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Description of a novel organ in the gametophyte of the fern *Schizaea pusilla* and its contribution to overall plant architecture

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Abstract: Plant architecture is determined by cell division and growth, thus simulation models describing these processes are ideal for determining how local development produces the overall plant form. Because fern gametophytes are structurally simple, they are ideal for investigating the effects of cellular growth and division on plant form. In this work we examine the gametophytic development of *Schizaea pusilla* Pursh., a small, bog-adapted fern whose gametophyte forms as a mass of single-celled filaments. Using light and scanning electron microscopy we made detailed observations of gametophyte development to generate data for a simulation mechanical model of *S. pusilla* gametophyte development. To examine how plant architecture is an emergent property of cell division, we constructed a simulation model expressed using the formalism of L-systems. While developing a model of growth in this fern we discovered a previously undescribed structure that contributes to the architecture of this plant, which we term knots. We document the development of knots and demonstrate how they contribute to the overall plant architecture.

Key words: Schizaea pusilla, fern, gametophyte, simulation model, L-system.

Résumé : La division cellulaire et la croissance déterminent l'architecture des plantes, de sorte que les modèles de simulation décrivant ces processus sont très utiles pour déterminer comment le développement local génère la forme générale de la plante. Compte tenu de leur structure simple, les gamétophytes des fougères permettent d'étudier facilement les effets de la croissance cellulaire et de la division sur la forme de la plante. Les auteurs ont examiné le développement du gamétophyte du *Schizaea pusilla* Purs., une petite fougère adaptée aux tourbières dont le gamétophyte est constitué d'une masse de filaments unicellulaires. À l'aide de la microscopie photonique et électronique par balayage, les auteurs ont conduit des observations détaillées sur le développement des gamétophytes pour obtenir les données nécessaires à la construction d'un modèle de simulation mécanique du développement du gamétophyte du *S. pusilla*. Afin d'examiner comment l'architecture de la plante provient de la division cellulaire, les auteurs ont construit un modèle de simulation exprimé à l'aide du formalisme de systèmes L. Tout en développant un modèle de croissance pour cette fougère, les auteurs ont découvert une structure jamais décrite qui contribue à l'architecture de cette plante et qu'ils nomment nœuds. Ils décrivent le développement des nœuds et montrent comment ils contribuent à l'architecture générale de la plante.

Mots-clés : Schizaea pusilla, fougère, gamétophyte, modèle de simulation, système L.

Introduction

Plant architecture is a result of cell division and growth, thus one can use cellular-level models of plant development to explore the effects of local development on global form (de Boer 1990; de Boer and de Does 1990; Prusinkiewicz et al. 1994; Holloway and Lantin 2002; Ruiz-Ramos and Minguez 2006) In theory, a model could reproduce the growth of an entire plant if the rules governing cell division at the local scale were understood. However, although successful developmental models of meristematic organization and phyllotaxis have been proposed (Jönsson et al. 2006; Smith et al. 2006), the modeling of even the simplest mature angiosperms is at present computationally intractable owing to prohibitively large numbers of cells.

Fern cells express similar biochemical and regulatory motifs as angiosperms (Banks 1999; Holloway and Lantin

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2002), but gametophyte development is much simpler. Furthermore, cellular structures that are difficult to observe in angiosperms, such as plasmodesmata, and mechanisms of intracellular communication, such as the establishment of apical dominance, may be more readily observed in fern gametophytes than angiosperms (Holloway and Lantin 2002). For example, a physiological model of intercellular signaling via plasmodesmata in *Onoclea sensibilis* proved sufficient to explain maintenance of apical dominance and response to removal of the apical region in this fern (Holloway and Lantin 2002). These results indicate that cellular processes in fern gametophytes can easily be modeled and are informative for both fern and angiosperm development.

