Where does the peanut smut pathogen, *Thecaphora frezii*, fit in the spectrum of smut diseases?

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Abstract

Smut fungi, such as *Ustilago maydis,* have been studied extensively as a model for plant-pathogenic basidiomycetes. However, little attention has been paid to smut diseases of agronomic importance that are caused by species of the genus *Thecaphora*, probably due to their more localized distribution. Peanut smut incited by *T. frezii* has been reported only in South America, and Argentina is the only country where this disease has been noted in commercial peanut production. In this work, important advances in deciphering *T. frezii* specific biology/pathobiology

in relation to potato (*T. solani*), wheat (*U. tritici*) and barley (*U. nuda*) smuts are presented. We summarize the state of knowledge of fungal effectors, functionally characterized to date in *U. maydis* and most recently in *T. thlaspeos*, as well as the potential to be present in other *Thecaphora* species involved in dicot-host interactions like *T. frezii*-peanut. We also discuss applicability and limitations of currently available methods for identification of smut fungi in different situations and management strategies to reduce their impact on agri-food quality. We conclude by describing some of the challenges in elucidating *T. frezii* strategies which allow it to infect the host and tolerate or evade plant immune defense mechanisms, and assessing other aspects related to pest control and their implications for human health.

Keywords: peanut smut, *Thecaphora frezii,* smut diseases, fungal life cycle, effectors.

Peanut smut: the pathogen *Thecaphora frezii* put at risk the Argentinean yields

In October 2020, the USDA reported a global peanut (*Arachis hypogaea* L.) production of approximately 46 million metric tons for 2019/2020. Argentina was eighth among producers, with production of 1.3 million metric tons (Figure 1A) (USDA 2020). In recent years, Argentina has commercialized processed peanuts, including shelled peanuts (the main product marketed worldwide), oil, pellets, snacks, peanut paste, chocolate peanuts, and other confectionery products. It was recently the world's third largest exporter of shelled peanuts, after India and the United States. The second most important product for this sector was prepared or preserved peanuts. Argentina served as a main exporter of preserved peanuts, with 42% of their total volume traded with the United States, Japan, Korea and Canada, among others. These data clearly show the importance that peanut cultivation has for the Argentine economy. Since 2010/2011, more than 80% of Argentina's peanut production has been cultivated in the province of Córdoba (Figures 1B and 1C).

However, peanut smut, caused by the fungus *Thecaphora frezii* (Carranza and Lindquist 1962), seriously affects production of commercial species (*Arachis hypogaea* L.) causing yield losses of 30% or more (Bressano et al. 2019). Although the first report of smut in wild peanut was in Brazil in 1962, it was detected for the first time in Argentina in 1994/1995, in peanuts from the north-central region of Córdoba (Marinelli et al. 1995). The disease became commercially relevant during 2001/2002, after which it grew exponentially. During 2015/2016 the production area was severely affected, with disease prevalence reaching 100% (Cazón et al. 2018). Other South American countries, such as Brazil and Bolivia, have reported presence of this disease, but only in wild peanut species (Carranza and Lindquist 1962; Soave et al. 2014).

The pathogen is disseminated primarily on seed. Spores of *T. frezii* can superficially contaminate healthy seeds after shelling or through small lesions (Cazón et al, 2016a). For export, preserving the phytosanitary status of producer regions is crucial. The US Animal and Plant Health Inspection Service (APHIS) restricts importation of peanuts from Argentina and Brazil for planting due to concerns associated with peanut smut (Animal and Plant Health Inspection Service, DA-2017-35). A Federal Order prohibits importation of raw peanut unless it is shelled, without red skin, and blanched for at least 12 minutes at 82℃. Processed peanuts (roasted peanuts or peanut butter) are still admissible under current regulations.

