmon, 2000). Additionally, asymmetry for PM I can be established even in mutants in which cell plate formation fails during meiosis and a coenocytic tetrad of nuclei is formed, such as in *Arabidopsis* STUD and TETRASPORE mutants (Spielmen *et al.*, 1997; Hülskamp *et al.*, 1997). Factors responsible for pseudomonad formation and PM I could be the same or equivalent, and due to the late cytokinesis, these factors may conduce asymmetric tetrad formation in the briefly coenocytic MMC stage. Initial degenerative microspore formation would resemble generative cell formation. Except degenerative microspores are not embraced in the functional microspore cytoplasm, fail to divide during PM I (Rocha *et al.*, 2016) and are aborted. Many genes have been known to act during PM I, especially from studies with other mutants such as the SIDECAR POLLEN (SCP), in

which nuclear migration for asymmetric division occurs, but the division still forms two equal cells, similar to the vegetative cell. SCP encodes a male gametophytic LATERAL ORGAN BOUNDARIES DOMAIN/ASYMMETRIC LEAVES 2-like protein essential for correct timing and orientation of PM I asymmetry (Oh *et al.,* 2010). In this case, it would be expected to find similar or equivalent genes acting in early pseudomonad development stages.

Relation between Cyperaceae pseudomonads and Juncaceae tetrads

Pseudomonads and permanent tetrads, as found in sedges and rushes, respectively, are formed through distinct processes leading to two different forms of pollen dispersion. However, they still present common features, such as simultaneous microsporogenesis (Furness & Rudall 1999) and pollen shed in the tricellular form (Brewbaker, 1967, Munro & Linder, 1997). Additionally, cytochemistry revealed that cellulosic and polysaccharidic walls (intine) separate individual pollen grains in Juncaceae tetrads, while the exterior is enveloped by sporopollenin (exine). This is also observed for Cyperaceae, in which TEM in *R. ciliata* revealed that degenerative microspores and vegetative cells are separated by intine while the pseudomonad is also enveloped by sporopollenin. The same is true for *Eleocharis palustris* (Dunbar 1973). It has been suggested that pseudomonads are derived from permanent tetrads as those in Juncaceae (Nijalingappa, 1976; Munro & Linder, 1997) and this is plausible considering their relation as sister families, as well as the morphological features mentioned above.

It is interesting to point out that some *Arabidopsis* pollen mutants present strikingly similar features to developing pollen of sedges and rushes. Besides the STUD and TETRASPORE mutants mentioned above, QUARTET mutants exhibit microspores individualized by cell plate but not released, forming pollen tetrads like in *J. tenuis*

(Preuss, Rhee & Davis, 1994; Rhee & Somerville, 1998). The similarities these mutants present in relation to Cyperaceae and Juncaceae pollen indicate that Juncaceae permanent tetrads are probably derived from regular monads found in other monocots. Pollen aggregation from monads, such as in rushes, have occurred independently several times over angiosperm evolution and might enhance the chance of fecundation, as most pollen in monads fail this task (Harder & Johnson 2008). Possibly, Cyperaceae pseudomonads appeared due to the abort of three microspores in a permanent tetrad. This way, we can imagine microsporogenesis in sedges and rushes being established if the expression of genes related to those mentioned above had been modified along their evolutionary history.

Programmed cell death and reserve accumulation

One question regarding the cell fates remains. Why are three microspores aborted in Cyperaceae pseudomonads while in Juncaceae tetrads they are viable? The pollen grain cytoplasm in Juncaceae is weakly stained and smaller compared to that of Cyperaceae pollen in general. This could indicate that three microspores are aborted in sedges in benefit of the functional one. As vacuolization starts, degenerative microspores begin PCD, which, as noticed in *R. ciliata* is a continuous process as degenerative microspore debris associated with vacuoles appeared in mature pollen. These debris are also found in mature pollen grains of *E. palustris* (Dunbar, 1973), suggesting this is a feature maintained in the family. The association between vacuoles and PCD is a strong indicative of autophagy (van Doorn, 2011), which could occur in benefit of pollen fitness. Pollen would be able to accumulate more reserves without real loss, since only one participate in the fecundation process in Cyperaceae, as flowers present one pistil with one ovule (Goetghebeur, 1998). This diverges greatly from Juncaceae gynoecium, which

also presents one pistil but with three to several ovules, and therefore all or almost all pollen grains from the tetrad can participate in fecundation (Munro & Linder, 1997). The greater investment in Cyperaceae pseudomonad compared to Juncaceae tetrads could be a factor responsible for sedges greater diversity and distribution. Additionally, selective microspore abortion in sedges might also be related to the non-random chromosome segregation during meiosis (meiotic drive), which could lead to the rapid fixation of certain mutations (Furness & Rudall 2011).

Cyperaceae members are among the few flowering plants in which pollen is released with two sperm cells (Brewbaker, 1967). Although the presence of bicellular or tricellular pollen is constant in members of the same genera or family, in some plants they coexist in the same anther. In *Annona cherimola* (Annonaceae), for instance, either bicellular or tricellular pollen can be released (Lora, Herrero & Hormaza, 2009). Under environmental circumstances that favor rapid pollen germination, tricellular pollen grains becomes more frequent in the anther. This is in accordance to the fact that, while tricellular pollen of angiosperms have lower longevity in general, it is ready for rapid germination (Brewbaker, 1967). In the case of the Cyperaceae tricellular pollen, the degenerative microspores PCD and its assimilation by the vegetative cell may have balanced the longevity problem, creating a long-lasting pollen that is ready to rapidly germinate.

Pollen grains accumulate reserves in the form of starch, other sugars or lipids (Baker & Baker, 1979; Franchi *et al.*, 1996), and in some sedges, pollen with no starch was detected. The most interesting case is the one of *R. ciliata* and *R. nervosa.* Both are closely related species (Luceño, Vanzela & Guerra, 1998), but while *R. ciliata* presented pollen with large starch granules, *R. nervosa* was basically starchless. Intraspecies

variation of pollen reserves have been reported (Franchi *et al.,* 1996), and variation between such close species is an indicative that pollen reserve anabolism is very dynamic. The breakdown of starch into soluble sugars protects the pollen grain against desiccation (Franchi *et al.,* 1996), and species with starchless pollen studied here are located preferentially in dry or well drained soils. Therefore, the presence or absence of starch in Cyperaceae pollen can be related to wet or dry environments, respectively. Lipids could also have a substantial role in reserve accumulation, since lipid droplets are found even in starch rich pollen, like in *R. pubera.*

Pseudomonad cell wall and the coexistence of multiple cell lineages

Cytochemistry and TEM revealed that Cyperaceae sporoderm is stratified into intine and tectate exine. Exine was uniform and no clear apertures were observed in sectioning. Additional data are necessary to determine the apertures presence and positioning, especially considering the exine thinness in Cyperaceae species. The sporoderm was not stained, or at least poorly stained, by basic fuchsin. The exine is commonly well stained by basic fuchsin (Knox, 1984), as observed in *J. tenuis*, which indicates that some components might be scarce, absent or preventing basic fuchsine staining in Cyperaceae sporoderm. The most interesting cytochemistry results, however, came from callose detection by aniline blue. This test revealed that degenerative microspores and generative cells are isolated from each other and from the vegetative cell cytoplasm by callose walls. The callose is thought to play a vital role during microspore differentiation in meiosis, acting as a molecular filter giving metabolic autonomy to haploid microspores, isolating them from interference of the surrounding sporophytic tissue (Bhandari, 1984; McCormick, 1993). Temporary callose walls are known to be formed in the generative cells after PM I, and their occurrence are considered an important

element conditioning the different course of development for generative and vegetative cells (Gorska-Brylass, 1967). This is observed as the “callose” stage (Gorska-Brylass, 1970). The callose is widely found during megasporogenesis in angiosperms, in which three out of four meiotic products also degenerate in benefit of the functional one (Bouman, 1984), what is similar to pseudomonad development. The presence of callose walls in both degenerative and generative cells highlights the important role of this polymer in cellular differentiation. Callose walls would ensure autonomous metabolism to degenerative, generative and vegetative cells, once they arise with their specific differentiation factors after asymmetric division. This makes it easier to imagine how a small structure such as the pseudomonad can harbor multiple cellular lineages: because once cells arise from asymmetrical division, they would follow autonomous preprogrammed development without influence from their neighbors.

Conclusions

Pseudomonad development can be described in five stages: i) post-meiotic tetrad stage, ii) PM I stage, iii) vacuolation stage, iv) PM II and v) mature pre-anthesis pollen grain stage. During microgametogenesis, PM I happens after meiosis and is possibly related to the establishment of pseudomonads. Afterwards, vacuolation is noticed, associated with degenerative microspores PCD. Regardless of their different positioning, degenerative microspores autophagy occurs in benefit of the functional cells, which accumulates reserves in form of starch or other substances, depending on environmental habit. In this process, multiple cellular linages coexist, which can be possible by isolation in callose walls. Pseudomonads can be considered a derivate feature from permanent tetrads, as found in sister family Juncaceae, except in Cyperaceae only one microspore remains viable. At first glance, it may be assumed that losing three microspores can cause

selective disadvantage. However, pseudomonads originate pollen grains with greater investment, possibly allowing the formation of longevous pollen that can rapidly germinate. This might have contributed to greater adaptative success in sedges. Also, aborting three meiotic products would not be a real loss in the Cyperaceae context, as the gynoecium presents only one ovule. Despite data shown here, studies with representatives of other genera, as well as with gene expression, are still needed to understand pseudomonad development and evolution.

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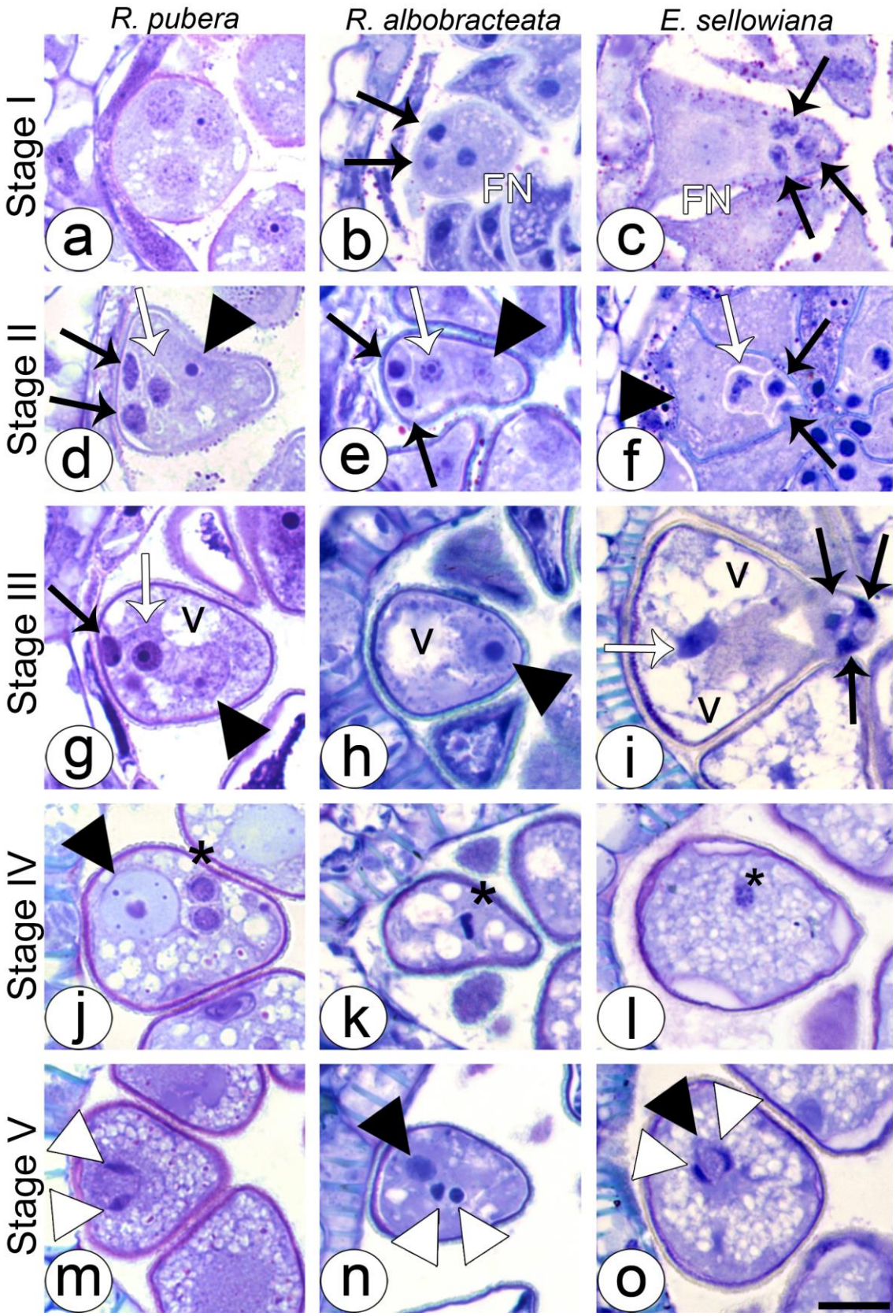


Figure 1. Transversal sections of *Rhynchospora* and *Eleocharis* anthers stained with toluidine blue showing pseudomonad development. Black arrow = degenerative

nucleus/microspore. FN = functional nucleus. White arrow = generative cell. Black arrowhead = vegetative nucleus. White arrowhead = sperm cell. V = vacuole. (a, d, g, j, m) *R. pubera*. (b, e, h, k, n) *R. albobracteata.* (c, f, i, l, o) *E. sellowiana.* Scale bar = 10 µm. (a-c) Stage I pseudomonads. In *R. pubera* (a), three undifferentiated nuclei can be noticed. In *R. albobracteata* pseudomonad, two degenerative nuclei occupy the basal region (b) while in *E. sellowiana* they are present in the apical region (c). (b-f) Stage II pseudomonads. Note that degenerative cells or microspores present no signs of programmed cell death. Next to them, the small generative is seen inside a large vegetative cell. (g-i) Stage III pseudomonads. Vacuoles can now be observed occupying most of the vegetative cell cytoplasm. Degenerative microspores, when observed, present different signs of PCD: from pyknotic nuclei (g) to cellular debris (i). Stage IV pseudomonads (j-l). Generative cells are undergoing pollen mitosis II (PM II) and distinct phases are seen: telophase (j), metaphase (k) and prophase in (l). Degenerative microspores can no longer be observed from this point on. Vegetative cell cytoplasm is filled with small vacuoles in *R. pubera* and *E. sellowiana* (j and l). In *R. albobracteata*, larger vacuoles can still be noticed (k). Stage V pseudomonads (m-o). The pre-anthesis mature pollen grain can be observed. Sperm cells formed after PM II are either round (n) or falciform (m, o). Vegetative cell cytoplasm is well stained with several small vacuoles and presented several small vacuoles.