Within the largely tropical primitive family of ferns Schizaeaceae, *Schizaea pusilla* Pursh. is the only member with a distribution from central America to northeastern Canada (Bartoo 1930; Atkinson 1965; Stolze 1987; Cody and Britton 1989; Goltz and Hinds 1993). It grows in bogs, an environment that is nutrient stressed. Within the genus, gametophyte morphology, which varies from filamentous to tuberous, is used to define different subgroups; consequently, pattern development in gametophytes has taxonomic significance (Bower 1926; Bierhorst 1967, 1971*a*, 1975; Wikstrom et al. 2002). The gametophyte of the fern *S. pusilla* grows as a clump of single-celled filaments, which may give rise to small multicellular organs. These include the male and female reproductive organs, antheridia and archegonia, and a large structure that houses a fungal symbiont, called a rhizoidophore (Britton and Taylor 1901). The rhizoidophores serve as the point of entry of mycorrhizal fungi, which improve the fern's ability to take up nutrients (Crotty 1967; Swatzell et al. 1996). While investigating the growth of the gametophyte, we discovered clusters of cells from which growth would radiate in many directions. These structures arise periodically and were found to be integral to the growth of the plant; we have chosen to call them knots. These structures are not recorded in the literature on this fern in any publications known to the authors.

A useful framework for modeling plant development in space and time is the formalism L-systems (Lindenmayer 1968, 1975; Prusinkiewicz and Lindenmayer 1990), which describes the growth of branching structures as a function of processes taking place in individual modules, for example, cells or organs. Thus spatial relationships are not described by reference to a global coordinate system, but instead emerge from interactions between cells at a local scale (Coen et al. 2004). L-system models are inherently dynamic: with the correct productions one can start from a single cell and simulate the development of an entire branching organism. In this sense, form is "an event in space-time, and not merely a configuration in space" (Thompson 1942). We use L-systems to model the division of filamentous cells in the simple fern gametophyte of *S. pusilla* and examine how local cell division determines the architecture of this gametophyte.

Materials and methods

Spore and gametophyte culture

Schizaea pusilla gametophytes were derived from spores collected in 1991 in Nova Scotia and were maintained in tissue culture until experiments carried out in 2000. To provide a starting point for modeling, spores collected in New Jersey in 1994 and 1995 (donated by Dr. J. Kiss, Miami University) were sterilized and germinated on Knudson's medium, as described by von Aderkas and Raghavan (von Aderkas and Raghavan 1985). Stock cultures of gametophytes were maintained on Knudson's medium at 23 °C at 35 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR with a 16 h (light) – 8 h (dark) photoperiod.

Microscopy

All observations of cell division and organ development were made with a Zeiss Axioplan compound light photomicroscope. Cell division events were classified as either subapical, diagonal leading to a branch, or longitudinal. Light photographs, as well as DAPI stained fluorescent photographs, of various stages of gametophyte development and of organs such as rhizoidophores, antheridia and knots, were taken with Fuji 64T film. Gametophytes were microwaved for 20 s at medium power with acetocarmine and dilute chloral hydrate to partially clear cell contents and stain nuclei. They were then incubated for 30 min with phosphate-buffered saline and 10 $\mu g \cdot mL^{-1}$ DAPI (4′,6-diamidino-2-phenylindole). Gametophyte filaments were also processed for scanning electron microscopy. Samples were fixed overnight in 2.5% glutaraldehyde with 0.05 $mol \cdot L^{-1}$ phosphate buffer, then post-fixed for 1 h in 1% osmium tetroxide in 0.05 $mol \cdot L^{-1}$ phosphate buffer. The samples were rinsed three times in phosphate buffer for 15 min, then dehydrated with a graded ethanol series (30%–100%). Half of these samples were critical-point dried (Bomar SPC 1500, Bomar Co., Tacoma Wash.) and the other half were dried in hexamethyldisilazane before being mounted on stubs with double-sided tape and gold coated (Edwards Sputter Coater S150B, Edwards, Mississauga, Ont.). Scanning electron microscopy was carried out at 15 MHz (Hitachi S-3500N, Hitachi, Pleasanton, Calif.).

Observations of light treated gametophytes were taken with a Leitz dissecting photomicroscope and Fuji 64T film.

