Characterization, In Vitro Culture, and Molecular Analysis of *Thecaphora solani*, the Causal Agent of Potato Smut

Orlando Andrade, Gastón Muñoz, Rafael Galdames, Paola Durán, and Rodrigo Honorato

Instituto de Investigaciones Agropecuarias, Centro Regional de Investigación Carillanca, Casilla 58-D, Temuco, Chile.
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ABSTRACT

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The fungus *Thecaphora solani* (syn.: *Angiosorus solani*), the causal agent of potato smut, was cultivated in vitro for the first time. Teliospores obtained from galls of infected potato plants were used to inoculate commonly used solid and liquid media. The teliospores produced two kinds of vegetative tissue depending on the nutrient status of the media. A very slow radial-growing, hyaline, and septate mycelium, as usually seen in most of the in vitro-cultivated filamentous fungi, was obtained in water-agar medium after 30 to 40 days. On the other hand, a white, sponge-like

mycelial mass was obtained in HCM + 1% activated charcoal, and on common potato dextrose agar or malt-yeast-peptone solid or liquid media, after 40 to 50 days under lab conditions. The identity among teliospores and the sponge-like mycelial mass was corroborated by DNA fingerprinting and partial sequencing of the large subunit (LSU) rDNA region. The sexual cycle of the pathogen was completed under lab conditions based on the development of teliospores on the sponge-like mycelial mass. The first attempt to reproduce the disease under controlled conditions was successful, inducing a gall in a cv. Desirée potato explant cultivated in vitro inoculated with radial-growing mycelia. Phylogenetic analysis of LSU rDNA data of the genus *Thecaphora* and other smut fungi confirmed the initial classification of the pathogen as *T. solani*.

Potato smut is one of the most destructive fungal diseases affecting the potato (*Solanum tuberosum* L.) in the Andean region of South America, producing yield losses of $\approx 85\%$ (30). Other than some geographical areas of Panama and Mexico where potato smut also has been found (28), there are no reports of potato smut elsewhere worldwide.

The main symptom is the development of galls specifically located on lower stems, stolons, and tubers on potato plants (28). Galls developed on stems may vary in size from a few centimeters up to 10 cm or more (31), and may weigh over 300 g (33). On tubers, galls vary in size from less than 1 mm to 4 cm or more in diameter (1), thus producing losses in yield and quality. A very distinctive symptom is the presence of oval to irregular locular sori of variable sizes within the galls which contain a reddish-dark, granular-powdery mass of teliospores (21,36). Teliospores are spore balls composed of two to eight moderately firmly united ustilospores (21). Visible symptoms on leaves and roots have not been described (28).

The disease affects various cultivated species of *Solanum*, such as *S. tuberosum*, *S. andigenum*, and *S. stoloniferum* (21). It also has been reported on genus *Lycopersicon*, and on some common weeds such as *Datura stramonium* (28).

The causal agent was originally described as *Thecaphora solani* by Barrus and Miller in 1943 (5). This description was invalidated due to the lack of Latin description. In 1974, O'Brien and Thirumalachar (24) renamed it *Angiosorus solani*, providing the Latin description of the fungus. However, its generic status remains controversial because an important taxonomically informative character, teliospore germination, is still unknown (21). Moreover, to date, no data are available describing several other charac-

ters considered in classification of smut fungi (7). Mordue (21) states that the characters taken into account by Thirumalachar and O'Brien to distinguish the genus *Angiosorus* from *Thecaphora* intergrade with those found in a number of species of *Thecaphora*. This statement is also agreed upon by Vanky (35). The unsuccessful artificial cultivation of the pathogen has been the main constraint to characterization of the causal agent; therefore, important biological and phytopathological aspects remain unknown.

In Chile, the disease was first reported in 1974 in region IV (29°53'S, 71°19'W), in the northern part of the country (14), where it was established in many soils, causing significant yield losses often exceeding 90%. In 1996, despite the internal quarantine barriers imposed by the Servicio Agrícola y Ganadero (Ministry of Agriculture, Chile) to the transport of potato tubers within the country, the presence of potato smut was detected in the Carahue area (38°40'S, 73°09'W), region IX, located $\approx 1,100$ km south of the primary focus. This serious threat to the main potato seed-producing area led to intensified efforts to develop strategies intended to eradicate the disease from region IX, and to study the basic biological aspects of the pathogen and the disease.