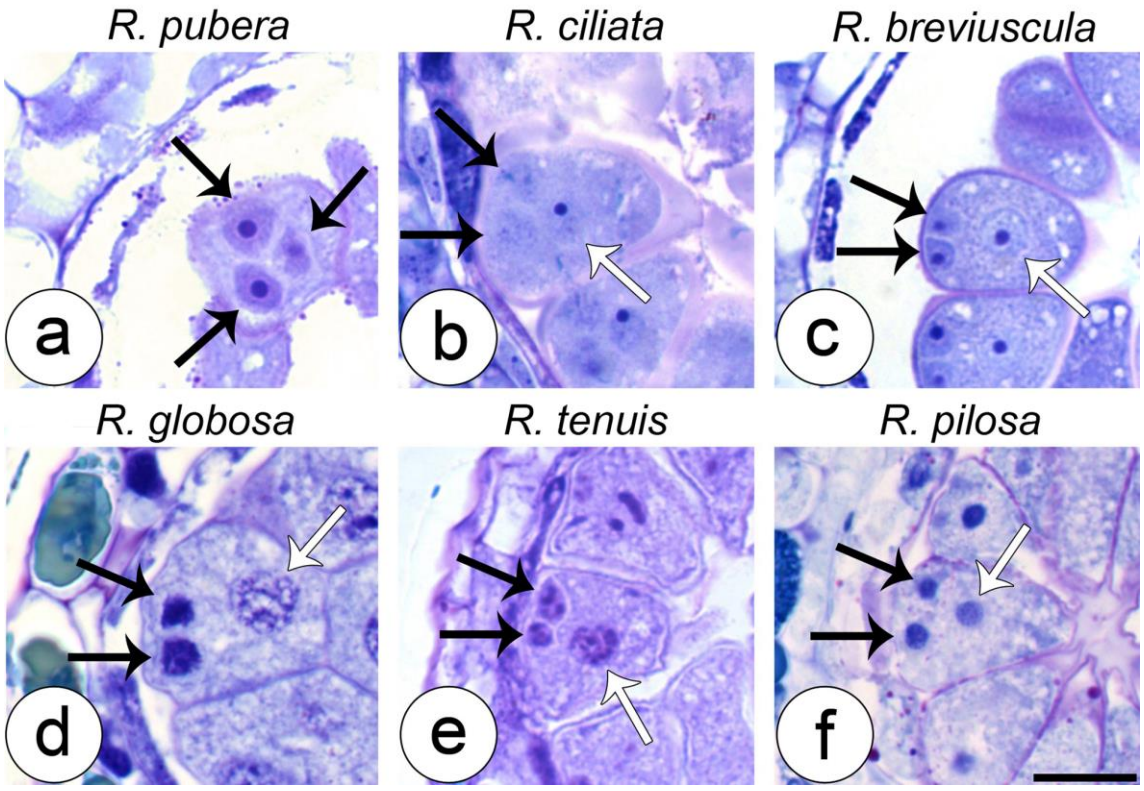


Figure 2. Longitudinal sections of *Rhynchospora* anthers showing stage I pseudomonads stained with toluidine blue. (a) *R. pubera.* (b) *R. ciliata.* (c) *R. breviuscula*. (d) *R. globosa*. (e) *R. tenuis.* (f) *R. pilosa.* Black arrow = degenerative nucleus. White arrow = functional nucleus. Scale bar = 10 µm. In all *Rhynchospora* species sampled, the degenerative nuclei were in the basal region. In a tangential section of a *R. pubera* pseudomonad basal region, all three degenerative nuclei can be observed in the same plane (a). In *R. tenuis*, cytokinesis occurred, forming the degenerative cells or microspores. In this moment, degenerative and functional nuclei appear to be condensing in response to pollen mitosis I entry (e). In *R. pilosa* vegetative nucleus is as condensed as the degenerative nuclei (f).

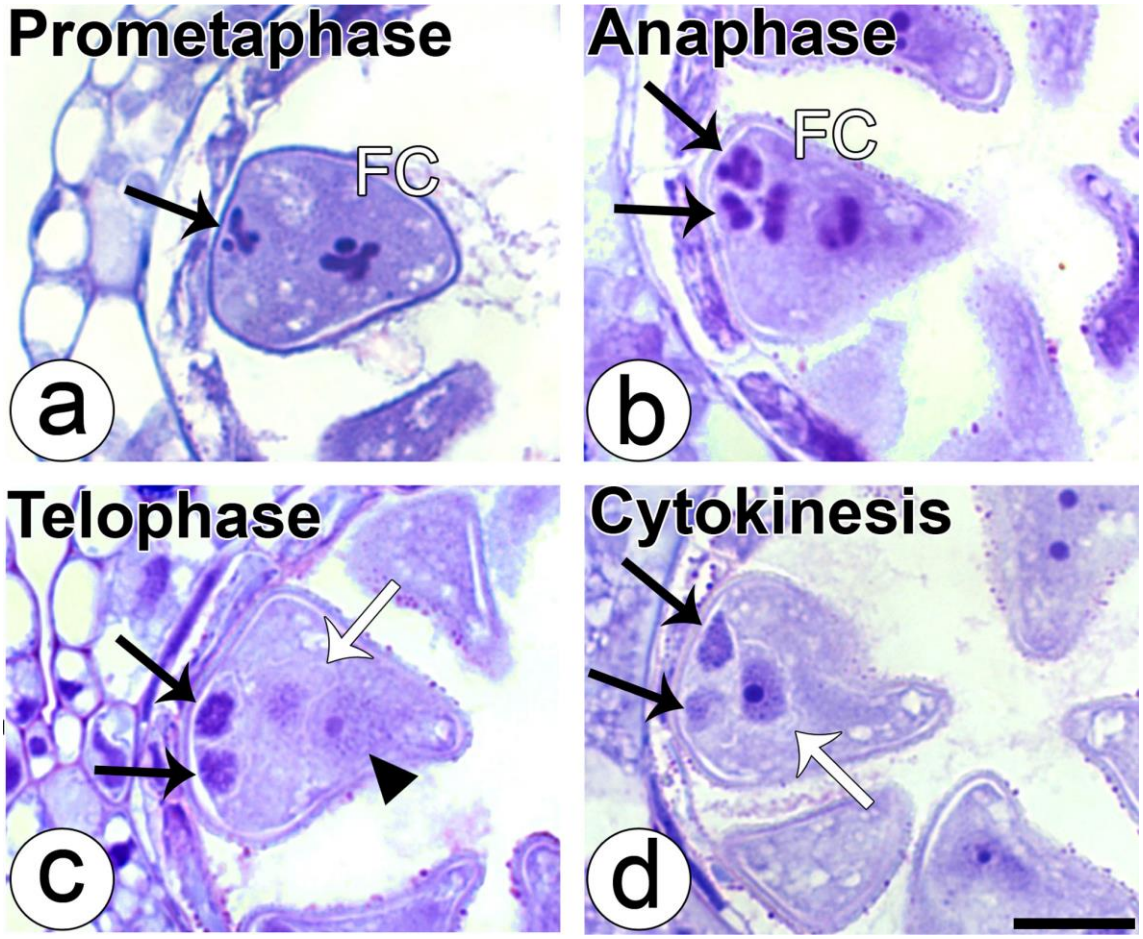


Figure 3. Longitudinal sections of *R. pubera* anthers showing stage II pseudomonads stained with toluidine blue in different phases of pollen mitosis I. FC = functional cell. Black arrow = degenerative cell/microspore. White arrow = generative nucleus/cell. Black arrowhead = vegetative nucleus. Scale bar = 10 µm. During stage II, the functional cell undergoes pollen mitosis I (PM I), giving rise to the generative and vegetative cells. PM I was seen in prometaphase (a), anaphase (b), telophase (c) and cytokinesis (d).

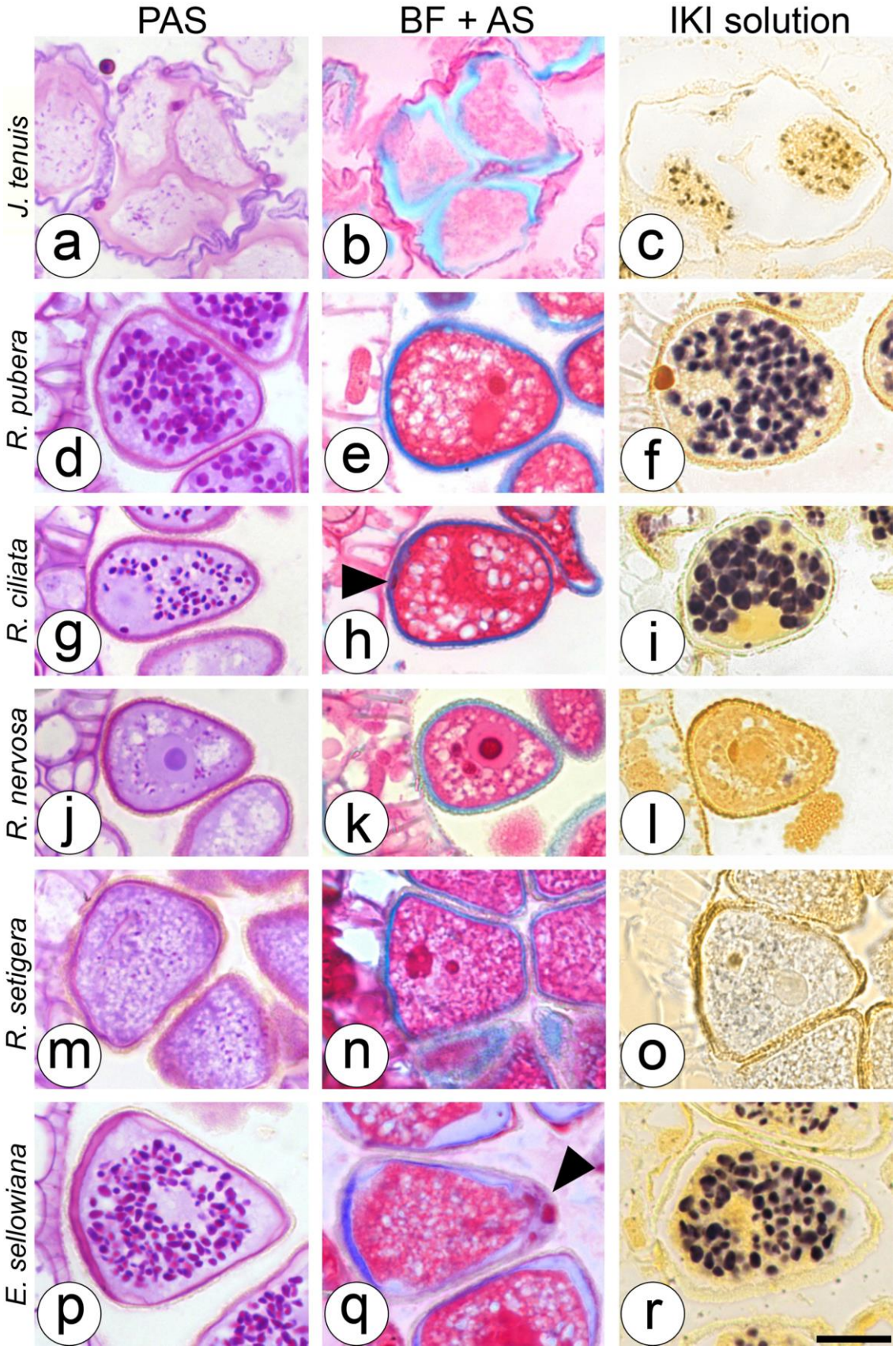

PAS
BF + AS
IKI solution
J. tenuis
R. pubera
R. ciliata
R. nervosa
R. setigera
E. sellowiana
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r

Figure 4. Bright-field detection of polysaccharides in transversal sections of Juncaceae and Cyperaceae anthers. (a-c) *Juncus tenuis.* (d-f) *R. pubera*. (g-i) *R. ciliata.* (j-l) *R. nervosa.* (m-o) *R. setigera.* (p-r) *E. sellowiana.* (a, d, g, j, m, p) Periodic acid–Schiff (PAS) stain for total polysaccharides. (b, e, h, k, n, q) Double staining with basic fuschin and astra blue (BF + AB). Astra blue stains polysaccharides of primary walls. (c, f, i, l, o, r) Starch staining using Lugol solution (IKI). Scale bar = 10 µm. PAS (a) and astra blue staining (b) revealed that individual pollen from the *J. tenuis* tetrad are separated from each other by primary walls containing polysaccharides, which corresponds to the intine. The outer wall enveloping the tetrad, the exine, is heavily stained by basic fuchsin, while cytoplasm was weakly stained (b). Granules detected by PAS are revealed by IKI to be starch granules (c). Pollen in Cyperaceae presents a great diversity in relation to polysaccharide accumulation. *R. pubera*, *R. ciliata* and *E. sellowiana* show numerous large granules scattered throughout the vegetative cell cytoplasm, as detected by PAS (d, g, p), which were revealed to be starch granules (f, i, r). Meanwhile, in other species, such as *R. nervosa* and *R. setigera*, almost no starch is detected by PAS (j, m) or IKI (l, o). Cyperaceae pollen grains present vegetative cells well stained by basic fuchsin, and intine is evidenced by astra blue (e, h, k, n, q). The exine is either not stained or weakly stained by basic fuchsin. Fragments of the degenerative microspore (black arrowheads) can be observed in *R. ciliata* (h) and *E. sellowiana* pollen (q).

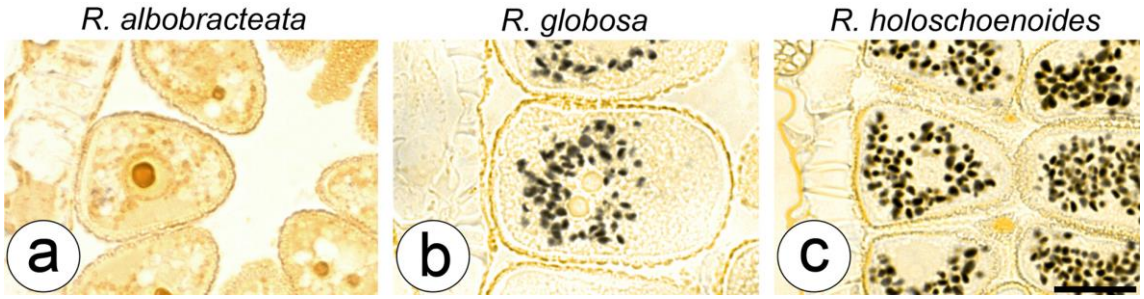


Figure 5. Differential **s**tarch accumulation in mature pollen of *Rhynchospora*. (a) *R. albobracteata*. (b) *R. globosa.* (c) *R. holoschoenoides.* Scale bar = 10 µm. Stage V pollen can be starchless (a), or rich in starch (b and c).