Considerable research has been published since the peanut smut pathogen was identified (Astiz Gassó and Marinelli 2003; Cazón et al. 2016a; Conforto et al. 2013; Marinelli et al. 2010), including an excellent review (Rago et al. 2017). The present article updates the major findings in understanding *T. frezii* biology and pathobiology in relation to other agronomically important smut fungi, such as the closely related pathogen *T. solani.* Effectors, which are key compounds produced by pathogens that determine host colonization and infection, have not been explored in *Thecaphora* spp. until recently (Courville et al. 2019). Here, we discuss roles and delivery mechanisms of fungal effectors in the interaction of the model system *U. maydis*-maize and in recent studies of *T. thlaspeos- Arabidopsis thaliana,* highlighting both conserved and unique mechanisms among the smut fungi. We also explore currently available morphological and molecular methods and difficulties associated with a paucity of morphological characters. We conclude with challenges associated with peanut smut management and future directions for research.

Where does the peanut smut pathogen fit in the spectrum of smut diseases?

Thecaphora frezii is a smut fungus. Therefore, it is part of a large non-monophyletic group of fungal biotrophs with similar life strategies. Smut fungi are obligate parasites of vascular plants that belong to the phylum Basidiomycota, subphylum Ustilaginomycotina, also known as "the true smut fungi" (Vánky 2012). Smut diseases affect monocots (such as rice, wheat, corn, barley and rye; and forage grasses) and dicots (peanuts, potatoes, onions) of agricultural importance worldwide.

Historically, smuts have impaired cereal production by reducing grain quality. More than 1500 species of smut fungi are recognized as plant pathogens; however, those that attack cereal crops are of major concern due to their economic impact (Zuo et al. 2019). Smut disease severity varies depending on weather conditions and agricultural practices. Interestingly, while some smuts are widespread, other are limited to specific regions. Widely distributed species include: *U. tritici* (loose smut, wheat); *U. nuda* (loose smut, barley); *Tilletia controversa* (dwarf bunt, wheat); *T. indica* (karnal bunt, wheat); *T. caries* (common bunt, wheat); *Urocystis agropyri* (flag smut, wheat); *U. maydis* (common smut, maize); *Sphacelotheca reiliana* (head smut, maize); and *U. hordei* (covered smut of barley) (EPPO 2020; GBIF 2020). Wheat and barley smuts are not devastating diseases; however, they are found wherever these grasses are cultivated (Abrahim 2019; Woldemichael 2019). Yield reductions are frequently proportional to the incidence of infected plants within a field. Nowadays, reported grain losses are less than 1%, but this can rise to >30% if highly contaminated seeds of susceptible varieties are sown in the absence of integrated management practices.

In contrast, *T. frezii* and the closely related species *T. solani* occur in geographically delimited areas (Figure 2). While peanut smut by *T. frezii* has been identified in cultivated species only in Argentina, potato smut caused by *T. solani* is found in the Andean region of South America and

parts of Mexico and Panama (EPPO 2020). Potato smut is a very destructive disease that has caused losses of up to 90% in both productivity and quality in Chile (Andrade et al. 2004). Potato is the main host, but this fungus also can infect tomato and wild solanaceous plants. Currently, a phytosanitary regulation has been applied to prevent the entry of *T. solani* to the European Union.

Symptoms and general life cycle of smuts (Ustilaginomycetes)

Smut diseases exhibit different symptoms depending on the fungal species. The common feature is an increase in size (hypertrophy) of plant tissues in the form of galls, where the affected cells are stimulated for division and/or growth (hyperplasia), causing deformity of plant organs (Harris and Pitzschke 2020). These altered cells are destroyed and replaced by thick-walled, dark- or light-brown colored and black, dust-like teliospores (Agrios 2005; Munkvold and White 2016). The infections usually are not detected until sporulation occurs on or in the invaded parenchymatous tissues. Even though most smuts may spread systemically through their hosts, the primary symptoms are often restricted to female or male inflorescences. Several smuts also affect stems and leaves; whereas in other cases the compromised tissues are limited to the belowground organs (Agrios 2005).