In this work, we report the successful in vitro cultivation of *T. solani* and the first attempts to characterize the pathogen by molecular analysis.

MATERIALS AND METHODS

Teliospore isolation. Teliospores were obtained from galls developed on lower stems of commercial cultivars of *S. tuberosum*. The infected plants were collected in seven locations in Chile (Table 1). The galls were surface disinfected by immersion in ethanol 90% and flaming three consecutive times. To assess germination and growth in vitro, the sterilized galls were sliced with a sterile surgical blade to expose the sorus, and the teliospores were removed under a stereoscope microscope with the tip of the sterile surgical blade. In order to obtain high amounts of teliospores for DNA extraction and long-term storage, thin (1- to 2-mm) gall slices were dried under sterile conditions in a glass desiccator with silica

Corresponding author: O. Andrade; E-mail address: oandrade@carillanca.inia.cl

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gel under vacuum for 5 days. Once dried, the slices were ground in sterile porcelain mortars, sieving the material through a sterile sieve (pore size, 100 µm), collecting the dried teliospores on sterile aluminum foil, and storing them in sterile glass tubes at 4°C. Teliospores obtained from a single gall were considered as one isolate.

In vitro culture. Fungal growth was evaluated in the following solid media described on a per-liter basis: (i) water-agar (WA + R) containing 13 g of agar (Arquimed S.A., Chile) and 10 mg of rifampicin (Fluka Chemie AG); (ii) potato dextrose agar + streptomycin (PDA + St) containing 6 g of dehydrated potato flakes (Maggi, Nestlé Chile S.A.) boiled for 3 min in 200 ml of distilled water and filtered through four layers of cheesecloth, 12 g of dextrose, 13 g of agar, and 200 mg of streptomycin sulfate; (iii) malt-yeast-peptone (MYP) (4) containing 7 g of malt extract (BBL, Cockeysville, MD), 1 g of tryptic soy broth (Merck, Darmstadt, Germany), 0.5 g of yeast extract (BBL), and 13 g of agar (Arquimed, Santiago, Chile); and (iv) Holliday's complete medium (HCM) (17) containing 1% activated charcoal. The liquid media were potato dextrose (PD) and MYP broth, both prepared as above, excluding agar and antibiotics.

To inoculate petri plates, teliospores were aseptically streaked on the surface of the agar media forming two or three separate straight lines of 5 to 6 cm on each plate, to dilute the teliospore masses. In some experiments, PDA plates with sterile 80-mm cellophane discs on the top of the medium also were prepared to facilitate harvesting of the biomass. Plates with cellophane were inoculated by streaking a sponge-like mycelial mass previously grown on the same medium for 50 days. All the plates were sealed with parafilm strips and maintained under lab conditions at

18 to 20°C. Once a sponge-like mycelial mass or radial-growing mycelium growth was obtained, these structures were subcultured to WA, PDA, or MYP media without antibiotics.

Broth media were seeded by immersing four to five small sections (2 mm in diameter) of a sponge-like mycelial mass from 50-day-old plate culture into four sterile, 250-ml flasks containing 50 ml of medium. Liquid cultures were incubated on a rotary shaker at 100 rpm at room temperature.

Colony growth and germination rates. The growth rate of the colonies growing on WA + R and PDA + St was determined by turning the plates upside down, measuring the colony diameter under a light microscope equipped with a micrometric rule, and performing regression analysis on the data. The germination rate of the teliospores was similarly assessed; however, in this case, determining the ratio of developing colonies and the number of teliospores on each streaked line.

Pathogenicity tests. The Carahue isolate (TS-21) was tested for pathogenicity using 20-day-old explants obtained from internode cuttings of cv. Desirée growing in 2% Murashagi & Skoog (MS) medium (23). Fifteen explants were inoculated with radial-growing mycelia obtained on WA after 70 days. Another 15 explants were inoculated with 1- to 2-mm-diameter sections of mycelial mass obtained from 60-day-old cultures on cellophane over PDA. Explants always were punctured at the base with a sterile hypodermic 21G1 1/2 needle carrying the radial-growing mycelia or the sponge-like mycelial mass sections at the tip. Five explants were inoculated with sterile PDA medium as a control. Inoculated explants were incubated at 25°C in growth chambers, with a photoperiod of 12 h of light and 12 h of darkness.