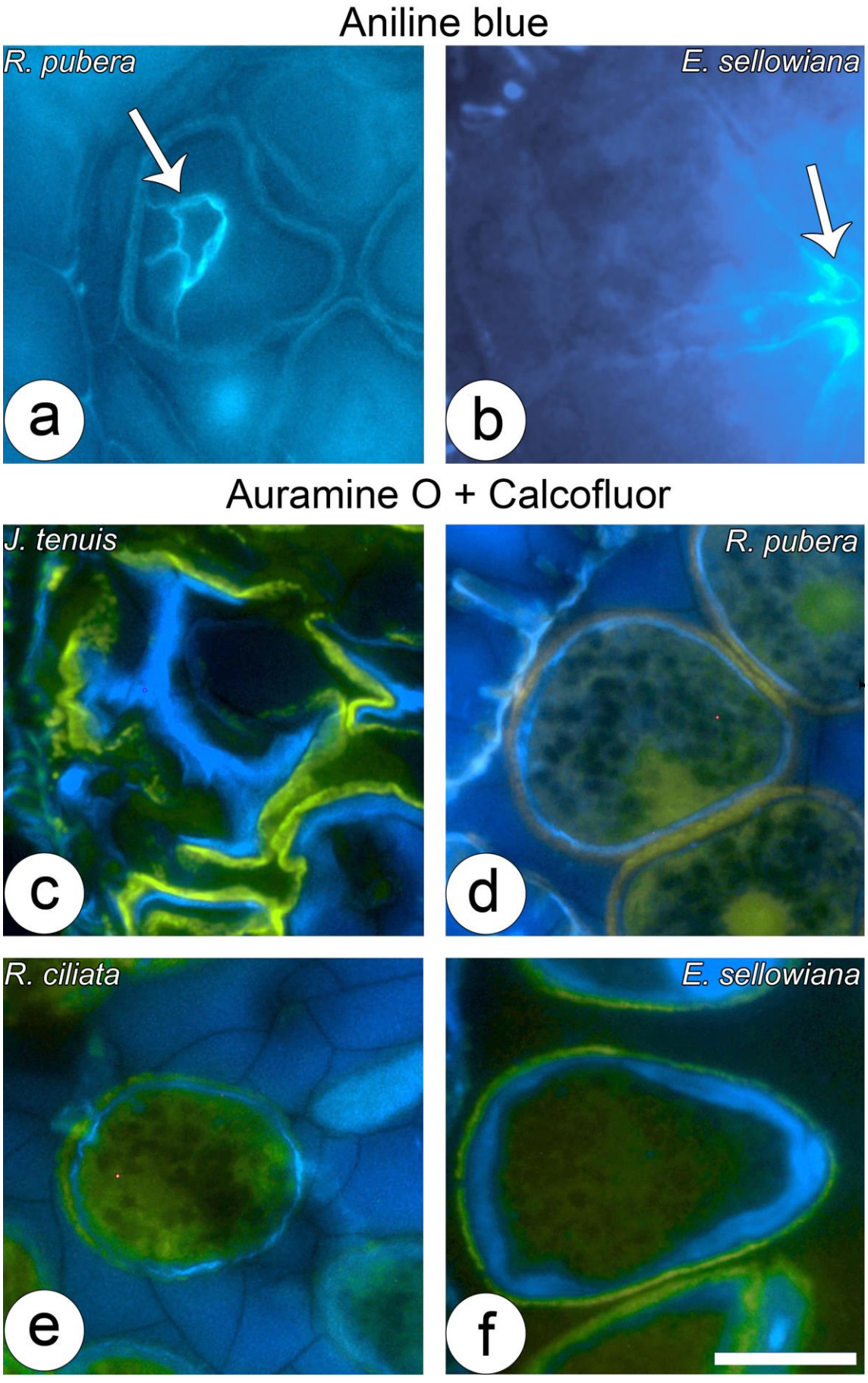

Aniline blue
R. pubera
E. sellowiana
a
b
Auramine O + Calcofluor
J. tenuis
R. pubera
c
d
R. ciliata
E. sellowiana
e
f

Figure 6. Fluorescent detection of cell wall components in transversal sections of Juncaceae and Cyperaceae anthers. (a and d) *R. pubera*. (b and f) *E. sellowiana*. (c) *J. tenuis.* (e) *R. ciliata*. (a and b) Callose detection using aniline blue in stage II pseudomonads. (c-f) Sporopollenin detection using auramine O and cellulose detection using calcofluor in stage V pollen grains. Scale bar = 10 µm. Aniline blue staining (a and b) revealed that the degenerative microspore and generative cells were enclosed by callose walls (white arrows). Fluorescence reaction with auramine O and calcofluor (c-f) could differentiate the cellulosic intine, in blue, from the sporopollenin of the exine, in green.

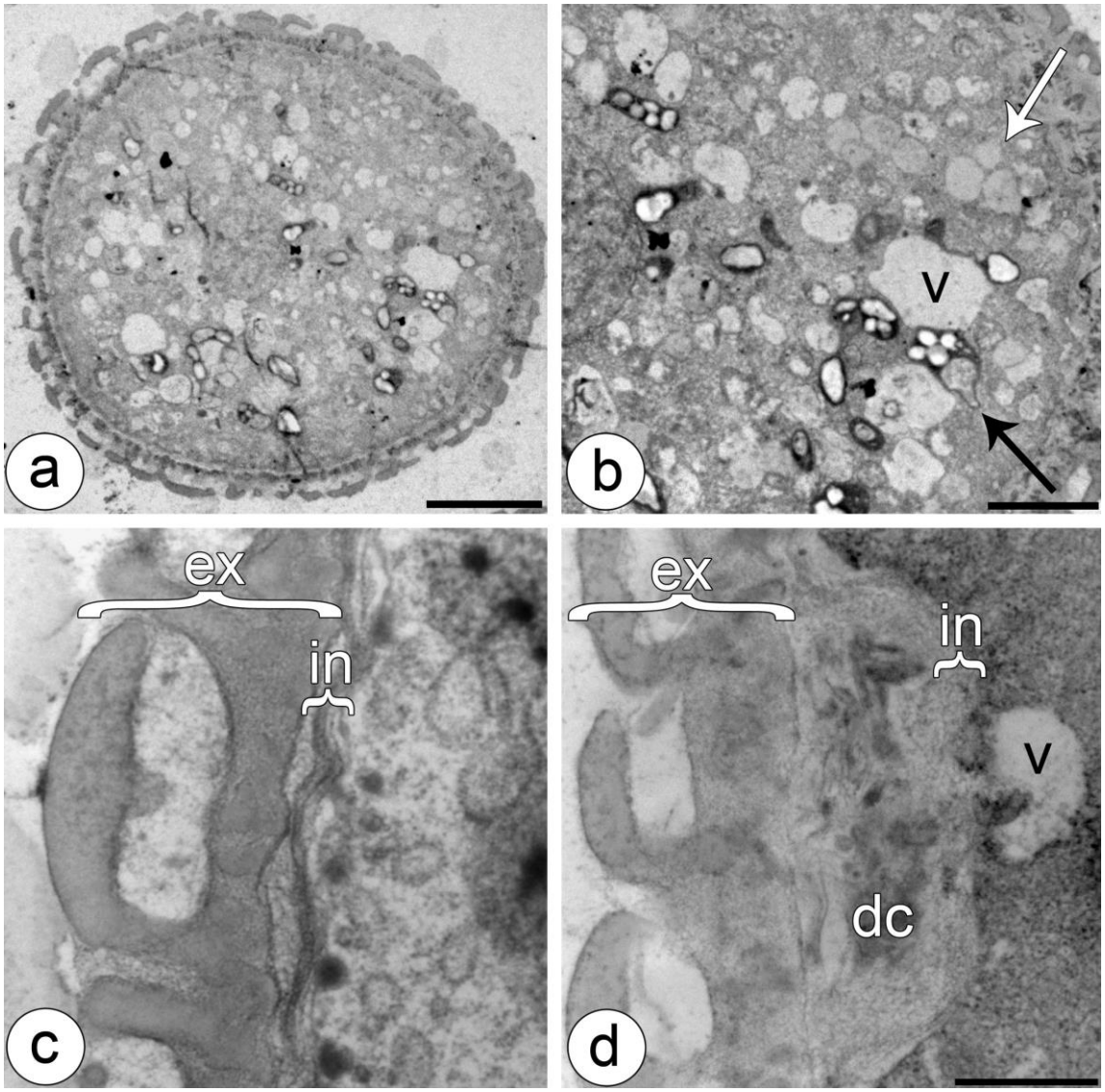
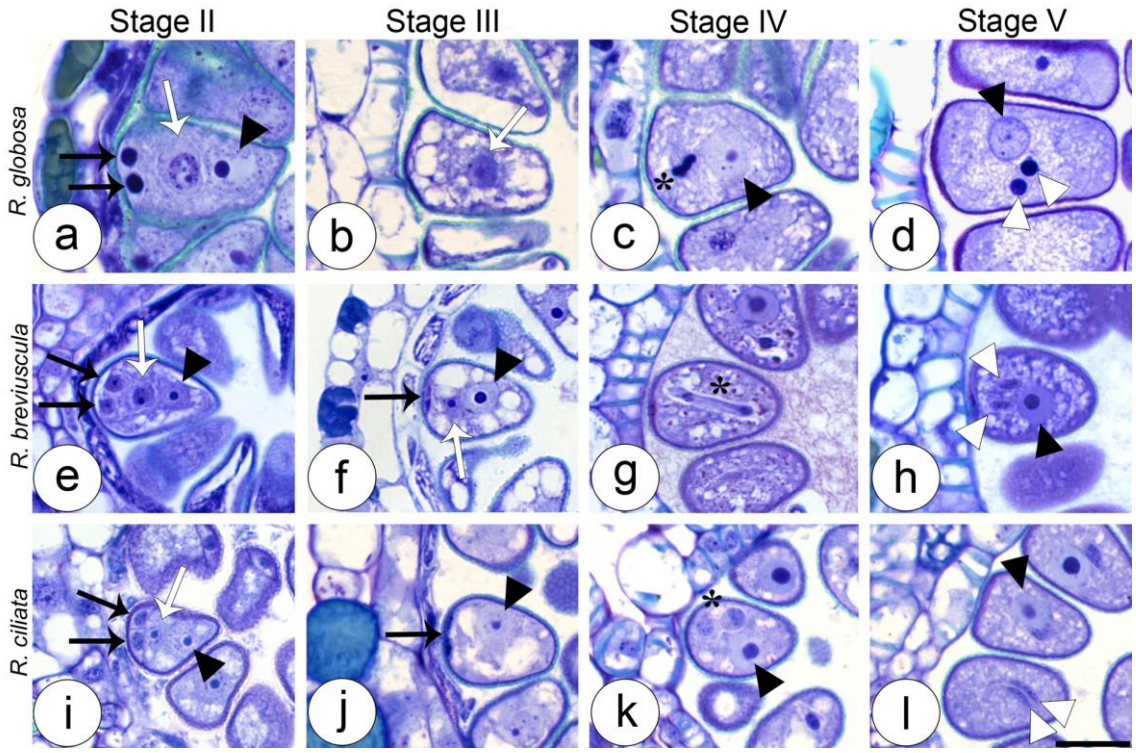


Figure 7. Ultrastructure of stage V pollen of *R. pubera* and *R. ciliata*. (a) Overview of a mature, pre-anthesis pollen grain of *R. pubera*. Scale bar = 5 µm. (b) Detail of the vegetative cell cytoplasm. Amyloplasts (black arrow), lipid droplets (white arrow) and vacuoles (V) can be seen scattered throughout the cytoplasm. Scale bar = 2 µm. (c and d) Pollen grain wall in *R. pubera* (c) and *R. ciliata* (d) can be clearly differentiated into a tectate exine (ex) and intine (in). In *R. ciliata,* heterogenous debris from the degenerative cells/microspores (dc) can still be observed between the exine and the intine. A small vacuole (v) can be seen in the vegetative cell cytoplasm, adjacent to the degenerative cell debris. Scale bar in (d), also for (c) = 500 nm.



Supplementary figure 1. Longitudinal sections of *Rhynchospora* anthers showing stage II to V pseudomonads stained with toluidine blue. (a-d) *R. globosa*. (e-h) *R. breviuscula.* (i-l) *R. ciliata.* Black arrow = degenerative cell. White arrow = generative cell. Black arrowhead = vegetative nucleus. White arrowhead = sperm cells. Asterisk = generative cell in PM II. Scale bar = 10 µm. Stages II to V represent microgametogenesis in Cyperaceae. During stage II (a, e, i), the generative cell and vegetative nucleus arise from the functional nucleus PM I. The degenerative cells nuclei were always small and with condensed nucleus. In some instances, they were even smaller and more condensed than the generative cells nuclei (a, i). The defining characteristic of stage III is the appearance of several vacuoles with different sizes in the vegetative cell cytoplasm (b, f, j). Generative cell and vegetative nuclei reassemble previous stage. Degenerative cells, when observed, are very reduced and present clear signs of PCD (f, j). In stage IV (c, g, k), PM II occurs in the generative cell. Vegetative nucleus is similar to that at previous stage, while the cytoplasm is now filled with several small vacuoles. The mature pollen

grain can be seen in stage V (d, h, l). The vegetative cell cytoplasm was filled with small vacuoles. Sperm cells can be seen in this stage. They are either falciform (l, h) or rounded (d). In (l) one can be seen next to the vegetative nucleus, forming the male germ unit.

Family	Genus	Especies	Location	FUEL voucher nº
Cyperaceae	*Eleocharis*	*sellowiana*	Tibagi PR	55363
	Rhynchospora sect. *Polycephalae*	*holoschoenoides*	Carrancas MG	55371
	Rhynchospora sect. *Pseudocapitatae*	*pilosa*	Carrancas MG	55368
	Rhynchospora sect. *Pluriflorae*	*albobracteata*	Carrancas MG	55369
	Rhynchospora sect. *Pluriflorae*	*globosa*	Carrancas MG	55364
	Rhynchospora sect. *Tenuis*	*tenuis*	Jaguariaiva PR	55365
	Rhynchospora sect. *Dichromena*	*setigera*	Carrancas MG	55370
	Rhynchospora sect. *Dichromena*	*pubera*	Recife PE	55374
	Rhynchospora sect. *Dichromena*	*nervosa*	Florianópolis SC	55366
	Rhynchospora sect. *Dichromena*	*ciliata*	Recife PE	55372
	Rhynchospora sect. *Dichromena*	*breviuscula*	Iporanga SP	55362
Juncaceae	*Juncus*	*tenuis*	Iporanga SP	55373

Table 1. The species studied with the corresponding genera, location and voucher number.

CAPÍTULO III

Are unusual ultrastructural features occurring in the endomembrane system of pollen cells in Cyperaceae and other angiosperms?

Artigo a ser submetido para publicação

Are unusual ultrastructural features occurring in the endomembrane system of pollen cells in Cyperaceae and other angiosperms?

Running Title: Unusual ultrastructural features in Cyperaceae pollen

Abstract

Cyperaceae individuals present peculiar microsporogenesis and microgametogenesis, even though they result in apparently regular tricellular pollen grains. However, there are few studies on the cellular components behavior in maturing pollen. To characterize pre-anthesis pollen grain in sedges, they were analyzed under light and transmission electron microscopy. In addition, cytochemistry using silver nitrate was performed. Maturing pollen in Cyperaceae presented unusual endomembrane behavior, which includes cortical and concentric endoplasmic reticulum, as well as unconventional secretion of multivesicular bodies. Endoplasmic reticulum and dictyosomes are concerned classic secretion pathways in vegetative cells, and possibly the late breakdown of degenerative microspores. However, endoplasmic reticulum may also assume storage, structural and signaling roles, as well as promoting cytoplasmic turnover. Unconventional secretion was observed several times and could be involved in a number of processes. Reports from the literature show that the unusual features observed here are present in other angiosperm pollen grains. This could represent an opportunity to reexamine our understanding on the endomembrane system in pollen cells in general.