Observation of knots

Filaments from 10 stock gametophytes established on Knudson's medium were randomly selected for analysis. Measurements included branching angles, frequency of different types of cell division, and organ frequency and position. From these measurements we estimated the distribution (mean and standard deviation) of branching angles and frequency of knot formation. To investigate the role of stress on knot formation, 50 apical cells were isolated by crushing proximal cells with Dumont No. 5 forceps, and observed until approximately the 10-cell stage, when survival and frequency of different types of cell division were determined.

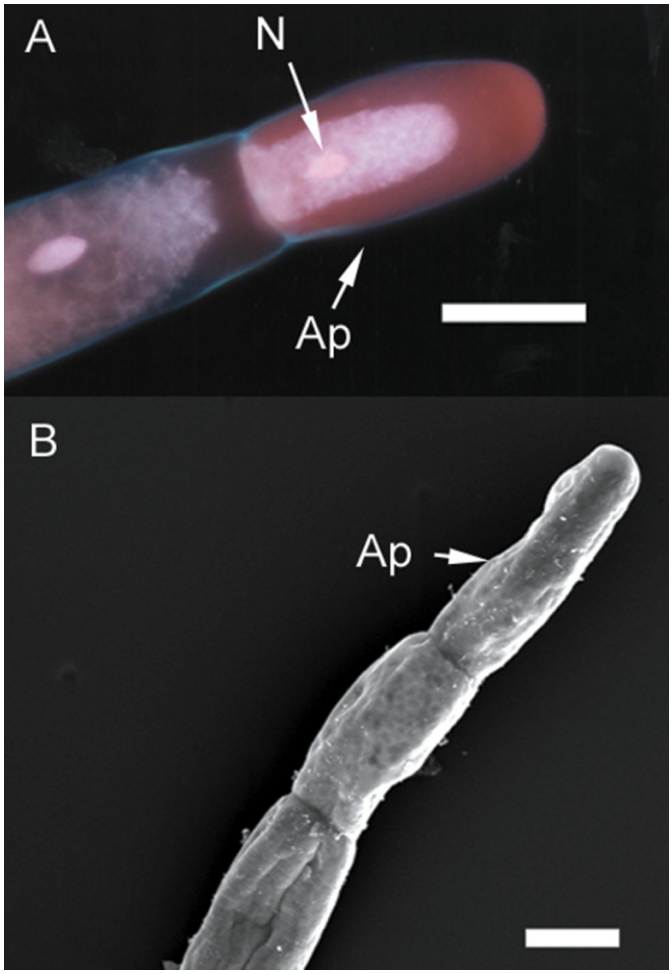
Model development

The model was developed using the L-system modeling environment L-studio (Prusinkiewicz et al. 2000). In the simplest case of deterministic productions without interactions, the syntax is as follows:

[1] predecessor → successor

Simulation begins with a structure of one or more modules, called the axiom. For example, with the axiom *A* and

Fig. 1. Apical division results in elongation of the filament. (A) DAPI-stained newly initiated apical cell. (B) Scanning electron micrograph of apical and subapical cell. Ap, apical cell; N, nucleus. Scale bar = 50 μm.



the production rules $A \rightarrow ABA$ and $B \rightarrow B$, in the first iteration A is replaced by ABA, and in the second, the string becomes $ABABABA$. Modules represent components of a linear or branching structure, and in our model of *S. pusilla*, represent cells or knots within a filamentous gametophyte. Modules can be associated with parameters (Prusinkiewicz and Lindenmayer 1990), for example representing size, age, or concentration of specific molecules. These parameters can be modified by productions.

In the model of *S. pusilla* gametophyte development, we distinguish between apical cells, A, active cells, C, and cells that have already undergone one cell division and are no longer dividing, D. Apical cells form the tips of filaments, and each division of an apical cell A gives rise to a subapical active cell C, which adds to the filament length:

$$[2] \qquad A \rightarrow CA$$

The division of a subapical cell C can be either intercalary,

$$[3] \qquad C \rightarrow D(0)\,C \qquad : p$$

or diagonal, giving rise to a new branch (enclosed in square brackets):

Fig. 2. Branches are initiated by diagonal or lateral division of an individual cell. (A) Fluorescent micrograph of DAPI-stained filament with new apical cell. (B) Scanning electron micrograph of new apical cell. (C) Light micrograph of new apical cell. Br, branch; N, nucleus; DP, division plane. Scale bar = 50 μm.