TABLE 1. List of *Thecaphora solani* isolates studied

Isolates	Cellular type	Location	Region	Year of collection	GenBank accession
TS-CQ01	Teliospores	Coquimbito	IV	2001	...
	Mycelium	...	...	...	...
TS-4	Teliospores	Pan de Azúcar	IV	2002	...
TS-5	Teliospores	El Romero	IV	2002	AY344049
TS-15	Teliospores	Altovalsol	IV	2002	...
	Mycelium	...	...	...	AY344055
TS-16	Teliospores	Don Nicanor	VIII	2002	...
	Mycelium	...	...	...	AY344051
TS-21	Teliospores	Carahue	IX	2001	AY344050
	Mycelium	...	...	...	AY344052
TS-25	Teliospores	Huidaquintue	IX	2002	...

Fig. 1. Teliospores of *Thecaphora solani* obtained from a gall of a naturally infected potato plant. **A**, Spore ball mass observed under the light microscope (bar = 30 µm). **B**, Single teliospore observed by scanning electron microscopy (bar = 5 µm).

Electron microscopy analyses. Samples for scanning electron microscopy (SEM; electron microscope Jeol, JSM-25-SII) of the sponge-like mycelial mass from the TS-21 isolate grown over cellophane on PDA plates for 60 days were prepared and examined as described by Muñoz et al. (22).

DNA extraction. A sponge-like mycelial mass, obtained by cultivating the teliospores for 40 days on PDA medium with cellophane as described above, and dried teliospores were ground in a sterile porcelain mortar with liquid nitrogen. The resulting powder was suspended in lysis buffer and extracted following the method of Bainbridge et al. (3). DNA from *S. tuberosum* cv. Desirée was obtained from leaves collected from a healthy plant growing in vitro on 2% MS medium following the method described by Doyle and Doyle (13). DNA quality was checked by electrophoresis on 0.8% agarose gels in Tris-borate EDTA and staining with ethidium bromide (27). The DNA was quantified by absorbance at 260 nm in a spectrophotometer (MBA2000; Perkin-Elmer, Norwalk, CT).

DNA fingerprints. DNA profiles were generated by polymerase chain reaction (PCR) using the following sequence-repeated primers: CAG5 (5'-CAGCAGCAGCAGCAGCAG-3'), GTG5 (5'-GTGGTGGTGGTGGTGGT-3'), TCC4 (5'-TCCTCCTCCTCCTCC-3'), GACA4 (5'-GACAGACAGACAGACA-3'), TGTC4 (5'-TG-TCTGTCTGTCTGTCTGTC-3'), and GACAC3 (5'-GACACGACAC-GACAC-3'). The primers were synthesized at Integrated DNA Technologies Inc. (Coralville, IA). All reactions were conducted in a final volume of 20 μ l containing 4 μ M primers, 200 μ M each dNTP, 1.5 mM MgCl₂, 10 ng of fungal DNA, and 1 unit of *Taq* DNA polymerase (Gibco-BRL, Rockville, MD). Reactions were carried out in a GenAmp 9700 thermal cycler (Applied Biosystems, Norwalk, CT), programmed as follows: 35 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C. The final cycle was 5 min at 72°C. Amplification products were separated by electrophoresis, stained, and photographed as described (27). All reactions were performed twice, and negative controls were always included (i.e., complete reactions mixes except for template DNA).

Fig. 2. Growth and morphology of *Thecaphora solani* observed by light microscopy. **A**, Hypha (h) emergence from teliospores (t) streaked on potato dextrose agar (PDA) (bar = 40 μ m). **B**, Coiling of hyphae (coil) and clamp connection-like structure (cc), after 25 to 30 days of incubation on PDA (bar = 15 μ m). **C**, Chlamydospore-like structures (black arrows) in the sponge-like mycelial mass after 90 days of incubation on PDA (bar = 20 μ m). The plate was inoculated with small sections of sponge-like mycelial mass previously developed for 50 days in PDA. **D**, Chlamydospore-like structures (cl) and teliospore (t) formation in the sponge-like mycelial mass after 30 days of incubation in liquid MYP medium (bar = 20 μ m). The culture was inoculated with small sections of sponge-like mycelial mass previously grown for 30 days in PDA.