KEYWORDS: Cortical endoplasmic reticulum ● Dictyosome ● Golgi Complex ● Unconventional secretion ● Multivesicular bodies ● Endosomes ● Exosomes ● Sperm cell ● Vegetative cell

Introduction

The endomembrane system consists on a group of membrane bound structures in eukaryotic cells which include endoplasmic reticulum (ER), dictyosomes and the many other structures arising from them, such as vesicles, endosomes, vacuoles, autophagosomes and others (Okita & Rogers, 1996). These organelles are usually concerned in endocytic and secretory pathways. In the classic secretory pathways, proteins and lipids are synthetized and processed in the ER and transported through vesicles to dictyosomes, where they can be further processed and sorted to their respective sites (Okita & Rogers 1996; Drakakaki & Dandekar, 2013). Besides the classic secretory pathway, there are other diverse secretory pathways that do not follow the traditional ER to dictyosome to plasma membrane route. These are usually referred to as unconventional or non-classic secretion (Ding *et al.*, 2012; Drakakaki & Dandekar 2013).

Classic and non-classic secretion, as well as the endocytic pathways are critical in cellular differentiation and plant development. In tracheary element differentiation, for instance, the endomembrane system must coordinate both secondary wall synthesis and programmed cell death (PCD) in order to achieve a functional cell corpse specialized in water conduction (Grover & Jones, 1999). For this to be possible, the developing cell uses both classic and non-classic secretory pathways, as well as lytic functions in the endocytic pathway (Robarts & Kidwai, 1969; Grover & Jones, 1999). The endomembrane system also plays key roles in the development of the gametophytic generation. In developing pollen, they promote cytoplasm turnover, contribute to generative cell morphogenesis, secrete sporoderm constituents, prepare the pollen for germination etc. (Bhandari, 1984; Knox, 1984). In a more specific case, the endomembrane system is one of the key elements in producing an asymmetric tetrad during microsporogenesis in Cyperaceae

(Brown & Lemmon 2000; San Martin *et al.*, 2013), which culminates in the formation of a structure called pseudomonad.

Cyperaceae (sedges) represents the third largest monocot family (Goetghebeur, 1998) and presents pollen shed in pseudomonads (Håkansson, 1954; Brown & Lemmon, 2000; Ranganath & Nagashree, 2000; San Martin *et al.*, 2013). In sedges, microsporogenesis is simultaneous, as it is in most monocotyledons (Furness & Rudall, 2009). However, after telophase II and before cytokinesis, three nuclei are dislocated to one region of the cell, while one remains in the center (Simpson *et al.*, 2003; San Martin *et al.*, 2013). Later, a displaced phragmoplast becomes visible, from which cell walls are laid down, with participation of the endomembrane system, forming three small degenerative microspores and one large functional microspore (Brown & Lemmon, 2000; San Martin *et al.*, 2013). The functional microspore undergoes a slightly different microgametogenesis, where the pollen mitosis I happens as early as cytokinesis during meiosis is finished (Rocha *et al.*, 2018). Despite peculiarities in both microsporogenesis and microgametogenesis, the resulting tricellular pollen grain at the end of gametogenesis, with two sperm cells inside a large vegetative cell, presents no unusual features compared to other angiosperm pollen (Rocha *et al.*, 2018), at least when analyzed in light microscopy. The formation of pseudomonads arising from asymmetric tetrads after meiosis is considered a synapomorphy in Cyperaceae, although a similar process is described in Epacridaceae (Furness & Rudall, 2011).

Since vegetative cells in pollen grains are known for presenting a well-developed endomembrane system and because of the peculiar aspects involved in Cyperaceae pollen grain formation, there is great interest in the cellular adaptations and modifications happening during microgametogenesis in sedges (Tanaka, 1941; Brown & Lemmon,

2000; Coan, Alves & Scatena, 2010; San Martin *et al.*, 2013, Rocha *et al.*, 2016). Besides this, there are still no significant studies of maturing pollen grains in Cyperaceae regarding its cellular components, such as the endomembrane system. Since microgametogenesis in sedges had shown indicatives of not following the standard development seen in other angiosperms, it would be of great cytological interest to describe and explain the cytological aspects occurring in the pollen grain development. In addition, many unusual endomembrane features have been indicated to occur during plant development pollen of other species, such as cortical endoplasmic reticulum (cER, Heslop-Harrison, 1968; Weber, 1989; Rodriguez-Garcia *et al.*, 1995; Lancele & Hepler, 1992) and possible unconventional secretion (Yamamoto *et al.*, 2003; Prado *et al.*, 2014). However, these occurrences have been overlooked or only briefly discussed so far.

In order to characterize the maturing pollen grain ultrastructural features in Cyperaceae, anthers of *Eleocharis geniculata* (L.) Roem. & Schult., *Rhynchospora pubera* Boeckeler, *Rhynchospora ciliata* (Vahl) Kük. and *Rhynchospora nervosa* Boeckeler were processed and analyzed under light and transmission electron microscopy. The question that guidelined this work was: is there any unusual ultrastructural features occurring in the maturing pollen grain of Cyperaceae? Here, we provide the answer to this and other questions.

Material and methods

Specimens of *E. geniculata, R. pubera, R. ciliata* and *R. nervosa* were collected in various locations and kept in the greenhouse of Laboratório de Citogenética e Diversidade Vegetal (LCDV) of the State University of Londrina (UEL). Vouchers were deposited in FUEL herbarium (see Table 1 for detailed information). For light and electron microscopy, pre-anthesis anthers of *E. geniculata, R. pubera, R. ciliata* and *R.*

nervosa were fixed by immersion in 2,5% glutaraldehyde in 0,05M phosphate buffer, pH=6.8, for 24 hours in constant agitation. Afterwards, anthers were washed in the same buffer and post-fixed in 1% osmium tetroxide for four hours. After washing, samples were dehydrated in a graded ethanol series and diaphanized in propylene oxide. After infiltration in propylene oxide:Araldite ® solutions (1:3, 1:1, 3:1, 24 hours each), material was blocked in Araldite ®. Thin sections (2µm) and ultrathin sections (70nm) were made in Leica Ultracut. For anatomical purpose, thin sections were placed in glass slides, stained using toluidine blue and mounted in Entellan. For cytochemical detection of dictyosomes and derived structures, thin sections were placed in glass slides and treated with 50% silver nitrate ($AgNO_3$) for 15 minutes at 60ºC. Afterwards, slides were plentifully washed in distilled water and mounted in Entellan. Ultrathin sections were collected in 200 mesh copper grids and contrasted with uranil acetate and Reynolds lead citrate (Reynolds, 1963), 15 minutes each. Samples were analyzed using a DM4500B light microscope and a FEI Tecnai 12 transmission electron microscope at 80 kV. Images were captured with the aid of Leica Application Suite and Soft Imaging System and Analysis Software, for light and electron microscopy, respectively.

Results

Mature pollen grains in *E. geniculata, R. pubera* and *R. ciliata* presented wedge shaped pollen with comparable sizes and some general similar cytoplasmic features. In these species, pre-anthesis anthers had tri-cellular pollen, with small elongated two sperm cells within a large vegetative cell, which presented cytoplasm well stained by toluidine blue. They contained strongly stained granules, small vacuoles and a thin well-developed sporoderm (Figures 1a, c, e). Pollen in *R. nervosa* was similar but presented larger vacuoles (Figure 1g). Cytochemistry revealed that some regions of the vegetative cell

cytoplasm reacted intensely to the silver nitrate, while others did not react with the same intensity. Several granules were also strongly marked by silver nitrate reaction (Figures 1b, d, f, h). Tonoplasts were also clearly evidenced in silver nitrate cytochemistry, as observed in *E. geniculata* (Figure 1b).

Ultrastructural analysis revealed that vegetative cells in all species studied exhibited a large nucleus with decondensed chromatin, and a cytoplasm rich in amyloplasts with mitochondria and some lipid droplets. The endomembrane system was well developed in the vegetative cell cytoplasm, with evident rough endoplasmic reticulum (rER) near vegetative nucleus and sperm cells, vesicles and vacuoles (Figure 2, 3a-b). Occasionally, vacuoles presented a wide range of cytoplasmic inclusions inside its lumen (Figure 3c). Portions of the rough rER and what apparently was smooth endoplasmic reticulum (sER) were seen near differentiated portions of the cytoplasm containing fibrillar material cell. Vacuoles and mitochondria also appeared in this region. The rER clearly recognizable by ribosomes adhered to the membrane and the lumen presented electron density similar to the cytoplasm. Apparent sER cisterns presented electron light lumen (Figure 3d). The two sperm cells exhibited small nuclei with condensed chromatin, a reduced number of organelles such as undifferentiated plastids in both *E. geniculata* and *R. pubera* (Figures 3b, e). Physical connections could be noticed between cytoplasms of sperm and vegetative cells (Figure 3f). Debris of the abortive or degenerative microspores could be observed in pre-anthesis pollen grains of all species studied. They often appeared as fragmented electron dense cellular structures (Figure 3g). The sporoderm was stratified in intine and exine. In three equidistant places, intine presented noticeable thickenings in *E. geniculata*, while in *Rhynchospora*, intine was always uniform. In all species, exine was uniform and no clear apertures could be noticed in any sections made in light or electron microscopy (Figures 1, 2, 3a).

The endomembrane system showed a particular arrangement in relation to some ER cisterns and dictyosomes in *E. geniculata*, as well as in *R. pubera*, *R. nervosa* and *R. ciliata* pollen grains (Figures 4 and 5) In addition to the ER cisterns that appeared in the inner portions of the cell (Figure 2), other ER cisterns appeared adjacent to the plasma membrane, either as small portions (Figure 4a) or as a complex network near the plasma membrane, and even reaching a physical contact to it, such as observed in *R. pubera* and *R. ciliata* (Figures 4b, c, 5a). In some instances, cisterns presented enlarged portions with electron light lumen, as seen in *R. pubera* (Figure 4c). Concentric networks of ER were seen in the vegetative cell cytoplasm as well. In some instances, their cisterns were clearly visible (Figure 4d), while in others they appeared collapsed in hardly distinguishable (Figure 4e). Dictyosomes presented also variable morphologies and location. Most presented the characteristic C shape with convex *cis* network and concave *trans* network, as observed in *R.pubera* and *R. ciliata* (Figures 5a, c, d) while others presented linear shape, as seen in *E. geniculata* (Figure 5b). Some presented their *trans* network facing inner regions of the vegetative cell, and, in such occasions, cortical ER could be seen nearby (Figure 5a). In *R. ciliata*, several dictyosomes occupied large portions of the cytoplasm and had their *trans* network facing multiple directions (Figure 5c). Dictyosomes presenting the classic trans network to plasma membrane configuration were also seen, including one facing its *trans* network towards debris between intine and exine (Figure 5d). However, in all species observed, vesicles were pouring out of the *trans* network in various directions (inwards and outwards of the vegetative cell, Figure 5).

Another unusual feature happening in pollen grains of the species studied was the apparent exocytosis of multivesicular bodies. This feature was seen in multiple occasions in *R. ciliata* (Figure 6a), *E. geniculata* (Figure 6b) and *R. pubera* (Figures 6c, d). These bodies contained multiple small vesicles and were frequently seen in the

vegetative cell cytoplasm fusing with the plasma membrane near sporoderm, apparently releasing their exosomes or paramural bodies content in the extracellular space between plasma membrane and intine (Figures 6a, b). Multivesicular bodies were also seen fusing with the plasma membrane releasing exosomes in the extracellular space between vegetative cell and sperm cells. Accordingly, many exosomes can be seen in this region (Figure 6c). Additionally, smaller multivesicular bodies could also be observed in the sperm cell cytoplasm near the plasma membrane (Figure 6d).

Discussion

Overall pollen features

In Cyperaceae, microsporogenesis give rise to an asymmetric tetrad of microspores (Ranganath & Nagashree, 2000). This process is well documented by light and transmission electron microscopy. After meiosis, the phragmoplast appears dislocated to the degenerative nuclei, which, in consequence, creates three small degenerative microspores and a large functional microspore (Brown & Lemmon, 2000). At stage of development (after meiotic cytokinesis), cytoplasm presents small organelles and vacuoles, but with few ER cisterns and dictyosomes (Brown & Lemmon, 2000; San Martin *et al.,* 2013), as it is also seen during early microspore development in other species such as *Arabidopsis thaliana* (Yamamoto *et al.,* 2003). After pollen mitosis I, which happens as soon as cell walls are laid down after meiosis (Rocha *et al.,* 2018), dictyosomes and autophagosomes become noticeable in vegetative cell cytoplasm, near the degenerative microspores, which later on presents clear signs of autophagy as well (Rocha *et al.* 2016). Microgametogenesis in Cyperaceae presents some peculiarities in comparison to most angiosperms, but still creates tricellular pollen with two sperm cells

inside a vegetative cell (Brown & Lemmon, 2000; San Martin *et al.*, 2013; Rocha *et al.*, 2018).

Sperm cells present is similar to those in other angiosperms, presenting, for instance, few small organelles and nucleus with condensed chromatin (Knox, 1984). In contrast, vegetative cell presents well developed cytoplasm, especially in comparison to earlier stages of pseudomonad development reported in the literature (San Martin *et al.*, 2013; Rocha *et al.,* 2016). Cytological features such as indicate large nucleus with prominent nucleoli, extensive networks of ER and several dictyosomes all indicate high metabolic activity, as expected for this cell. This activity involves many aspects of pollen physiology, such as reserve anabolism, as observed in the form of starch and lipid droplets, and cell wall development (Mepham & Lane, 1970; Knox, 1984). Unique to Cyperaceae, the vegetative cell could be involved in assimilating material from the degenerative microspores. These cells are aborted and fragmented in small bodies, similar to apoptotic bodies in animal cells, as shown in *E. geniculata*, and in the vegetative cytoplasm adjacent to them there are dictyosomes with their trans network facing the plasma membrane, as observed in *R. ciliata*. This could be indicative that vesicles arising from dictyosomes are involved with the late breakdown of cellular debris from the abortive microspores. This way, degeneration of the nonfunctional microspores and their assimilation by the vegetative cell can play an important role in late stages of pollen development (Rocha *et al.*, 2018). However, immunocytochemical analysis are needed to reveal the contents of the vesicles arising from the trans Golgi network at this point.