Ustilaginomycetes (the true smuts) parasitize angiosperms, mostly belonging to Poaceae and Cyperaceae families. Interestingly, the teliospore-forming species invade herbaceous hosts almost exclusively (Zuo et al. 2019). The general life cycle of smut fungi (Figure 3) displays saprophytic and parasitic phases, with different ploidy (number of sets of chromosomes) and a variable number of nuclei (1 or 2) in each cell (Bauer et al. 2008). Teliospores are dormant structures produced by smuts that allow the fungi to survive in the environment for long periods until they meet a susceptible host to invade. Under suitable conditions of temperature and humidity, but especially in the presence of a host, the mature diploid teliospore (which represents the fungal diplophase)

germinates and undergoes meiosis. A young promycelium with haploid cells and sexual budding yeast, known as sporidia, begins haplophase. Occasionally, instead of sporidia, the promycelium forms haploid hyphae. The fusion (plasmogamy) of two sporidia or hyphae with compatible mating types (determined at the fungal MAT *locus*) restores the dikaryotic state and gives rise to parasitic mycelium (dikaryophase). The fungus grows intra- or intercellularly inside the plant, mainly in the meristematic tissues, without causing significant tissue damage until sporulation. Spore development occurs primarily in the host's reproductive organs, in galls. Sporogenesis begins with hyphal fragmentation. The developing spores (simple or aggregated) are covered with a thick, echinulate (with small spines) wall and are commonly black or dark brown. Teliospores form masses, also known as sori, that look like coal dust. Nuclear fusion (karyogamy) in immature diploid spores produces mature diploid monokaryotic teliospores (Zuo et al. 2019).

Features of *Thecaphora frezii* biology that are specific or shared with other agronomically important smuts

Peanut smut:

The peanut plant blooms above the ground, but the fruit develops underground. After self-pollination, the flowers lose their petals and the fertilized ovules, future seeds and fruit; penetrate into the soil by a specialized organ called a gynophore or peg. During this underground stage of rapid metabolic activity and plant cell division, *T. frezii* invades (Cazón et al. 2018). Peanut smut is a monocyclic (one infection cycle per growing season) and polyetic (inoculum accumulation affects subsequent seasons) disease (Rago et al. 2017). *T. frezii* causes partial or total destruction of peanut fruit, which is evidenced by appearance of “brown flour” or smut (masses of teliospores) replacing seed tissue inside mature pods (Figure 4). Information concerning the details of fungal

infection and dissemination is sparse. But it is clear that the burial of the peanut gynophores or pegs in the soil is a critical stage for infection. The molecular mechanisms involved in this process are currently unknown. In 2017, Mary et al. evaluated the ability of peg extracts to induce spore germination in an *in vitro* experiment. The authors used pegs collected from peanut plants grown in soils contaminated or not by *T. frezii* teliospores. Spore germination occurred earlier and at higher percentages in extracts of those developed from soils contaminated by the fungus. These results reinforce the hypothesis that *T. frezii* teliospore germination is triggered by the pegs through the release of chemical mediators. Infection takes place after overcoming several physical and chemical barriers, in the absence of effective defense mechanisms to limit fungal invasion. Briefly, teliospores surrounding pegs germinate and produce sporidia (Figure 5A). These cells need to fuse with a compatible partner to produce the infective form (the dikaryotic mycelium). Upon invasion, the latter reach the developing seeds and produce galls, to partially or totally transform them into a carbonaceous mass of teliospores (Marinelli et al. 2010; Rago et al. 2017). Marraro Acuña et al. (2012) studied fungal localization in histological sections of pegs in the early stages of development and reported that initially, the infective mycelium invaded the outer and middle layers of parenchymal cells of the developing fruit, inducing cellular hyperplasia. The authors observed the presence of haustoria. Later, hyphae infect seeds through the funiculus, a connecting tissue that holds them to the fruit, and spread throughout the integument. In the most advanced stages of infection, the fungus develops intra- and intercellularly in the cotyledon reserve parenchyma, attacking the phloem and then the xylem (Astiz Gasso et al. 2010). As in other smuts, teliospore formation begins with fragmentation of hyphae and proceeds until seeds transform into carbonaceous masses. Strongly bound teliospores (2-7) form glomeruli. Deformation and

hypertrophy are commonly observed in smutted mature fruit, although the lack of such symptoms is not always associated with absence of infection (Marinelli et al. 2010).