Sequencing and phylogenetic analysis. The 5' region of the nuclear large subunit (LSU) of the ribosomal DNA gene was amplified by PCR using NL1 and NL4 primers (11). The DNA fragment was gel purified and cloned into the vector TOPO-TA for sequencing (Invitrogen, Carlsbad, CA) according to the manufacturer's suggestions and sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase (Perkin Elmer) and the ABI PRISM 310 Genetic Analyser (Applied Biosystems). The obtained sequence was analyzed searching for homology to known sequences with the BLAST program and the nucleotide databases (2) and then aligned with published (9,10) LSU sequences from ustilaginomycetous fungi using the ClustalX program (29). The phylogenetic analysis was finally made using the MEGA2.1 program (20). The aligned sequences were analyzed by the neighbor-joining method of the distance matrix (Kimura 2-parameter model, transition to transversion rate: 2.0) with 1,000 bootstrap replicates. Maximum parsimony analysis also was carried out using the MEGA2.1 program with the default settings (random addition trees option turned on 10 and 100 bootstrap replicates).

RESULTS

In vitro culture. Teliospores were obtained as spore ball masses from naturally infected plants (Fig. 1A). They were frequently

ovoid (Fig. 1B) and composed of six to eight ustilospores (Fig. 1). When spore balls were pressed between glass slides and lightly hit, they never separated into single ustilospores. The same results were found when a teliospores suspension was vortexed at high speed in the presence of glass beads.

When plated in solid culture, teliospores produced two types of mycelial structures depending on the medium. On WA + R medium, we found a radial-growing, septate, and hyaline mycelium as commonly observed in filamentous fungi (Fig. 2A). This mycelium appeared after 24 to 36 h, and the mean growth rate was estimated at 0.03 mm per day, reaching only 3.88 mm after 128 days. Thereafter, the growth is almost undetectable. On rich media, such as PDA + St and HCM + 1% activated charcoal medium, a white, firm, irregular, and sponge-like mycelial mass was obtained. On both these media, the mycelia started developing as a radial-growing, hyaline mycelium but, after 25 to 30 days, the hyphae coiled at the branch tips (Figs. 2B and 3C), becoming more dense and forming a sponge-like mycelial mass of 2 to 3 mm of diameter in ≈ 45 to 60 days (Fig. 3A and B). However, the growth rate of the radial-growing, hyaline mycelium during the first 25 days was 66% faster in PDA + St than in WA + R. Notably, when radial-growing, hyaline mycelium from WA + R or sections of sponge-like mycelial mass from PDA + St were transferred to PDA + cellophane, the growth of the sponge-like structure

Fig. 3. Morphology of sponge-like mycelium; sponge-like mycelial mass of *Thecaphora solani* observed in **A**, a petri plate and **B** to **D**, under the scanning electron microscope after subculturing small sections of the same fungal tissue onto a new potato dextrose agar plate. **A**, Petri plate with white, sponge-like mycelial mass after 45 to 60 days of growth. **B**, Mycelium of 75 days of growth (bar = 10 μ m). **C**, Hyphae of the sponge-like mycelial mass showing coiling at the hypha tips (coil). **D**, Teliospores of *T. solani* with their characteristic form of reticulated spore balls developing in the sponge-like mycelial mass (bar = 10 μ m).

increased significantly, obtaining two- to threefold of biomass in only 30 to 40 days.

The sponge-like mycelial mass transferred to liquid medium was able to grow at a faster rate than in solid cultures. After 15 to 20 days on a rotary shaker, the sponge-like mycelium mass grew 5- to 10-fold.

The development of fungal biomass from teliospores occurred mainly when teliospores looked dry and dusty once galls were cut. Even so, the teliospores seemed to have a very low germination rate. In fact, we determined that 0.3 to 0.5% of the teliospores obtained from dusty-looking sori yielded fungal colonies.