Endoplasmic reticulum and dictyosome arrangements

In addition to ER located near the vegetative nucleus, the ER presented some unusual configurations in the vegetative cell. Concentric layers of ER showing clear

tubules were seen in some cell while in others, ER cisterns were flat and electrondense. Concentric ER are reported during microspore and pseudomonad development, and they are also present in megaspore mother cells (Mepham & Lane, 1970; San Martin *et al.*, 2013; Rocha *et al.*, 2014). The appearance of this structure can represent many processes, one of each is the cytoplasm turnover from sporophytic to gametophytic generations (Bhandari, 1984; Knox, 1984). Similar concentric structures with collapsed aspect could represent late concentric ER, which, in addition to the different cytoplasmic regions observed in silver nitrate cytochemistry and transmission electron microscopy, corroborate to the cytoplasmic turnover hypothesis. In other instances, Cortical ER (cER) networks adjacent to the plasma membrane of vegetative cells next to the sporoderm were observed in Cyperaceae. Although sedges present peculiar microsporogenesis and microgametogenesis, this feature appears to be common in pollen development in general (Rodriguez-Garcia & Fernández, 1990). Similar reports are available about this feature in the literature for microspores (Heslop-Harrison, 1968), pollen grains (Weber, 1989; Rodriguez-Garcia *et al.*, 1995) and pollen tubes (Lancele & Hepler, 1992). However, little attention has been given to the occurrence of cER and its biological meaning in pollen cells so far.

cER is reported to appear during microspore development as early as tetrad stage, and was thought to act as a physical barrier, preventing deposition of cell wall in the adjacent region (Heslop-Harrison, 1968). However, in maturing pollen, networks of cER are in some instances very extensive. If the cER would be preventing cell wall deposition in the region near it, it would mean that large portions of sporoderm would be lacking intine, which was not observed. This way, it is plausible that the cER network here is involved in other roles rather than playing a physical barrier for secretion. The occurrence of dilated cisterns of ER in maturing pollen of *Apium nodiflorum* indicates a

possible storage function (Weber, 1989). Dilated ER cisterns in pollen were seen here in *R. pubera* and reported in *Arabidopsis thaliana* (Yamamoto *et al.,* 2003) and, therefore, storage function could be common in some maturing pollen. In epithelial cells of onions, cER is thought to play both structural and signaling roles (Hepler *et al.,* 1990). By acting as calcium reserves, cER can promote signaling pathways (Hepler *et al.,* 1990; Wang, Hawes & Hussey, 2016), as this ion is a well-known intracellular signalizer. Calcium is known to play an important role in pollen germination and pollen tube growth (Steinhorst & Kudla, 2013), therefore, cER observed in Cyperaceae pollen could possibly be related to germination. In a mechanical role, cER can act as anchoring sites to the cytoskeleton and therefore facilitating the application of forces to move cell compartments and organelles (Hepler *et al.,* 1990). This way, cellular movements seen in pollen, such as the migration of the sperm cells near the vegetative cell or the movement of endosomes and multivesicular bodies could be anchored in the cER network. Additionally, cER might be involved in cargo transport directly to the plasma membrane, endocytosis, biotic stress response and other functions in plant cells (Rodriguez-Garcia & Fernández 1990; Hepler *et al.*, 1990; Wang, Hawes & Hussey, 2016).

Even though cER networks may assume storage, signaling and mechanical roles in maturing pollen, it is safe to say that it still presents its classic biosynthetic-secretion roles due to the presence of ribosomes adhered to its membranes, which could also be the reason dictyosomes presented a variety of positionings in the vegetative cell. Similarly, to the ER, dictyosomes are scarce in early stages of microgametogenesis (Yamamoto *et al.,* 2003; San Martin *et al.,* 2013; Rocha *et al.,* 2016), but become numerous during pollen maturation, as seen here and in *A. thaliana* (Yamamoto *et al.,* 2003). Many dictyosomes were observed with their *trans* network not facing the plasma membrane, but instead, facing the interior regions of the cell. Several small vesicles, probably arising

from the *trans* network were seen in these regions as well, especially in *R. ciliata*, in which significant portions of the cytoplasm were composed of dictyosomes arranged in different orientations, accompanied by small vesicles. Although some dictyosomes near the plasma membrane can be related to secretion towards the sporoderm, it is probable that many are involved in vesicle trafficking to cell wall formation between vegetative and sperm cells. Additionally, dictyosomes are related to vesicle trafficking to components of the endomembrane system, such as endosomes (which includes multivesicular bodies) and vacuoles. This is evidenced by positive reaction of the tonoplast with silver nitrate – which reacts positively to proteins arising in dictyosome compartments (Elftman, 1952). Vesicle trafficking to form sperm cell wall and to endosomes can explain the observation of dictyosomes with *trans* networks facing the interior of the pollen.

Unconventional secretion pathways

It has been observed here in the vegetative cell of Cyperaceae pollen that in more than one instance and in more than one species, some cellular compartments, such as vacuoles or multivesicular bodies, are dislocated to the plasma membrane and their components are apparently secreted to the sporoderm. Similar situations were observed in cryofixation of *Arabidopsis thaliana* pollen, in which the exocytosis of a small vacuole is visible (Yamamoto *et al.*, 2003). The several observations made here and in cryofixated pollen of *A. thaliana* shows that this is not an artifact from glutaraldehyde fixation, but instead stands for a form of unconventional secretion.

Known mechanisms of unconventional secretion involve translocation of proteins across the plasma membrane or fusion of membrane bound bodies with the plasma membrane with the release of cargo to the extracellular space. The latter may

include vesicle trafficking ER directly to the plasma membrane and the exocytosis of vacuoles, endosomes, multivesicular bodies, lysosomes and even autophagosomes (Ding *et al.*, 2012). Since cER forms an extensive network near the plasma membrane, there could be unconventional secretion occurring as material being transported directly from the ER to the plasma membrane (Rodriguez-Garcia & Fernández, 1990). Additionally, as it was shown in *Arabidopsis* (Yamamoto *et al.*, 2003) and in Cyperaceae, vacuoles, multivesicular bodies and possibly other related structures can apparently be secreted in the form of unconventional secretion. This way, membrane bound structures may receive material from the cytoplasm by vesicles arising from dictyosomes (as evidenced here by silver nitrate), translocation or inward budding of the cytoplasm (An, van Bel & Hückelhoven, 2007) and release them to the extracellular space. This type of secretion was observed mainly in vegetative cells but also in sperm cells. The presence of secretion in sperm cells and its physical connection with the vegetative cell through plasmodesmata indicate metabolic activity despite the presence of condensed chromatin. Unconventional secretion is described in other plant cell in defense mechanisms, such as in barley and tabacco (An, van Bel & Hückelhoven, 2007; Ding *et al.*, 2014), and during development, such as in differentiating tracheary elements of species such as beech and red oak (Robards & Kidwai, 1969). Here, both conventional and unconventional secretion are seen taking place in secondary wall synthesis. This situation is similar in the maturing pollen grain, where the vegetative cell must produce and secrete the intine precursors (Knox, 1984). However, evidence of unconventional secretion occurring in developing pollen is elusive in the literature. It is probable that unconventional secretion in maturing pollen is concerned with cell wall formation, as it does in tracheary elements. Still, more data should be collected in order to stablish the role unconventional secretion has in pollen development.

Conclusions

Maturing pollen in Cyperaceae presented unusual endomembrane behavior, which includes cortical ER, concentric ER and unconventional secretion of multivesicular bodies. Cortical ER near the plasma membrane next to the sporodem is concerned with classic secretion pathways. However, they may also assume storage, structural and signaling roles, and possible others. Meanwhile, concentric ER could be involved in cytoplasmic turnover. Numerous dictyosomes were observed, involved in classic secretion pathways to sporoderm and sperm cell wall formation, and, unique to Cyperaceae, they could be involved in late breakdown and assimilation of degenerative microspores. Unconventional secretion was observed several times in vegetative and sperm cells. This occurrence in the later is an indicative that they are not as metabolically inactive as assumed. Despite the occurrence of peculiar microsporogenesis and microgametogenesis, reports from the literature show that none of the unusual features observed here are exclusive from sedges. Instead, many other angiosperm pollen grains are reported to present them. This could represent an opportunity to reexamine our understanding on the endomembrane system in pollen cells in general.

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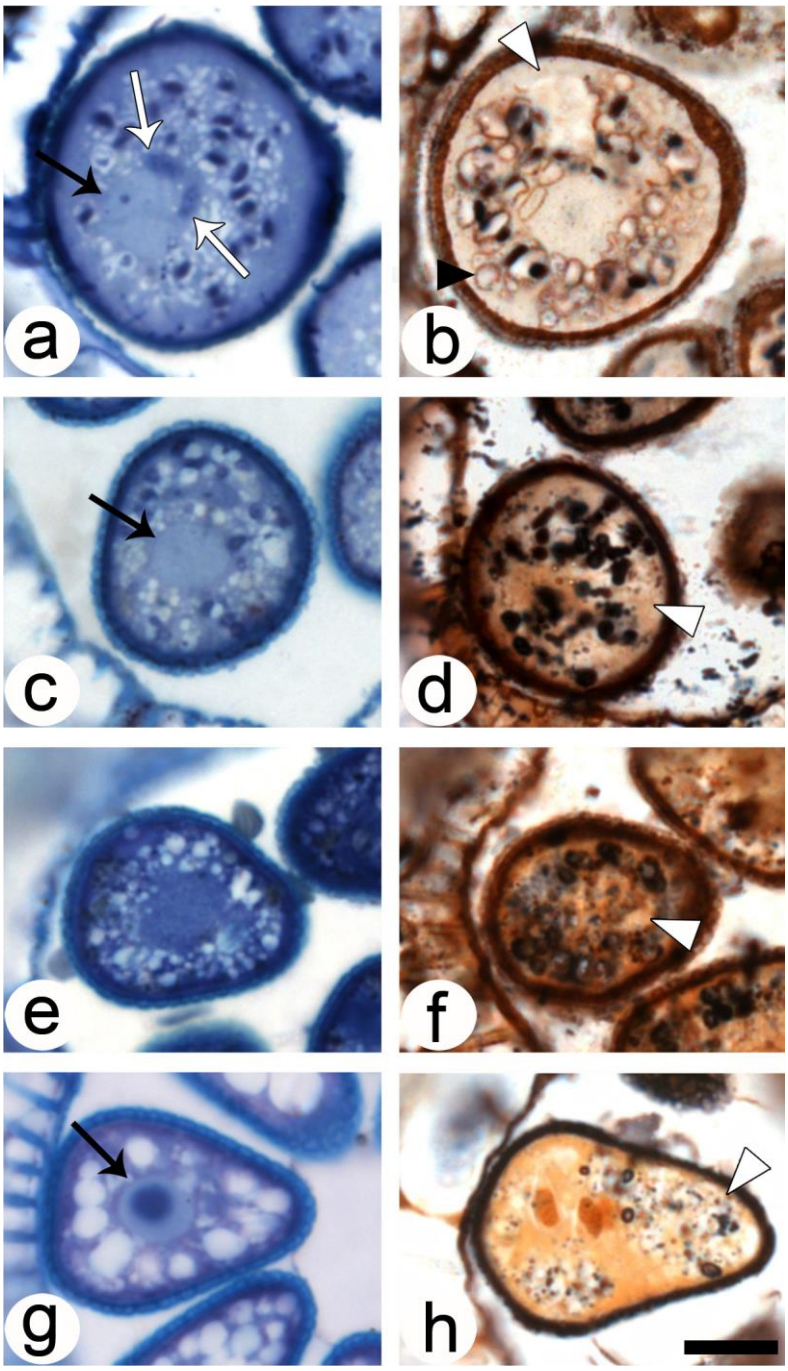

Figure 1. Transversal thin sections showing the overall features of pre-anthesis pollen in Cyperaceae. (a and b) *Eleocharis geniculata*, (c and d) *Rhynchospora pubera*, (e and f) *Rhynchospora ciliata* and (g and h) *Rhynchospora nervosa*. (a, c, e, f) Toluidine blue staining. (b, d, f, g) Cytochemistry with silver nitrate. Black arrow = vegetative nucleus,

white arrow = sperm cells, white arrowheads = Cytoplasmic regions of the vegetative cells with less silver nitrate oxidation, black arrowhead = tonoplast oxidized by silver nitrate Scale bar= 10 µm. All pollen grains are wedge shaped and present similar size. Small vacuoles can be seen scattered throughout the vegetative cell cytoplasm. In *R. nervosa,* larger vacuoles can be seen. Note that in both toluidine blue staining and silver nitrate reaction, small granules were evident.

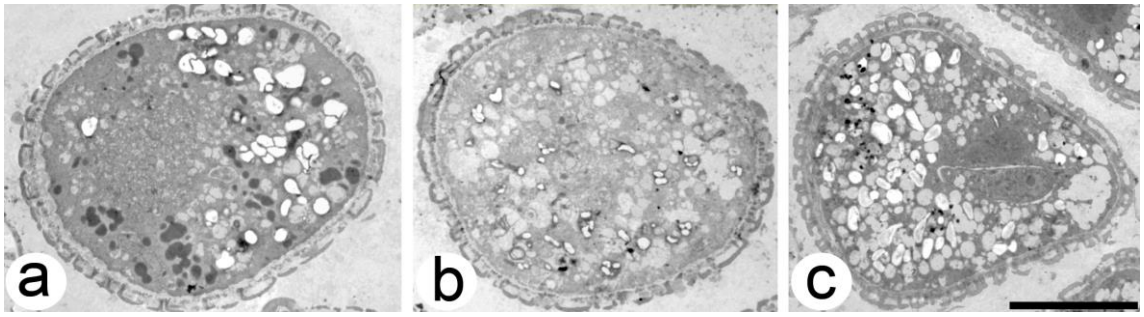

Figure 2. Transversal section showing the overview of pre-anthesis *Rhynchospora* pollen. (a) *R. ciliata,* (b) *R. pubera* and (c) *R. nervosa*. Scale bar = 10 µm.