Most soil contamination occurs during the peanut picking and threshing process, where the peg of the spore-laden fruit is frequently broken or cut, increasing inoculum with each growing season. Although seed contamination with teliospores does not endanger seedling development in the next growing season (Cazón et al. 2018), it serves as a vehicle for spreading the disease over long distances. Wind contributes to peanut smut dissemination, transporting teliospores to formerly *T. frezii*-free fields. The spores survive in the soil and crop residue, where they can remain viable for years (Cazón et al. 2016b).

Potato smut:

Potato (*Solanum tuberosum)* production is carried out mainly by vegetative multiplication of tubers used as seeds. Seed potatoes are grown in regions considered safe from the point of view of pest and disease transmission. Potato smut, caused by *T. solani,* is one of the most damaging fungal diseases affecting potato crops. The pathogen is transmitted primarily by infected seed pieces (EPPO 2020). Many aspects of the infection process remain unclear. In infected potatoes, the fungus grows internally through the underground stems until it reaches the developing tubers (Figure 5B). Fungal penetration of the host plant cortex is followed by ramification toward the phloem and parenchyma. The hyphae stimulate proliferation of cells in the cambium, causing hypertrophy in stems and tubers (Andrade and Muñoz 2005). The fungus invades potato tubers, stolons, and underground stem parts but not the roots. Each gall develops from a separate infection. The size and shape of galls depend mostly on the time of infection. They contain dark brown to black spores, whose carbonaceous appearance give the disease its name (Andrade 2005). Alike

peanut smut, symptoms of infection are not found on aerial parts of plants. Spore sori are easy to identify in malformed tubers, but their absence in those that are asymptomatic does not necessarily correlate with health. Andrade and Muñoz (2005) reported that some healthy tubers can carry the fungus without causing visible symptoms until the next harvest, thereby increasing the chances of spread and introduction of the pathogen to disease-free areas. Undetectable levels of spores can be transported on the surface of healthy tubers favor pathogen spread. Although the fungus can persist for up to 7 years in gall fragments (Torres 2002), growth of non-infected seed potatoes in contaminated soil is not considered the principal mode of transmission. Other routes of transmission such as shoes, machinery, and tools contaminated with soil from infected areas, can occur. Natural dispersion of the fungus by water or wind is also a possibility; however, the potential is very low (EFSA 2018).

Other solanaceous species, such as *S. lycopersicum* (tomato) and wild solanaceous plants (*Datura stramonium, S. stoloniferum*) are hosts of *T. solani*, and in consequence are considered potential reservoirs of the pathogen. For example, *D. stramonium* that grows in potato production fields can contribute to the fungal contamination of soil (CABI 2019).

The need for pathogen-free seed tubers for potato production is acute. Cultivation of resistant varieties, the implementation of sanitation measures, and compliance with a quarantine period are mandatory to avoid disease dissemination by seed tubers.

Loose smuts of wheat and barley:

Loose smut of wheat caused by *U. tritici* (Pers.) Rostr. (*Triticum aestivum* L.) and loose smut of barley (*Hordeum vulgare* L.) caused by *U. nuda* are seed-borne monocyclic diseases. The biology of both pathogens is similar (Figure 5C), but shows some differences with that of *T. frezii* (Figure

5A). The disease cycles show that primary infection takes place when teliospores, released by smutted heads, fall into healthy flowers. Following spore germination and mating, the young infective mycelium penetrates the developing seed, where it will lie dormant. In the next growing season, the mycelium grows in parallel to seedling development, producing a systemic infection, which reaches maturity and sporulates to give smutted heads, completing the cycle.

Both diseases result from a complex interplay between the pathogen, the host, and the weather conditions that occur during bloom. The pathogenicity of *U. tritici* is dependent on its physiological race. Race in this context is defined as members of a spore collection sharing a relatively conserved virulence against a defined set of differential wheat varieties. An in-depth knowledge of such pathogenic behavior is essential for the development of wheat varieties resistant to smut.