The sexual cycle of the fungus seemed to be completed in vitro culture, as can be deduced from the teliospore-like structures observed within the sponge-like mycelial mass (compare Fig. 1A and B with Figs. 2D and 3D). On PDA, after 60 to 75 days of incubation (with or without cellophane), chlamydospore-like structures were observed on the sponge-like mycelial mass (Fig. 2C) and, after 90 to 100 days, many teliospores were found (Fig. 3D). Concomitantly, the white sponge-like mycelium mass turned light brown. The same response was obtained each time the sponge-like mycelial mass was plated on new media, and

by no means were they remnants of the teliospores obtained originally from galls. This putative development of teliospores and the color change of the sponge-like mycelial mass was observed on solid PDA and MYP media, with or without cellophane, and in liquid MYP medium (Fig. 2D). In liquid, the chlamydospore-like structure and teliospore formation was much faster. These teliospores remained firmly attached to the sponge-like mycelial mass, making their mechanical separation from mycelia almost impossible.

Pathogenicity tests. Our first attempt to artificially induce smut on healthy potato plants under controlled conditions was successful. A firm, round gall of ≈ 9 mm in diameter (Fig. 4B), similar to those observed in naturally infected plants (Fig. 4A), was obtained 75 days postinoculation in one out of 15 explants inoculated with mycelium obtained on WA + R agar medium. Thin-section light microscopy of lactophenol-stained gall tissue obtained through our inoculation experiment revealed the presence of hyphae similar to those observed on naturally infected potato tubers (Fig. 4C and D).

DNA fingerprinting of teliospores and mycelia. To establish whether teliospores and the sponge-like mycelial mass originated

Fig. 4. Natural and artificially induced galls on cv. Désirée potato plants. **A**, Galls (g) observed in a naturally infected potato plant in the field. **B**, Gall (g) induced in cv. Désirée potato explant 75 days after inoculation with mycelium of *Thecaphora solani* obtained on water-agar medium. **C**, Mycelium of *T. solani* without staining growing in the inner tissue of a naturally infected potato tuber (bar = 10 μ m). **D**, Mycelium of *T. solani*, stained with cotton blue, growing in the inner tissue of a gall induced as a result of the pathogenicity test (bar = 10 μ m).

from these teliospores belong to the same organism, DNA was extracted from both structures and used to generate PCR DNA profiles. Moreover, and as a first attempt to assess the genetic variability of *T. solani*, DNA from different teliospores collected throughout Chile also were included in this analysis. All primers used generated DNA amplification products. The profiles obtained were the same for all the teliospores and mycelia analyzed, but different from those obtained from *S. tuberosum* DNA (Fig. 5), indicating a close genetic relationship among all the fungal isolates. This result suggests that teliospores and the sponge-like mycelial mass cultured in vitro correspond to the same fungus.

Sequencing and phylogenetic analysis. LSU sequencing was chosen as a second approach to assess the identity between teliospores and the sponge-like mycelial mass obtained in vitro, and to determine the phylogenetic position of the pathogen as well. The GenBank accessions of the obtained sequences and the isolates used in the analysis are shown in Table 1. In all cases, the same 658-bp sequence was obtained, again confirming that the teliospores isolated from galls of different geographical origin and the sponge-like mycelial mass artificially cultivated correspond to the same fungus. Homology searches revealed similarities just to LSU sequences of ustilaginomycetous fungi, identifying *Thecaphora* spp. such as *T. amaranthi*, *T. semini-convolvuli*, and *T. saponariae* as those with the highest similarity (E values of $<10^{-168}$).

The LSU sequence of *T. solani* was analyzed along with 18 other LSU sequences of representatives of ustilaginomycetous fungi (7–9). Phylogenetic analysis derived from the neighbor-joining method resulted in the topology shown in Figure 6. The same topology was obtained when maximum parsimony analysis was used (data not shown). In agreement to the homology searches described before, *T. solani* appears closely related to other *Thecaphora* spp. In fact, all the *Thecaphora* spp. form a

monophyletic group, the family *Glomosporiaceae* (Fig. 6), which is statistically well supported by bootstrap values. They appear separated from the families *Tilletiaceae*, *Ustilaginaceae*, and *Urocystaceae*, but belong to the subclass *Ustilaginomycetidae* (Fig. 6).