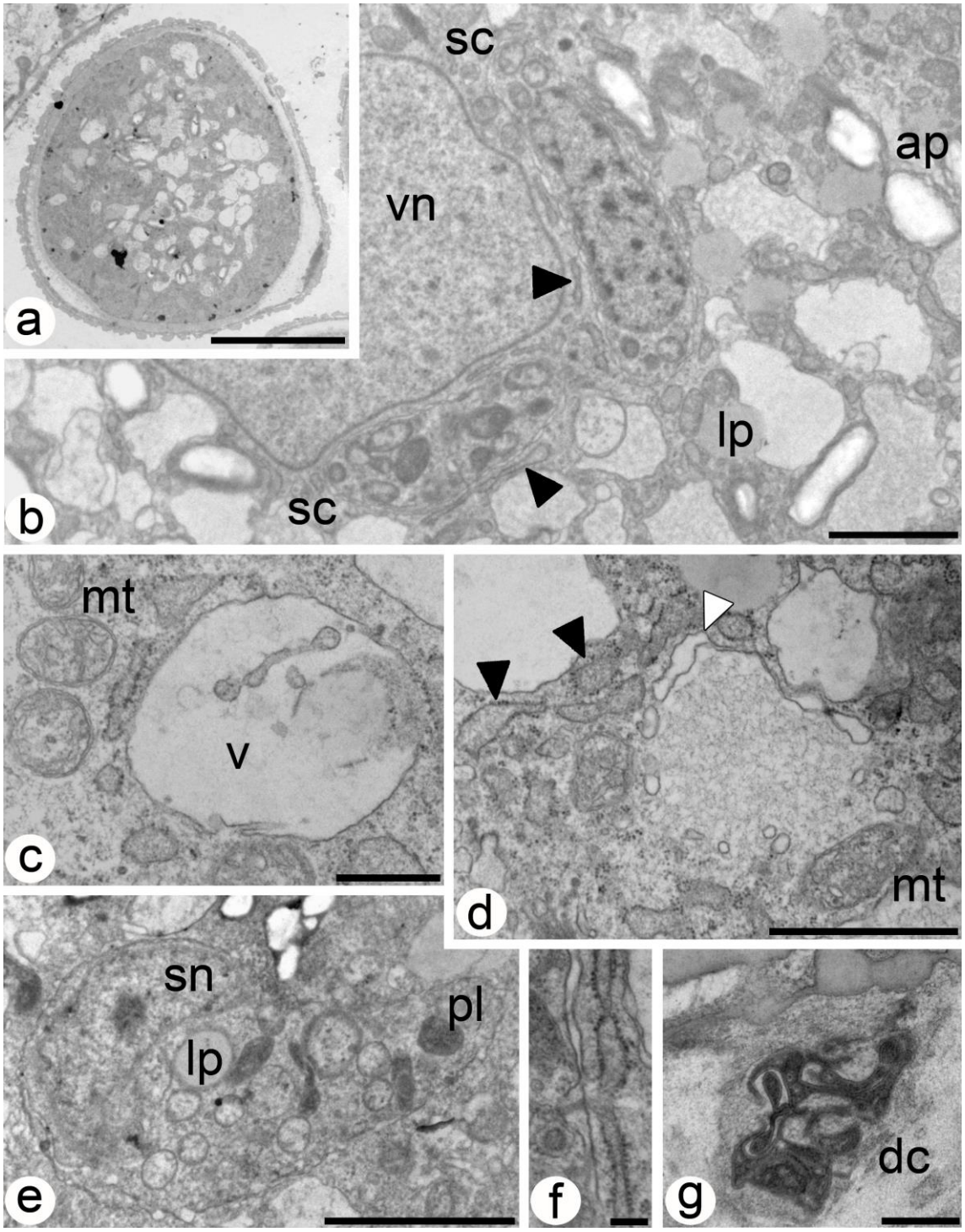

Figure 3. Transversal ultrathin sections showing the ultrastructural features of *E. geniculata* and *R. pubera* pre-anthesis pollen. (a-d, f and g) *E. geniculata.* (e) *R. pubera.* (a) Overview of the pollen grain. Scale bar = 10 µm. (b) A large section showing vegetative nucleus (vn) and sperm cells (sc) with endoplasmic reticulum (ER, black

arrowheads) in their proximities. The vegetative cell cytoplasm is rich in amyloplasts (ap) and small vacuoles. Some lipid droplets can be seen, one in close association with a vacuole (lp). Scale bar = 2 µm. (c) A vacuole with cytoplasm inclusions can be seen (v). Some mitochondria are present with well visible cristae (mt). Scale bar = 500 nm (d) Another region of the cytoplasm shows an electron light region with fibrous material. Next to it, cisterns of rough endoplasmic reticulum (rER, black arrowheads) and smooth endoplasmic reticulum (sER, white arrowhead) are present, as well as some mitochondria (mt) and vacuoles. Note that the rER lumen is electron dense in comparison to the sER lumen. Scale bar = 1 µm. (e) A detail of one sperm cell, with small nucleus (sn), a lipid droplet (lp) and some organelles like undifferentiated plastids (pl). Scale bar = 2 µm. (f) A plasmodesmata is seen between sperm cell and vegetative cell. Scale bar = 100 nm. (g) Debris from the degenerative cells can be seen in the apical or abaxial region of the pollen grain. These debris are seen as several electron dense bodies (dc). Scale bar = 200 nm.

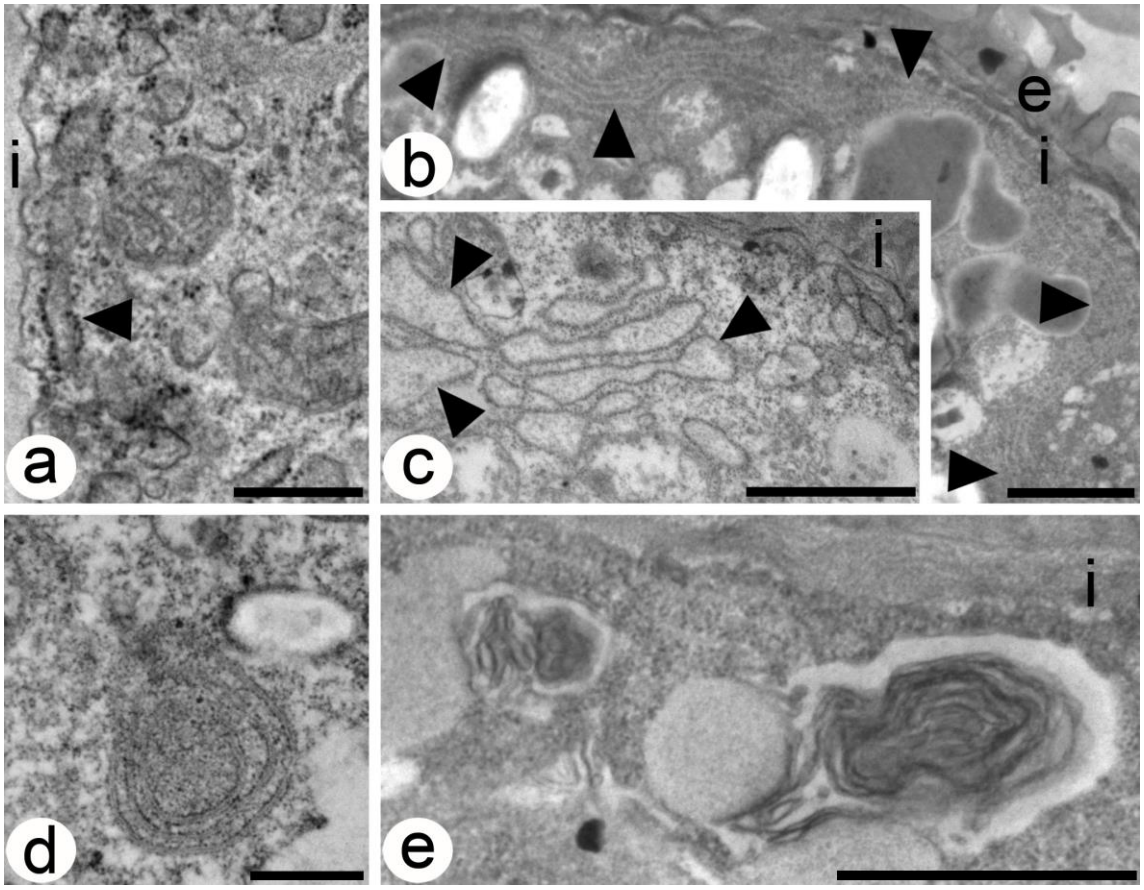

Figure 4. Transversal ultrathin sections showing different endoplasmic reticulum configurations in vegetative cells. (a) *E. geniculata*, Scale bar = 500nm, (b and e) *R. ciliata*, Scale bar = 1 µm in b and e, (c) *R. pubera*, Scale bar = 1 µm, (d and e) *R. nervosa*, Scale bar = 1 µm. (a-c) Cortical endoplasmic reticulum (cER, black arrowheads). While a small cistern of endoplasmic reticulum can be seen near the plasma membrane adjacent to the intine (i) in *E. geniculata* (a), a vast network (black arrowheads) of cER is present in *R. nervosa* along the intine (i) and exine (e). (b) In *R. pubera*, cisterns of cER appear dilated (black arrowheads) with electron light lumen (c). (d-e) Concentric layers of ER can be noticed in the vegetative cell of *R. nervosa* pollen. In some instances, cisterns have well defined limits (d), in others (e) they could be hardly distinguished and presented a collapsed aspect.

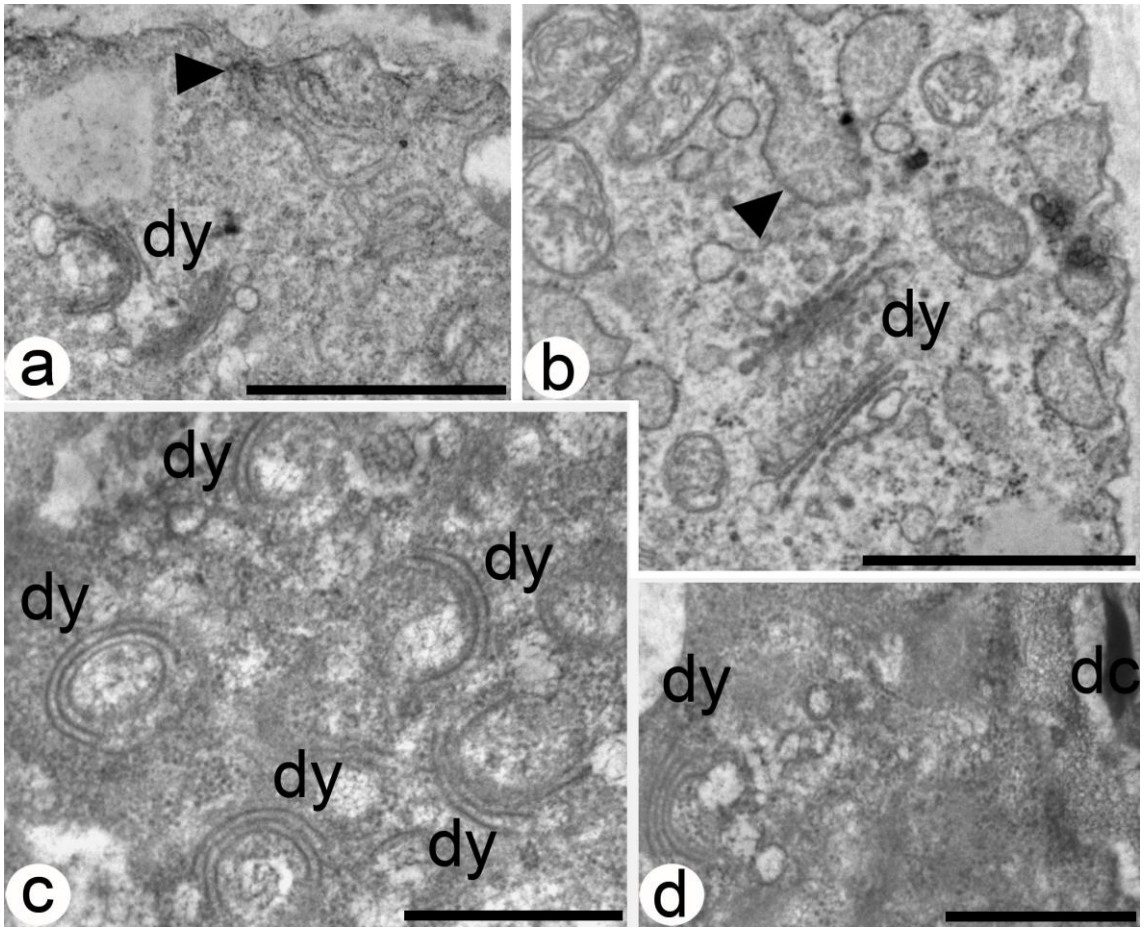

Figure 5. Transversal ultrathin sections showing cortical endoplasmic reticulum and dictyosomes in vegetative cells. (a) *R. pubera*, (b) *E. geniculata*, (c and d) *R. ciliata.* (a) Two dictyosomes (dy) can be seen with their *trans* network facing the inner portions of the cytoplasm. Above, next to their *cis* network, cER is present adjacent the plasma membrane near the intine (i). In some places, cER appears to be in very close association with the plasma membrane (black arrowhead). Scale bar = 1 µm. (b) Dictyosomes can be seen again with *trans* networks facing different portions of the cytoplasm. Note that these dictyosomes present linear configuration. Cistens of ER can be seen nearby (black arrowhead). Scale bar = 1 µm. (c) Several dictyosomes (dy) occupy a large portion of the cytoplasm. Each one presents a different orientation. Scale bar = 500nm (d) A dictyosome (dy) is seen with its *trans* network facing the plasma membrane adjacent to intine. In this

region of the extracellular space, debris of the degenerative cell (dc) can be seen. Scale bar = 500 nm.

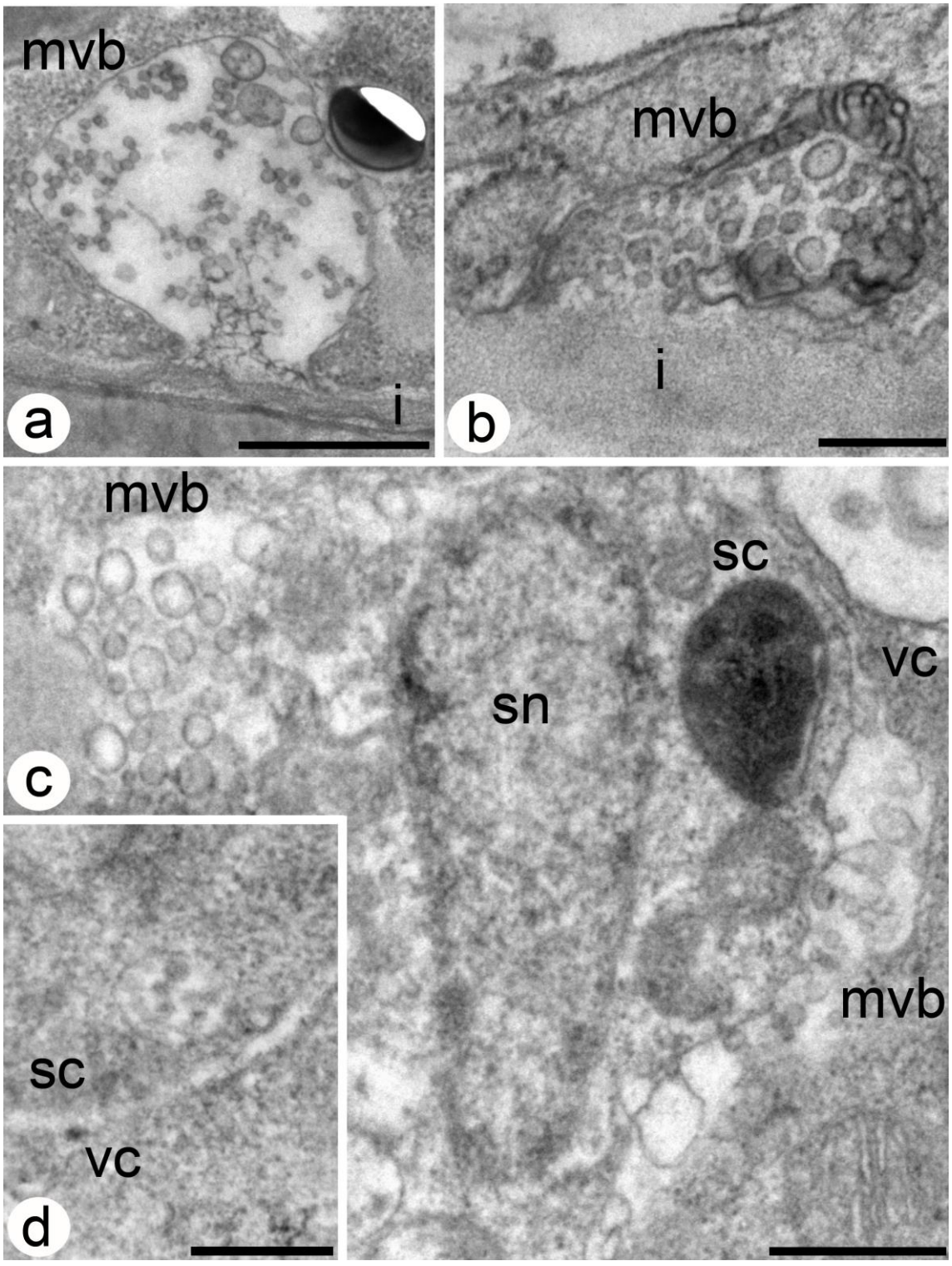

Figure 6. Transversal ultrathin sections showing unconventional secretion in vegetative and sperm cells. (a, c and d) *R. ciliata*, (b) *E. geniculata.* (a) A large multivesicular body (mvb) is seen fusing with the plasma membrane near the intine (i). Note that vesicles present different sizes. Fibrous material is seen in the multivesicular body (mvb) near the

extracellular space. Scale bar = 500 nm. (b) Another large multivesicular body (mvb) fusing with the plasma membrane near intine (i). Again, vesicles present different sizes. Scale bar = 200 nm. (c) Two multivesicular bodies (mvb) are seen in the vegetative cell cytoplasm fusing with the plasma membrane, releasing their vesicles or exosomes in the extracellular space between vegetative cell (vc) and sperm cell (sc). Sperm cell nucleus is also seen (sn). Scale bar = 500 nm. (d) A small multivesicular body is seen in the sperm cell cytoplasm (sc), near the plasma membrane (vegetative cell=vc) Scale bar = 200 nm.