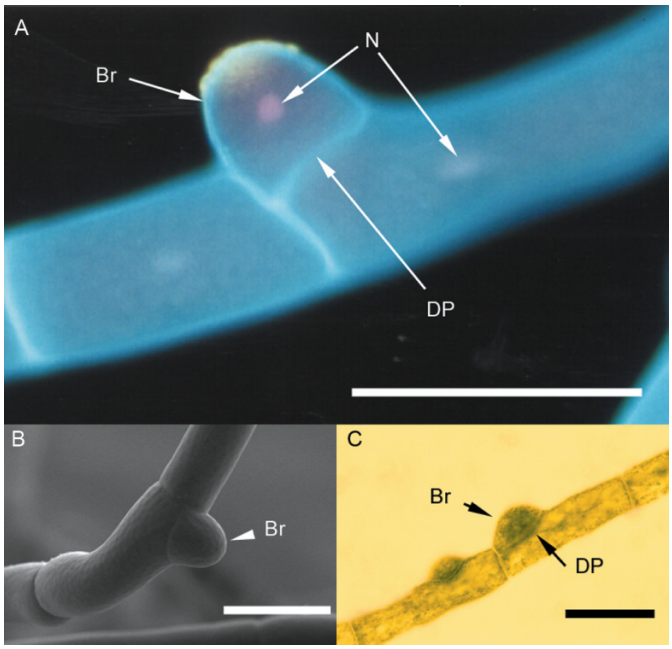
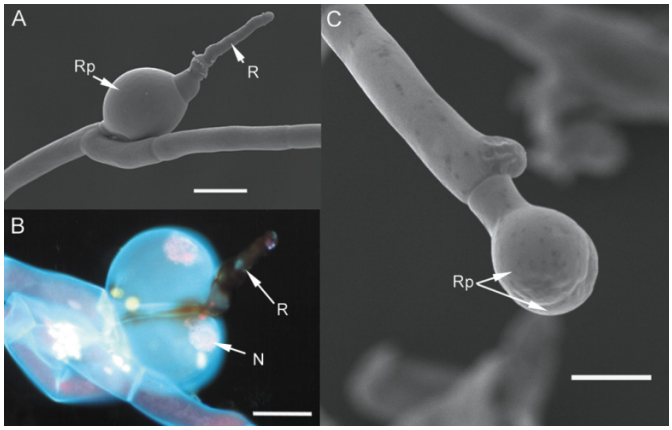


Fig. 3. Rhizoidophores are organs for the housing of an obligate mycorrhizal fungus, and are commonly found near the substrate. (A) Scanning electron micrograph of single rhizoidophore. (B) Fluorescent micrograph of DAPI-stained double rhizoidophore. (C) Scanning electron micrograph of double rhizoidophore. Rp, rhizoidophores; R, rhizoid; N, nucleus. Scale bar = 50 μm.



$$[4] \qquad C \rightarrow D(0)\,[A] \qquad : 1 - p$$

We assume that these developmental fates are controlled by a stochastic mechanism, with the probability of each production application indicated by the parameter after the colon. The division of a cell C yields a nondividing cell, D, which in the model is associated with age, initially set to 0.

Nondividing cells may also have two fates: grow older, or differentiate into knots. Knots were observed at the base of failing filaments and were nucleation sites of numerous new