DISCUSSION

Despite its importance as the causal agent of potato smut, since the first description of *T. solani* in 1943 (6) and its taxonomic re-examination published in 1974 (24), no other studies on basic fungal biology have been reported. For the first time, we report culturing mycelium of *T. solani* in commonly used solid and liquid media. Four lines of evidence support this conclusion. First, the sponge-like mycelial mass developed in artificial media produced teliospores indistinguishable from those observed inside galls of naturally infected plants. Second, the single gall induced on a potato explant as a result of our pathogenicity test looked similar to natural galls and possessed hyphae typical of *T. solani* internally. Third, DNA fingerprints showed the genetic identity of both teliospores and the sponge-like mycelium developed from those teliospores. Finally, the LSU sequencing demonstrated that both teliospores and the sponge-like mycelial mass belong to the genus *Thecaphora*.

The lack of previous success in isolating the pathogen in artificial media could be related to the following conditions: (i) low germination capability of the teliospores, (ii) the extremely low growth rate of the mycelium, and (iii) the absence of suitable indicators for detecting the physiological stage at which teliospores are able to germinate and, therefore, to grow in culture. Despite the experience gained during the course of this work, culturing of *T. solani* remains as a difficult task. In fact, at this date, we have been able to artificially cultivate only 7 of 26 isolates. We conclude that the low germination capability of teliospores is the main factor limiting in vitro culturing of the pathogen.

Germination has not been studied in detail within the genus *Thecaphora*. According to Vánky (36), teliospore germination within the genus is variable, ranging from holobasidia to aseptate or septate hyphae that sometimes bear basidiospores (25). We tried to follow the germination process of *T. solani* teliospores in water agar, a humid chamber, and nutrient-rich media. We primarily observed the development at the very early stage of a single hypha, sometimes two hyphae, growing from a single spore ball. We never saw yeast production as has been reported in the case of *T. haumannii* (25) or any structure similar to typical basidia (36). Moreover, in opposition to results described by O'Brien and Thirumalachar (24) but in agreement with results described by Barrus (6), we found that *T. solani* spore balls cannot be separated into single ustilospores. Therefore, it was technically impossible to follow the time course of germination from a single cell, limiting derived information about the germination process. The variability of teliospore germination in *Thecaphora* spp. has been interpreted as atypical germination as a result of suboptimal environmental conditions (7,10). We suggest that this also could be related to the physiological stage of the isolated teliospores.

That *T. solani* can produce teliospores in culture is very obvious. It has been stated that the heterobasidiomycetous plant-pathogenic fungi require specific plant hosts for the completion of their sexual cycles, even though they are not obligate parasites (5,26). A few cases have been reported related to in vitro teliospore production. These are the cases of the *Tilletiaceae* *Tilletia caries* (38), *T. indica* (16), and *T. controversa* (32). More recently, it was reported of the teliospore production and genetic recombination in vitro of the *Ustilaginaceae* *Ustilago maydis* (26). However, in the later case, growing the fungus over the top of embryogenic cultures of *Zea mays* separated by a porous membrane was required. The authors suggested that some diffusible compounds

Fig. 5. DNA profiles obtained by polymerase chain reaction with sequence repeated primers for teliospores and mycelium of *Thecaphora solani* isolates. Primers **A**, GTG5 and **B**, GACAC3 were used to amplify DNA fragments using genomic DNA obtained from teliospores and mycelium of isolates TS-CQ01 (lanes 1 and 8), TS-15 (lanes 2 and 9), TS-16 (lanes 3 and 10), and TS-21 (lanes 4 and 11), as well as DNA obtained from teliospores of isolates TS-4, TS-5, and TS-25 (lanes 5, 6, and 7, respectively). Lane 12 corresponds to amplification obtained with DNA from *Solanum tuberosum*. At right side, migration of DNA markers (lane M) is indicated in basepairs.

produced by the embryogenic maize cells were needed for the completion of the pathogen sexual cycle. As we observed here, *Thecaphora solani* does not seem to have special requirements for complex host-derived compounds for teliospore production. Rather, the teliospores were produced on solid or liquid common media such as PDA or MYP.