Family	Genus	Especies	Location	FUEL voucher nº
Cyperaceae	*Eleocharis*	*geniculata*	Tupã SP	55225
	Rhynchospora	*pubera*	Recife PE	55374
	Rhynchospora	*nervosa*	Florianópolis SC	55366
	Rhynchospora	*ciliata*	Recife PE	55372

Table 1. The species studied with the corresponding genera, location and voucher number.

CAPÍTULO IV

Expression of a heat shock protein-like gene in inflorescences of *Rhynchospora pubera* (Cyperaceae) and its possible relevance in pseudomonad development

Short communication a ser submetido para publicação

Expression of a heat shock protein-like gene in inflorescences of *Rhynchospora pubera* (Cyperaceae) and its possible relevance in pseudomonad development

Running title: Expression of a heat shock protein-like gene in *Rhynchospora pubera* (Cyperaceae)

Abstract

Programmed cell death (PCD) is present in many aspects during plant development and defense against pathogens. In Cyperaceae, PCD is also a part of pollen grain formation, during the abortion of three of the four microspores after microsporogenesis. In this structure, called pseudomonad, multiple cellular lineages coexist during pollen development. However, not a single gene has been implicated in this process. In this short communication, we analyze by bioinformatics and molecular tools some of the genes expressed in *Rhynchospora pubera* inflorescence development. A HSP70-like gene was well distributed in the sequenced transcriptome and, after RNA extraction and reverse transcription, PCR and electrophoresis showed greater expression compared to a reference gene: actin. HSP70, along with other genes found in the transcriptome, are implicated with ROS related HR-like cell death that could be associated to PCD in pseudomonads.

KEYWORDS: Pollen ● Microspore ● Microsporogenesis ● Microgametogenesis ● Gene expression ● PCR ● cDNA ● Electrophoresis ● Programmed cell death ● Microspore

Introduction

In a multicellular organism, some cells are selectively eliminated in a deliberate and controlled processes called programed cell death (PCD), which can be triggered by

several events (Pannel & Lamb, 1997; Gray & Johal, 2018). Interaction between pathogen gene products and host resistance genes can trigger the generation of reactive oxygen species (ROS) that lead to PCD, in a process called hypersensitive response (HR, Heath, 2000). Meanwhile, during regular development, different forms of PCD are an essential aspect in the development of many plant tissues, such as in tracheary element maturation, leaf senescence and megasporogenesis (Pannel & Lamb, 1997; *Bajon et al.*, 1999; Groover & Jones, 1999).

Microspore formation does not usually concern PCD, as in most angiosperms, microsporogenesis gives rise to four viable microspores (Bhandari, 1984). However, in all members of the sedge family Cyperaceae studied so far, microsporogenesis gives rise to an asymmetric tetrad of microspores, in which three degenerative microspores undergo PCD while the functional microspore forms the pollen grain (Furness & Rudall, 2011). Since pollen arises from a tetrad but only one microspore forms the pollen grain, pollen is shed as a false monad, thus its name: pseudomonad (Håkansson, 1954; Strandhede, 1965).

Although many genetic aspects of microsporogenesis have been elucidated in model organisms with regular microspore tetrads (Spielmen *et al.*, 1997; Hülskamp *et al.*, 1997; Oh *et al.*, 2010; Deveshwar *et al.*, 2011), not a single gene has been implicated on pseudomonad formation or microspore PCD in Cyperaceae. Given that pseudomonads present an excellent model of PCD and development, since degenerative and functional cellular lineages coexist in the limited space of a pollen grain, it would be of great interest to uncover the genetics involved in pseudomonad development. In this short communication, we use bioinformatics and molecular tools to investigate the expression

of genes related to cell death and their possible role during pseudomonad formation in sister species *Rhynchospora pubera* and *Rhynchospora breviuscula* (Cyperaceae).

Material and Methods

Plant material

Rhynchospora pubera Boeckeler and *Rhynchospora breviuscula* H.Pfeiff. were collected in Recife, Pernambuco, Brazil and Iporanga, Sao Paulo, Brazil, respectively. Specimens were kept in the greenhouse of the Laboratory of Cytogenetics and Plant Diversity at the State University of Londrina at ambient temperatures. Vouchers were deposited in FUEL herbarium (voucher number 55374 for *R. pubera* and 55362 for *R. breviuscula*).

Transcriptome analysis

The sequenced transcriptome of *R. pubera* pollen mother cells was obtained from the European Nucleotide Archive (ENA, https://www.ebi.ac.uk/ena), accession number PRJEB9645 (Marques *et al.,* 2015). A database containing several amino acid sequences from GeneBank (https://www.ncbi.nlm.nih.gov/genbank/) was built. BLASTx (Altschul *et al.*, 1990) was performed using the sequenced transcriptome against the database using the following parameters: identity above 80%, size above 150 amino acids, with E-value inferior than 10^{-5}. The results were plotted in a graph showing the reference from database against the number of contigs aligned with that reference.

HSP70 phylogeny

From the BLASTx results, contigs were selected based on having the greatest nucleotide lengths. A consensus sequence was made from the HSP70 genes aligned with sequences obtained from BLASTx run. A new BLASTn was made using this consensus

sequence and afterwards, HSP70 aligments from representatives of each plant family were selected. The selected sequences and the consensus sequence were aligned in MUSCLE. Aligments were verified in Gblocks and organized in a maximum likelihood phylogenetic tree using PhyML applying the model of substitution GTR with bootstrap value of 100. In addition, a Bayesian phylogenetic tree using MrBayes, applying the GTR likelihood substitution model and 10 000 generations were made and a tree was sampled every ten generations. Trees were rendered in TreeDyn and edited using Figtree 1.4.4.

RNA extraction, reverse transcription, amplification and electrophoresis

Total RNA from young *R. pubera* inflorescences containing several anthers with different sizes was isolated using phenol/SDS method (Ghawana *et al.*, 2011). Afterwards, DNAse I treatment (Sigma Aldrich) and reverse transcription (M-MLV, Promega) were made according to the manufacturer's specifications. Primers for PCR amplification were design using GenScript Online PCR Primers Designs Tool (https://www.genscript.com/tools/pcr-primers-designer). PCR with 32 replication cycles was made using Platinum Taq DNA Polimerase (Thermo Fisher) and primers for HSP 70 (F-ATCAGTGGCAACCCAAGGGC and R-AAACCTGGCACGGGTGATGG) and Actin (F- ATACGGTCGGCAATGCCAGG and R- TGCGGAGCGGTTCAGATGTC). Amplified products were submitted to electrophoresis in a 1% agarose gel with a 100 base pair ladder DNA (Invitrogen).

Results

Transcriptome analysis showed that 29 different coding sequences aligned with several contigs from the sequenced transcriptome PRJEB9645 (Marques *et al.*, 2015), which are represented in Figure 1. Eight different contigs aligned with a heat shock protein 70 (HSP70) from the GeneBank database. That was the most alignments any gene

from the database could get. As reference, the mRNA for actin aligned with six different contigs. Six contigs had correspondence with a Rac-like GTP-binding protein. Six contigs had correspondence with Map quinase and other six with Actin. TATA-box binding protein, mannose-1-phosphate guanylytransferase and callose synthase sequences from database aligned with five different contigs each. Four different contigs had correspondence with ubiquitin conjugating enzyme and other four had correspondence with ATP-citrate synthase. Prohibitin-3, exportin 1A and a B2DCD domain sequences aligned with two different contigs each. Several other sequences from the database aligned with only one contig from the sequenced transcriptome (Figure 1). Primers were designed to amplify a 150 base pairs fragment of *Rhynchospora* HSP70-like and a 186 base pairs fragment of *Rhynchospora* actin (Figure 2). Electrophoresis of the amplified PCR products showed that HSP70-like was amplified in a greater amount in PCR, in comparation to actin (Figure 3).

Both trees resulting from maximum likelihood analysis and Bayesian analysis showed that *R. pubera* HSP70-like presented a greater similarity with other HSP found in monocotyledons such as representatives of Asparagaceae, Bromeliaceae and Poaceae. In the Bayesian analysis, the monocot clade was sister to a clade containing several individuals from the Solanaceae family, including genera *Nicotiana, Solanum* and *Capsicum* (Figure 4). In the maximum likelihood analysis, they were farther apart. The relationship of *R. pubera* HSP70-like with other angiosperm families was also slightly different in each tree (Figure 5).

Discussion

Several coding sequences were found to be expressed in inflorescences of *R. pubera.* Among these sequences, some can be closely associated with PCD in plants.

Among all genes found in BLAST, the one with most alignments was a HSP70, with a total of eight contigs aligning with multiple HSP 70 in BLAST analysis. Electrophoresis analysis from semi-quantitative PCR inferred that HSP70-like shows a greater expression when compared to our reference gene actin. In addition, since RNA isolation for cDNA synthesis and PCR was performed in inflorescences containing anthers with different sizes, it could be inferred that HSP70-like is present in other stages of development apart from pollen mother cell stage, from which the transcriptome analysis was made.

The HSP70 is a multigene family found in many eukaryotic organisms that encodes a 70kd HSP associated with a wide variety of cellular processes (Lindquist & Craig, 1988). There is record in the literature of a HSP70 cognate being expressed constitutively in ovaries and vascular bundles (Duck, McCormick & Winter, 1989), as well as other HSP being expressed during microspore and pollen development (Frova, Taramino & Binelli, 1989). However, their relation to *R. pubera* HSP70-like is unclear. As the phylogenetic trees showed, *R. pubera* HSP70-like shows similarity to many other HSP70 sequences, especially with other monocots, as expected since they share a more recent common ancestor in relation to other dicot family members. A great number of those are predicted sequences, in which there is still few information about their function. However, the HSP 70-like found in *R. pubera* shows similarity with the CaHSP70, found in pepper (*Capsicum annum*), as indicated by Bayesian analysis. In pepper, this HSP interacts with Type III Effector AvrBsT, a protein produced by pathogen *Xanthomonas campestris*, forming a CaHSP70-AvrBsT complex. This interaction induces defense mechanisms such as reactive oxygen species (ROS) accumulation, which eventually leads to a form of HR cell death (Kim & Hwang, 2015).

To our knowledge, there is no record of a similar HSP70 expression in anthers or inflorescences of other plant groups. Additionally, reference aligned sequences from GeneBank for HSP70 did not concern any work made with anther or inflorescence development. This is despite PCD being common in tissues such as the tapetum in anthers, which could indicate these genes could be related to pseudomonad development in *Rhynchospora*. In addition, other genes that might have relation to PCD were also found. One of these is prohibitin. Literature indicates that silencing the prohibitin gene in plants causes plants to present greater senescence and higher levels of calatase and HSP expression (Chen, Jiang & Reid, 2005). A conserved protein domain named DCD (development and cell death) also appeared in the transcriptome analysis. As the name suggests, proteins with this domain were found to be related in development and PCD processes (Tenhaken, Doerks & Bork, 2005). For instance, a B2 protein containing the DCD domain was found to be overexpressed during embryogenic development in carrot (Schrader, Kaldenhoff & Richter, 1997). In soybean, the expression of a N-rich protein named NRP, with a DCD domain corresponding to the mRNA sequence found in *R. pubera*, is moderate in unstresses plants but is induced during pathogen related HR cell death (Ludwig & Tenhaken, 2001).

These genes appear to be linked with ROS signaling or HR response. Therefore, it could be that some of the mechanisms acting in HR are repurposed or conserved with PCD or other developmental processes that are happening during pseudomonad and pollen grain formation. However, more studies are needed to confirm and elucidate the possible relation between these genes and microspore abortion in *Rhynchospora*, as they could possibly play other roles in inflorescence development. If these genes were to be related to PCD in pseudomonads, it would be clear that others are required to this process,

as, for instance, CaHSP70 and NRP alone do not trigger PCD in pepper and soybean, respectively (Ludwig & Tenhaken, 2001; Kim & Hwang, 2015).

Conclusions

The transcriptome analysis of *R. pubera* inflorescences revealed a group of genes related to PCD which have not yet been described in anthers and inflorescences of other plant species with regular monads, including HSP70. A segment of this protein was amplified by PCD after RNA isolation and reverse transcription, which confirmed by electrophoresis that HSP70 is overexpressed in relation to the reference gene used. This gene, along with others such as prohibitin and a DCD domain protein, are implicated with ROS related HR-like cell death that could be related to PCD in pseudomonads. Despite our efforts, more studies are still needed to confirm the involvement of these genes in microspore abortion and the gene expression network underlining PCD in pseudomonads during pollen development in Cyperaceae.