Unlike what is seen in peanut smut, *U. tritici* and *U. nuda* infections affect aerial parts of plants. Both wheat and barley loose smuts were recently reviewed (Abrahim 2019; Woldemichael 2019). The vegetative growth of infected plants can be slightly increased (barley plants may be slightly taller than unaffected neighbors) or mostly unaltered (wheat). Symptoms generally become evident at the phenological stage of spike emergence. Characteristic narrow linear sori can eventually be detected on the flag leaves, sheath, and culms of barley. Healthy and *U. tritici*-infected wheat heads emerge almost simultaneously, but *U. nuda* infection seems to slightly accelerate the maturation process in barley.

Teliospores from smutted spikes are transported by several carriers, including insects and wind, to the flowers of non-infected heads (Figure 5C). It is believed that wind spreads spores to nearby healthy heads with recently opened florets. The initial steps of fungal invasion occur from early

and mid-anthesis up to two days after flowering, while the female reproductive tissues are susceptible to infection. Environmental conditions can lengthen or shorten the period that flowers stay open, and therefore modify the probability of infection. Another difference with *T. frezii* is that *U. tritici* and *U. nuda* teliospores do not show a dormancy period. With favorable relative humidity and temperature inside the glumes, the spores that land on open flowers quickly germinate. Excessive heat or low humidity can inhibit seed invasion. After several rounds of meiosis and mitosis in the teliospore diploid nuclei, a promycelium (slightly curved in *U. tritici*), initially formed by four monokaryotic haploid cells, emerges. Typically, *U. tritici* and *U. nuda* promycelia do not form sporidia. Young cells in the germ tube divide and lead to the formation of haploid hyphae that elongate and fuse through short conjugation tubes. The dikaryotic infective hyphae rebranch, grow along the stigma, and proceed between and through the cells to penetrate the ovary and infect the embryo.

Independently from entry through the style, *U. tritici* can penetrate the ovary wall directly or enter from any part of the pericarp to reach the embryo (Ram and Singh 2004). Regardless of the infection mechanism, the mycelium reaches the growing point of the embryo, where it will lie dormant in the plumular bud of the asymptomatic mature kernel until seed germination.

In some cases, seed infection may weaken or kill seedlings before emergence. If germination occurs, the dormant dikaryotic mycelium (diplophase) revitalizes and starts to grow. It invades meristem tissue and is transported in the crown node throughout the seedling, colonizing the seed primordia. The mycelium develops sporiferous hyphae and sporulation proceeds in parallel with the development of the spike, whose tissues are largely replaced by sori containing teliospores. The two nuclei in the initial young dikaryotic spores fuse together to give mature diploid monokaryotic spores. The sori of *U. nuda*, but not *U. tritici*, are covered with a thin and fragile

membrane, whose rupture shortly after emergence leaves the teliospore mass naked and free for dispersion in the environment. Almost all parts of young spikes except the rachis and the awns (barley) are destroyed and replaced before their emergence by a powdery mass of the characteristic light olivaceous-brown teliospores.

Effectors: Chemical weapons of smut fungi against their hosts

Smut fungi are biotrophs, which means that they need living host cells in order to survive. As a result, they minimize damage to the host during infection. Plants usually react in response to the biotrophic pathogen's "attack" with a first line of defense including physical cell wall reinforcement, production of reactive oxygen species (ROS), or oxidative burst and production of antifungal compounds, among others. In addition, salicylic acid (SA) signaling leads defense response against biotrophs, which induces the expression of defense genes and localized cell death (Glazebrook 2005). Plant local cell death, also called hypersensitive response (HR), can effectively circumscribe the pathogens and limit their access to nutrients and water.