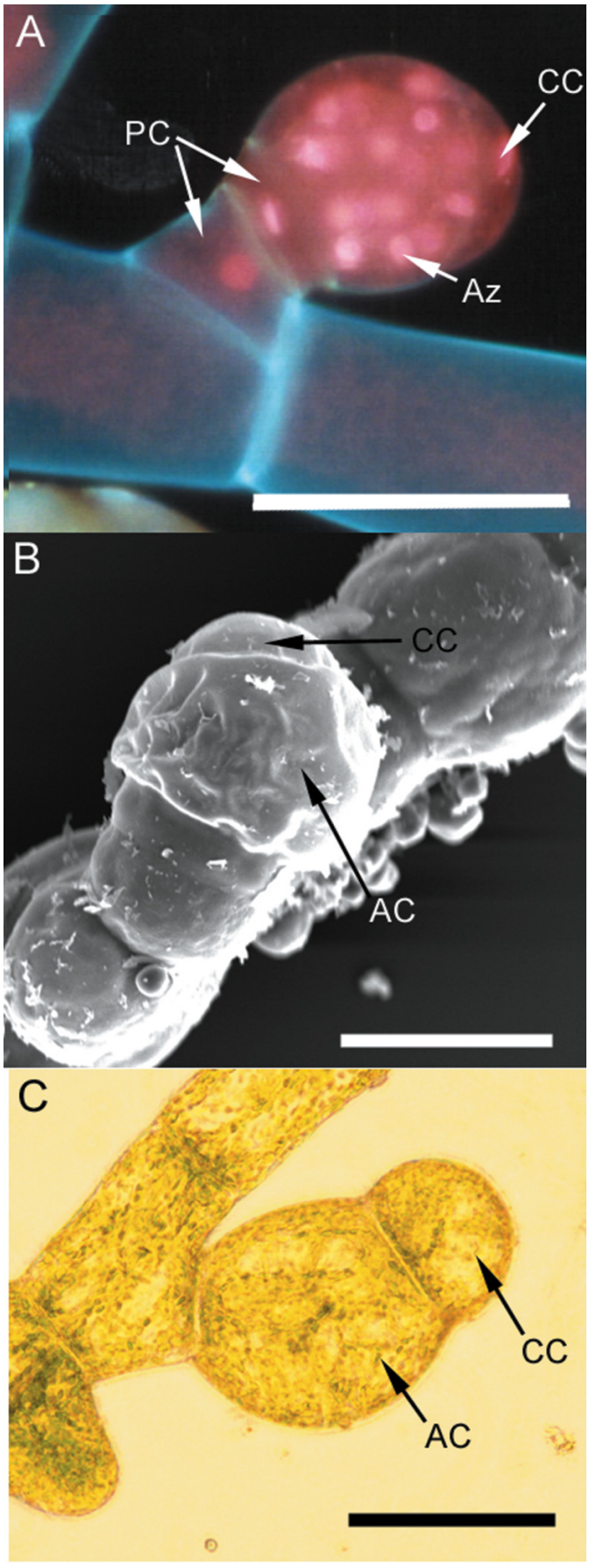


Fig. 4. Antheridia are male reproductive organs that are found throughout the filament. (A) DAPI-stained antheridium, showing spermatozoids. (B) Scanning electron micrograph of antheridium. (C) Light micrograph of antheridium. CC, cap cell; PC, pedestal cell; Az, antherozoid; Ac, antheridial chamber. Scale bar = 50 μm.

branches. Their basal position within failing filaments suggests that they arise from older nondividing cells. We therefore modeled knot initiation as a function of age. To this end, we estimated the age threshold for the initiation of knots as a random variable, with the mean and standard deviation derived from observations of knots in maintenance cultures. Knot initiation was then simulated using conditional productions:

[5] $D(a) : a \leq \text{threshold} \rightarrow D(a+1)$

[6] $D(a) : a > \text{threshold} \rightarrow K$

The above productions state that a nondividing cell D with age a less than or equal to the threshold will grow older, whereas a cell with an age exceeding the threshold will differentiate into a knot K.

Knots initiate new branches according to the rules:

[7] $K \rightarrow L\,[A][A][A] \quad : 0.8$

[8] $K \rightarrow L\,[A][A][A][A] \quad : 0.2 \quad (7)$

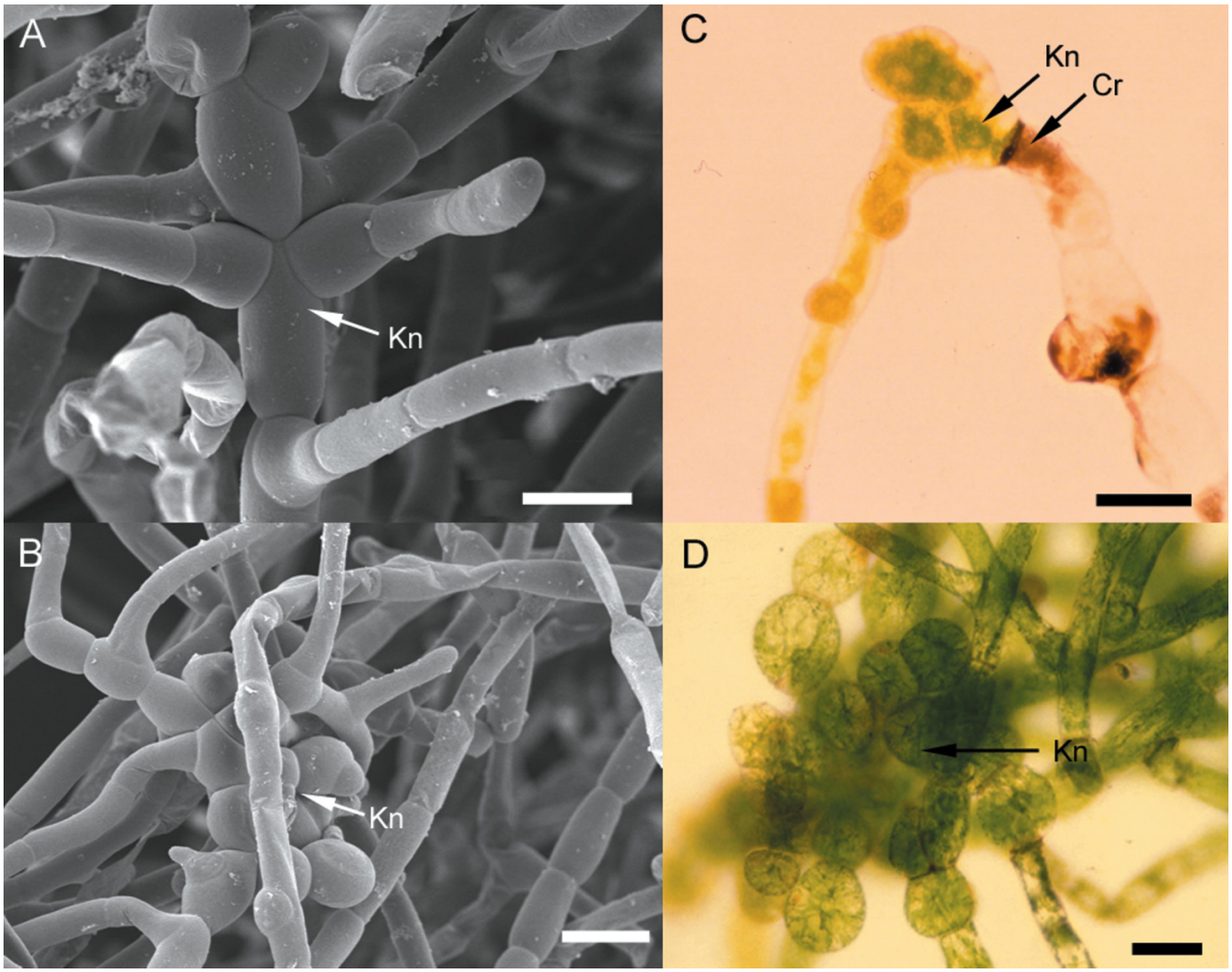
According to these rules, the activity of knots is limited to a one-time production of branches. In production 7, the knot gives rise to three new branches and assumes an inactive state, L. In production 8, the knot gives rise to four branches. Rules 2–8 capture the essence of the stochastic model of *S. pusilla* gametophyte development. The complete model, included in the supplementary materials, also specifies branching angles, which were determined from measurements of stock gametophytes and have been omitted in the above discussion for simplicity.

Results and discussion

The gametophyte of *S. pusilla* consists of photosynthetic cells that grow in branching filaments, forming an undistinguished clump of cells close to the substrate. Apical cells give rise to subapical cells (Fig. 1), which are capable of either intercalary division, which lengthens the filament, or branching to initiate a new filament (Fig. 2). We did not observe more than one branch originating from a single cell, which suggests that each subapical cell is capable of only one division event.