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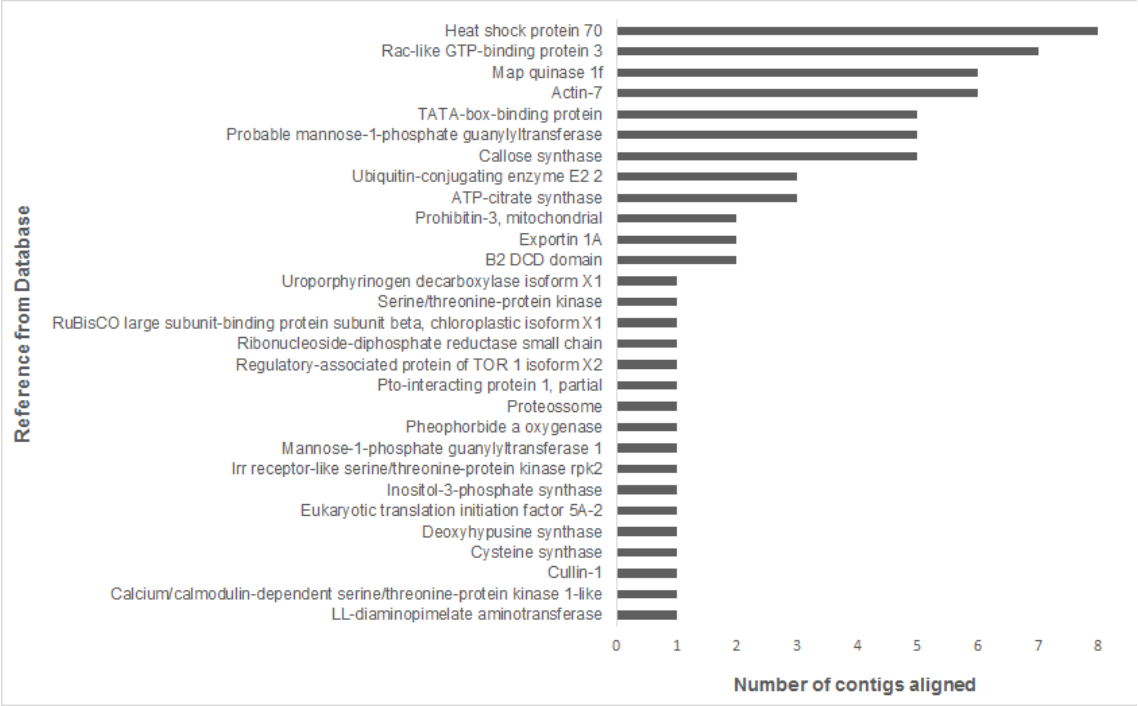

Figure 1. BLASTx result in using *Rhynchospora pubera* transcriptome against database.

Note that eight different contigs had correspondence with a heat shock protein 70.

>*Rynchospora* HSP70

1.....|.....|.....|.....|.....|.....|.....70

ATCAGTGGCAACCCAAGGGCTCTGAGGAGGCTCAGAACTGCTTGCGAGAGGGGAAGAGAACTCTCTCATC
AACTGCCCAGACCACAATTGAGATTGATTCTCTCTTTGAGGGTATTGATTTCTACTCTACCATCACCCGT
GCCAGGTTT

>*Rynchospora* Actin

1.....|.....|.....|.....|.....|.....|.....70

ATACGGTCGGCAATGCCAGGGAACATGGTAGATCCACCACTGAGCACAATGTTGCCATACAGGTCCTTCC
TGATATCCACATCACACTTCATGATCGAGTTGTAGGTCGTCTCATGAATACCGCCAGCTTCCATCCCTAT
CAAGGACGGCTGGAAGAGCACCTCTGGACATCTGAACCGCTCCGCA

Figure 2. Partial DNA sequences of HSP70-like and actin genes taken from *R. pubera* sequenced transcriptome. Primers used for PCR are underlined. The DNA sequence corresponding to HSP70 is 150 base pairs long. The DNA sequence corresponding to actin is 186 base pairs long.

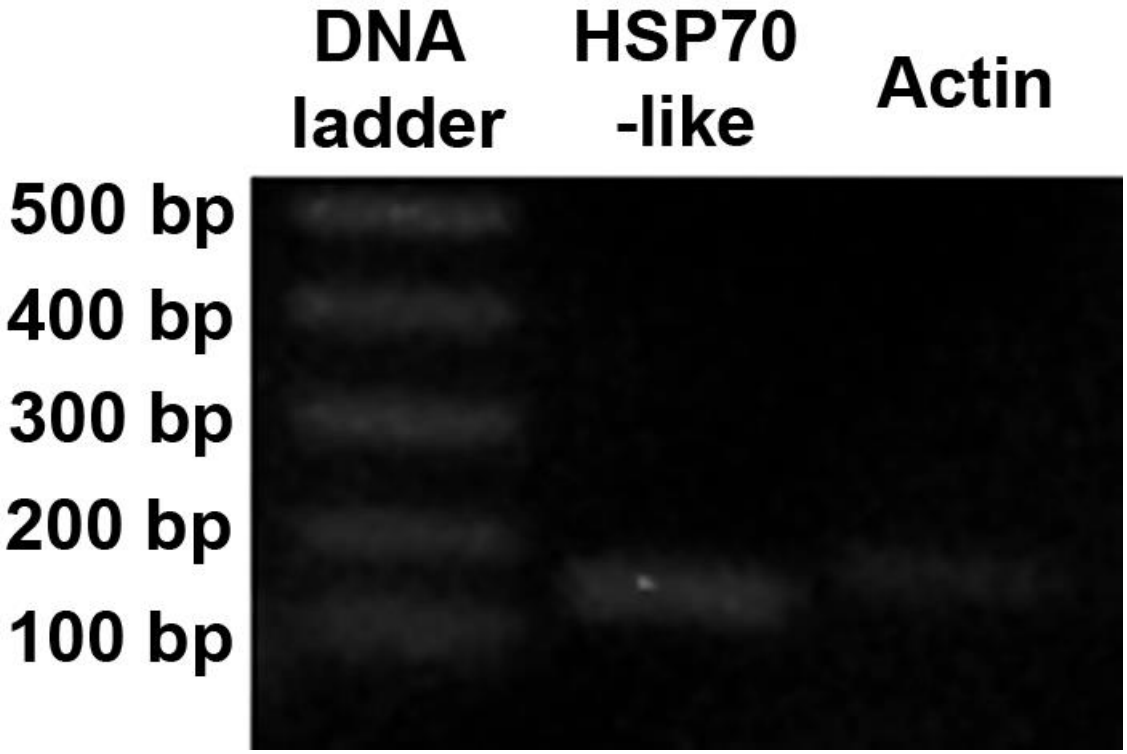

Figure 3. Eletrophoresis of the amplified DNA products for HSP70-like and actin in *R. breviuscula*, obtained from cDNA of inflorescences containing anthers with different sizes. Both fragments appear in between 100 and 200 base pars, according to the DNA ladder used. Note that the HSP70-like shows greater amount of DNA in relation to actin.

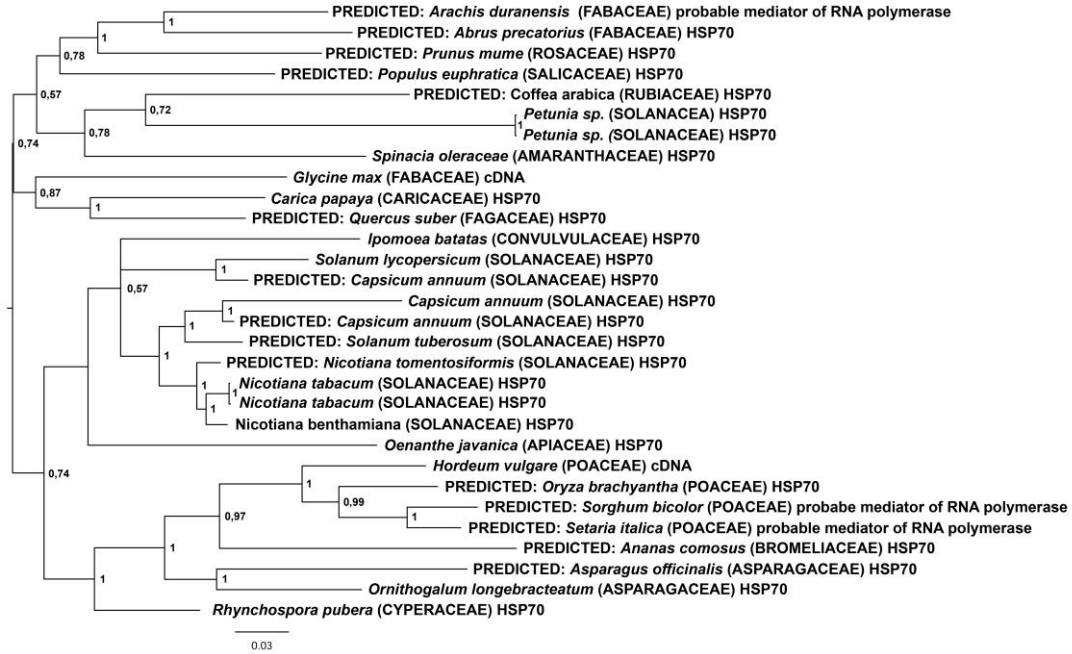

Figure 4. HSP70 phylogeny using Bayesian analysis. HSP70 in *Rhynchospora* appears close to the ones in other monocotiledons. In a sister clade, genes from individuals of Solanaceae family such as *Capsicum annuum* appear.

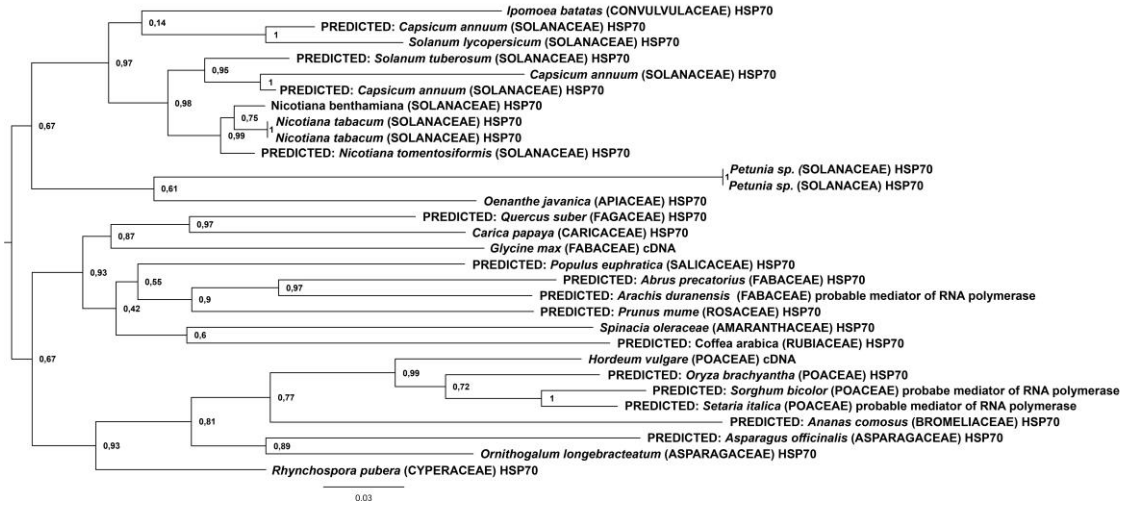

Figure 5. HSP70 phylogeny using maximum likelihood analysis. Similar to the Bayesian analysis, HSP70 in *Rhynchospora* appears close to the ones in other monocotiledons.

CAPÍTULO V

Considerações finais e perspectivas futuras

Considerações finais e perspectivas futuras

Os presentes trabalhos buscaram expandir o conhecimento sobre o desenvolvimento do grão de pólen em Cyperaceae, principalmente no que diz respeito aos estádios de desenvolvimento do final da microsporogênese e microgametogênese, os aspectos ultraestruturais do grão de pólen e alguns aspectos relacionados à expressão gênica envolvida nesse processo. De maneira geral, destacam-se as seguintes conclusões:

I) Pôde-se distinguir cinco estádios de desenvolvimento após a meiose assimétrica da célula mãe de micrósporo, que incluem: tétrade, mitose polínica I, vacuolação, mitose polínica II e grão de pólen maduro pré-antese. Esse desenvolvimento difere da maioria das angiospermas, em que a vacuolação do micrósporo ocorre antes da mitose polínica I.

II) Independente do seu posicionamento na pseudomônade, os micrósporos degenerativos são abortados por PCD durante o período de vacuolação, o que está associado a autofagia em primazia do micrósporo funcional, que ao mesmo tempo começa a acumular reservas na forma de amido ou outras substâncias, dependendo das condições ambientais. Durante esse processo, a coexistência de diferentes linhagens celulares pode ser possível pelo isolamento por calose.

III) Pseudomônades em Cyperaceae parecem ser originadas de tétrades permanentes, como aquelas em indivíduos da família irmã Juncaceae. Essa derivação aparentemente gerou um grão de pólen longevo e de rápida germinação, sem perda real de eficiência reprodutiva, já que em Cyperaceae os gineceus apresentam apenas um único rudimento seminal.

IV) O grão de pólen nas espécies estudadas apresentou características ultraestruturais peculiares, como a presença de retículo endoplasmático cortical, que pode estar associado com papeis estruturais, de armazenamento e de sinalização, além dos papeis tradicionais na via clássica de secreção.

V) As análises de bioinformática e biologia molecular revelaram que alguns genes relacionados a morte celular programada estão presentes em células mães de micrósporo e inflorescências de *Rhynchospora*. Entre eles, o gene mais encontrado no transcriptoma, que também é expresso em quantidades relativamente maiores do que actina em inflorescências jovens, é uma proteína de choque térmico que, segundo a literatura, está relacionada ao acúmulo de espécies reativas de oxigênio e resposta hipersensitiva.

Em relação as hipóteses levantadas no início do trabalho, podemos fazer os seguintes levantamentos:

-O perfil de desenvolvimento de pseudomônades é similar nos diferentes indivíduos da família Cyperaceae, exceto pela posição dos núcleos degenerativos, pelo menos nos gêneros *Rhynchospora* e *Eleocharis*.

-A microgametogênese em Cyperaceae não segue os padrões vistos nas demais angiospermas, visto que a vacuolação ocorre após a mitose polínica I e o grão de pólen apresenta diversos aspectos ultraestruturais peculiares.

-Genes já descritos que atuam em outros eventos do desenvolvimento da antera ou de outros tecidos das plantas estariam relacionados com as alterações morfológicas encontradas em pseudomônades, já que o gene de choque térmico é sintetizado em outras situações ou tecidos.

Diante dessas conclusões, ainda se tornam necessários mais estudos para elucidar alguns aspectos sobre o desenvolvimento de pseudomônades. Um deles seria a identificação de quais substâncias estão sendo secretadas nos corpos multivesiculares durante o processo de secreção não-convencional do grão de pólen. É provável que esse tipo de secreção esteja envolvido na formação de parede celular. Porém, apenas um estudo relacionando imunocitoquímica e ultraestrutura poderia responder essa questão.

Além disso, ainda é necessário mais dados sobre a regulação da expressão gênica envolvida durante o desenvolvimento das pseudomônades. No trabalho contido nessa tese, pudemos ver apenas alguns genes que poderiam estar relacionados com esse processo. Entretanto, seria necessário ainda quantificar a expressão desses genes em diferentes estádios do desenvolvimento por RT-qPCR e ainda localizar esses genes por hibridização *in situ* nas pseudomônades para confirmar a localização desses RNAs mensageiros.