To deal with the host defense response, pathogens have a secret "army" called effectors. These compounds help the microorganisms to manipulate the immune response of the plant and can determine the outcome of the interaction. Effectors are comprised of secreted proteins but may also include secondary metabolites and sRNAs (Rodriguez-Moreno et al. 2018). The protein effectors are classified as apoplastic or cytoplasmic according to their zone of action in the host plant. The effectors could be either secreted to the intercellular space (apoplast), i.e., stay in the space between plant and pathogen cells, or be translocated into the cytoplasm of host cells (Lanver et al. 2017). Sequencing the fungal genome has provided significant insight into the study of protein effectors in smut fungi, especially in *U. maydis* but also *Sporisorium reilianum, U. hordei, S. scitamineum* (Schuster et al. 2018) and *T. thlaspeos* (Courville et al. 2019). Interestingly, the

genome of *U. maydis*, the model of biotrophic plant- pathogenic basidiomycetes, is very similar to that of *T. thlaspeos*, but they behave very differently (Frantzeskakis et al. 2017).

Although several effectors have been functionally characterized, particularly for *U. maydis* (Xia et al. 2020), little is known about the genus *Thecaphora*. A smut fungus effector in the early stages of infection is Pep1 (Protein essential during penetration 1), which is essential for entering both monocot and dicot hosts (Figure 6). This functionally conserved compound is required by *U. maydis, S. reilianum, U. hordei and T. thlaspeos* to penetrate maize, barley or *A. thaliana,* respectively (Courville et al. 2019; Doehlemann et al. 2009; Hemetsberger et al. 2012). In the *U. maydis*-maize pathosystem Pep1 inhibits an apoplastic plant peroxidase enzyme (POX12) that is one of the key components of the oxidative burst, suppressing the host defense response. Similarly, *T. thlaspeos*' Pep1 could potentially block an apoplastic *A. thaliana* peroxidase (PR33/34) function (Courville et al. 2019). Noticeably, Pep1 is a pathogenicity factor for *U. maydis* and *U. hordei.* Without it, biotrophic infection would not be possible (Doehlemann et al. 2009).

Nlps (Necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins) are effectors that inhibit defense responses typically seen in dicot hosts (Oome et al. 2014) but not in monocots, such as cereal grasses. For example, a non-cytotoxic Nlp1 in *T. thlaspeos*, does not cause necrosis in cells but may potentially suppress the function of the dicot- specific enzyme NADPH oxidase, inhibiting the oxidative burst (Courville 2018). Although more studies are needed to define the function of Nlp1, this finding suggests that effector genes identified in *T. thlaspeos* (Courville et al. 2019) may also be identified in other *Thecaphora* species involved in dicot-host interactions like *T. frezii*-peanut and *T. solani*-potatoes.

Smut fungi can use multiple strategies to damper host reactions. For instance, *U. maydis* secretes Rsp3 and UmFly1 proteins to protect itself from plant-derived antifungals (Ma et al. 2018; Ökmen et al. 2018). Moreover, the pathogens can outsmart phytohormone signaling in different ways. While SA protects plants against biotrophic pathogens, ethylene (ET) and jasmonic acid (JA) lead responses against necrotrophs (Glazebrook 2005). The virulence factor of *U. maydis*, Cmu1 (Chorismate mutase 1), reduces synthesis of SA by lowering the level of its precursor chorismate (Djamei et al. 2011). In the shikimate pathway, Cmu1 detours the synthesis of SA towards the production of other compounds (phenylalanine, tyrosine) (Lanver et al. 2017). Recently, Tue1 (*Thecaphora* unique effector 1), a novel effector candidate secreted by *T. thlaspeos*, was described (Courville et al. 2019). Interestingly, Tue1 may activate the expression of ERF6 (Ethylene Response Factor 6), a transcription factor involved in response to abiotic stress (Huang et al. 2016). However, ERFs participate in the regulation of the genetic expression of JA/ET response (Huang et al. 2016). ERF6 could potentially induce PDF1.2 expression, hence triggering JA/ET response, effective for necrotrophic but not biotrophic pathogens. Although effectors have not yet been described in *T. frezii*, Mary et al. (2019) found that the fungus could manipulate plant immunity though jasmonate signaling pathway. The authors found that the jasmonate levels in peg extracts showed a direct correlation with susceptibility to *T. frezii* smut and were increased by presence of teliospores in the ground. These results show that plant defense mechanisms can be modulated by teliospores and the young promycelium in soils, or compounds released by them, potentially favoring fungal invasion.