As the filaments grow cells near the substrate or shadowed by neighbouring filaments become chlorotic and develop rhizoidophores (Fig. 3), which are organs specialized for housing a mycorrhizal fungus (Kiss and Swatzell 1996). Other organs, such as the male reproductive organ, the antheridium, can be initiated throughout the filament (Fig. 4). Archegonia are rare and only one was observed during the course of this study. In chlorotic, failing filaments, nondividing cells can initiate a structure characterized by numerous diagonal and longitudinal divisions, resulting in a thickening

Fig. 5. Knots are thickened sections of filament characterized by multiple branching divisions from the same cell. (A and B) Scanning electron micrographs of knots. (C) Light micrograph of knot formation in an isolated apical cell. (D) Light micrograph of knot. Kn, knot; Cr, crushed distal cells. Scale bar = 50 μm.



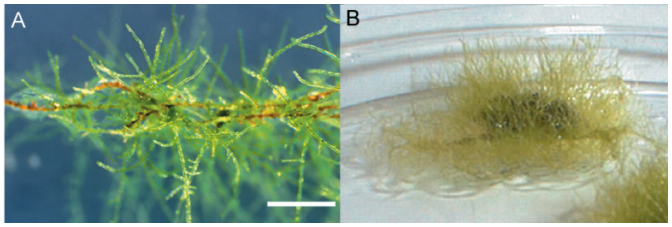
of the filament and initiation of numerous new branches. We termed the thickened sections of filament, knots (Fig. 5). Knots act as initiation sites for new branches, which develop once an older filament loses vigour and becomes chlorotic. They have not been previously described in the literature.

To investigate the role of knots in response to stress, we isolated apical cells by crushing proximal cells. This treatment produced considerable stress: only 44.1% of the isolated apical cells survived and went on to produce gametophytes. In these gametophytes, 20.2% of all cell divisions resulted in knot formation (Fig. 5B), compared with 12.2% in maintenance cultures. This suggests that knot formation may be an adaptive response to stress.

Spores collected from New Jersey were germinated to provide a starting point for observing growth parameters for the model. These cultures represented individual genotypes from a population far removed from those in continuous culture. Knots were also observed in these cultures at a similar frequency (data not shown), indicating that they were not an artifact of long-term tissue culture. It may be that knots are an artifact of culture even among those recently germinated from spores. However, no one, to our knowledge, has undertaken a systematic survey of wild Schizaeaceous ferns, and the remoteness of the field location made this untenable for this work. Atkinson (1965) documented the survival of a *S. pusilla* gametophyte in culture for 13 years; this indicates that the organism is capable of long term growth in its gametophytic form, and the demonstration that recently germinated spores developed knots suggests that our observations are not the result of adaptation to long-term culture. Furthermore, the Knudson's medium on which the cultures are maintained is minimal, simple in composition, and does not contain growth-regulating substances. That knot formation has not been noticed in other systems may be due to the fact *S. pusilla* gametophytes are not observed long past germination and are not a common laboratory organism.

Filamentous gametophytes are described in other ferns (Bierhorst 1971*b*), and among the Schizaeaceae the filamentous nature of gametophytes is the basis for their division into subgroups (Bierhorst 1967, 1971*a*, 1975). Another spe-

Fig. 6. Light micrograph of gametophyte filaments. (A) One filament at edge of gametophyte. Scale bar = 1 mm. (B) Whole gametophyte showing spreading habit.