The stage at which teliospores are capable of germination seems to be related to the dusty or powdery aspect of teliospores within the sori. This stage may be the last step of the teliospore development. The reason why not all teliospores reach this stage is unknown. However, the culture systems described here could be useful for studying the cellular processes involved in teliospore development.

The establishment of a suitable and reproducible system to induce the disease under controlled conditions is key to understanding the mechanisms involved in pathogenicity. On a single plant, we were able to reproduce the disease after inoculating several potato explants with the radial-growing mycelium developed in WA.

DNA profiles obtained by PCR and repeated sequence primers provided a useful approach to show a close genetic relationship between teliospores and the sponge-like mycelial that originated from them, thus indicating that both structures are the same. This kind of analysis has been used to identify genetic differences between genera such as *Penicillium* (18) and *Trichoderma* (19,34). It also has been used to assess population diversity in other fungi (12,15). As a preliminary result, we found low genetic variability among the isolates, suggesting that population structure of the pathogen in Chile is relatively simple, perhaps a single clonal lineage.

Due to the availability of axenic cultures of *T. solani*, we were able to obtain the LSU sequence and evaluate the phylogenetic relationship with other smut fungi. The actual classification of smut

fungi is based predominantly on characteristics of host-parasite interactions and septal pore apparatus structure, and is supported by the mode of teliosporegenesis, the ultrastructure of teliospore walls, and LSU sequences analysis (7). Our analysis of this sequence showed that the causal agent of potato smut is closely related to the other species of *Thecaphora*; in fact, they form a group clearly distinguishable from other related ustilaginomycetous fungi. The genus *Thecaphora* includes more than 50 species and does not represent a homogeneous natural group due to the variability of the ustilospore germination (36). The genus was emended, including *Sorosporium saponariae*, which was renamed *T. saponariae* (37). Because of their morphological relationship to genus *Glomosporium*, the family *Glomosporiaceae* was erected to separate the genus *Thecaphora* from the family *Ustilaginaceae* and tentatively placed in the order *Ustilaginales* (8,10). In agreement with previous, more limited, reported studies (9,10), where only three available LSU sequences for *Thecaphora* members were included, our expanded results show that the genus forms a clade within the order *Urocystales*.

LSU rDNA sequence analysis clearly indicates that the causal agent of potato smut belongs to the genus *Thecaphora*. This is new evidence that supports Vanky's (35,37) and Mordue's (21) claims for inclusion of this pathogen in the genus *Thecaphora* and confirms the initial name as correct.

Finally, our findings place *T. solani* in a new position, opening new lines of research to get a better understanding about its biology, epidemiology, and genetics. However, characteristics such as the very low germination and growth rate, and the lack of an optimized and reproducible infection assay, represent a significant challenge to further study of this host-pathogen system. We currently are trying to better define nutritional requirements for growth, genetic variability among isolates, and the induction of the disease by artificial inoculation.

Fig. 6. Topology obtained by neighbor-joining analysis of large subunit rDNA sequences of 26 ustilaginomycetes. Topology was rooted with *Entorrhiza* sp. Bootstrap values are given at each furcation and values under 50% are not shown. The fungal species considered in the analysis and their corresponding accession numbers are *Cintractia axicola*, AF009847; *Entorrhiza aschersonia*, AF009851; *E. casparyana*, AF009852; *Erratomyces patelii*, AF009855; *Moeziomyces bullatus*, AF009868; *Mundkurella kalopanax*, AF009869; *Pseudozyma flocculosa*, AJ235299; *Thecaphora saponariae*, AF133579; *Sporisorium culmiperdum*, AF133580; *T. amaranthi*, AF009873; *T. semini-convolvuli*, AF009874; *Tilletia caries*, L20285; *T. ayresii*, AJ235308; *Tolysporium junci*, AF009876; *Urocystis colchici*, AF009878; *U. ranunculi*, AF009879; *Ustacystis waldsteiniae*, AF009880; and *Ustilago maydis*, L20287. Bar indicates 0.05 expected changes per site.

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