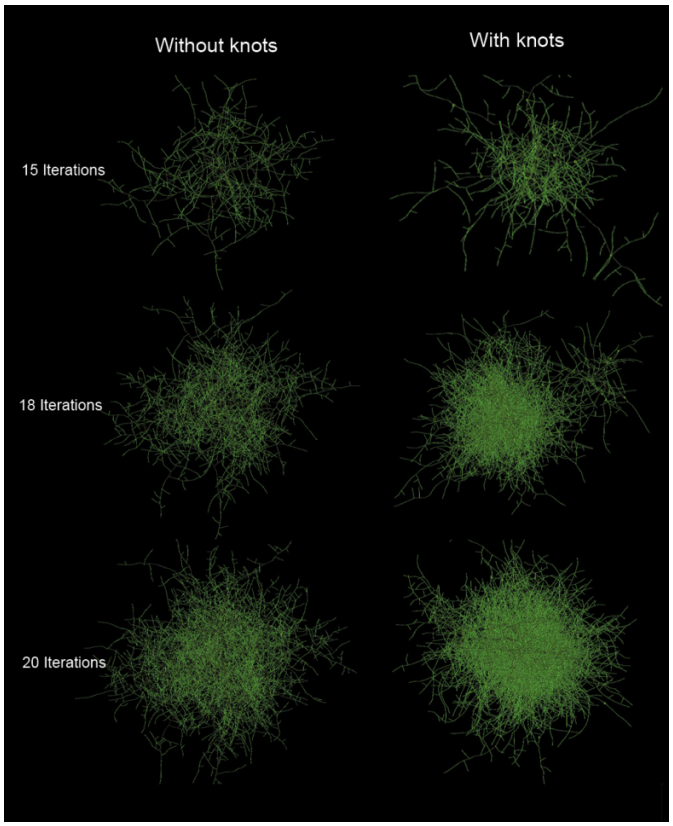


cies, *Schizaea elegans* (Vahl) Swartz, which is in the same section of the family, Section *Schizaea*, is classified as a modified filamentous type owing to the formation of localized areas of three dimensional growth in an otherwise two-dimensional filament (Bierhorst 1975). All other members of (Sect. *Pectinatae*) are tuberous (Bierhorst 1967, 1971*a*). However, close examination of the knots shows that they may be organs that are not entirely filamentous (Fig. 5). While Britton and Taylor (Britton and Taylor 1901) observed occasional thickening of the filament due to one or two longitudinal divisions, this was thought to be rare and associated with archegonial formation. The presence of non-filamentous structures is important because the gametophyte structure, and in particular, the degree of filamentous growth, has taxonomic importance (Bierhorst 1967, 1971*a*).

To further elucidate the architectural role of knots, we constructed a simulation model of *S. pusilla* gametophyte development. The L-system model describes simple rules of division at the level of individual cells. Thus cells are free to divide and age according to frequencies observed in vitro to test whether the gametophyte architecture can be predicted from these simple processes. A visual comparison of real *S. pusilla* gametophytes (Fig. 6) and their models (Fig. 7) indicates that simple rules governing cell division and knot initiation are sufficient to reproduce the overall architecture of the plant. Simulations show that the gametophyte is capable of generating a structure that appears as abundantly branching as the real gametophyte without the contribution of knots. However, the simulations also show that knots may contribute to the spread of the gametophyte by providing nucleation sites for the development of new branching structures (Fig. 7). In the actual gametophyte, areas of new clumps beside the existing gametophyte were observed (Fig. 6) indicating that in the gametophyte spreading may occur by this process. The observation of knot formation as a response to stress and the implications from simulation models suggests that knots may represent a survival strategy of the gametophyte seeking a more favourable location.

Simulation modeling is a powerful tool for testing hypothesis regarding the emergence of form from cellular level processes. Not only does the development of a "virtual" plant allow for the testing of specific hypotheses that are difficult in vivo, but it also requires a high level of detailed observation. In fact, it was during the cellular-level observation of the gametophyte that we observed knots, which at first can appear as areas of dying plant. However, we never observed a filament longer than approximately 10–15 cells without a knot, leading us to hypothesize that these structures are not simply artifacts of age or tissue culture, but

Fig. 7. Model output from stochastic L-systems model, showing gametophyte development over 15–18 iterations, in model including knots and without knots.



structures integral to the growth of the gametophyte. The simulation models demonstrated that knots contribute to the spreading habit of the gametophyte. Stress induction resulted in an increased formation of knots, suggesting that this organ may be a response to stress that facilitates spreading along the substrate. Because gametophyte structure is a basis for the taxonomy of this genus, the demonstration that knots are integral to the structure of the gametophyte has taxonomic implications.

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