While effectors of necrotrophic pathogens kill host cells in order to get nutrients, effectors of biotrophs “manipulate death” or localized cell death. Most smut fungi colonize their host without causing drastic symptoms. Many smuts of grasses, such us *S. reilianum,* and *T. thlaspeos*, develop

systemically and disease symptoms are restricted to the inflorescence of the host plant after several weeks or months. Conversely, *U. maydis* and *T. frezii* infect locally and produce symptoms in aboveground or belowground parts of the plant, respectively. The effector repertoires of each smut fungus evolve according to its lifestyle. For instance, the effector Tin2 (Tumor inducing 2), common to a number of smut fungi, displays different functions when *S. reilianum* or *U. maydis* infect maize (Tanaka et al. 2014; Zess et al. 2019). *S. reilianum* follows the typical model of smut fungi that infect grasses, whereas symptoms like tumors can be visualized after systemic infection. In *S. reilianum,* Tin2 inhibits critical proteins in maize: plant kinases TTK2 and TTK3 (Tanaka et al. 2019). For *U. maydis*, in contrast, Tin2 induces plant anthocyanin production (red pigment) by stabilization, but not inhibition, of the plant kinase enzyme (TTK1) (Tanaka et al. 2014). Induction of the anthocyanin pathway may inhibit the biosynthesis of lignin. Deficiency in lignification of the cell wall could explain the development of noticeable tumors in aerial vegetative tissues during the early stages of the infection, in addition to tumor formation in the inflorescence. Tanaka et al. (2019) showed evidence that *U. maydis*' Tin2 has gone through an evolutionary process called "neofunctionalization" related to tumor formation. The authors observed that the ancestral Tin2 effector can substitute virulence function in *S. reilianum,* but not in *U. maydis,* suggesting that Tin2 has acquired a new function in *U. maydis*, according to its lifestyle. See1 (Seedling-efficient effector 1) is another cytoplasmic effector that, along with Tin2, is crucial for tumor formation (Redkar et al. 2015). Interestingly, although See1 has been described in other smut fungi, such us *U. hordei*, it induces tumors only in *U. maydis* (Redkar et al. 2015). This seedling-specific effector intervenes in reactivation of maize DNA synthesis and cell division in leaves, contributing to tumor progression (Xia et al. 2020). See1 reactivates plant DNA synthesis through interaction with a cell

cycle suppressor (SGT1), during tumor formation in maize leaves. Future research will be crucial to determine if See1, like Tin2, has acquired a new function in *U. maydis*.

Disease assessment and methods for isolation and identification of *Thecaphora* smuts

Field evaluation of disease symptoms of peanut and potato smuts is difficult because they are absent in aerial parts. Therefore, they can be evaluated only at harvest time. To assess peanut smut, it is necessary to open mature pods. Astiz Gassó et al. (2008) proposed the following five-level scale to characterize disease severity: 0, healthy pods; 1, normal pod with a small sorus in a single kernel; 2, deformed or normal pod with half of the kernels affected; 3, deformed pod and a completely smutted kernel; 4, deformed pod with two completely smutted kernels. Later, Paredes et al. (2014) calculated a disease intensity index, combining incidence (proportion of infected pods) and severity of infection in a representative pod sample.

Fungal detection can be required to confirm infection, and is essential to facilitate removal of infected but asymptomatic pods. Moreover, it is a useful tool to evaluate the persistence of smut fungi span in seeds and soil. The methods currently available for fungal detection include microbiological cultures, teliospore identification and counts, and recognition and quantification of fungal-specific nucleic acids, among others. As is expected, some methods fit better than others depending on the test purpose.

Culturing enables initial isolation and morphological characterization of pathogens. Cultures are part of *in vitro* evaluation tests to detect potential antifungal substances and in studies aimed at unveiling fungal genetic traits. In order to avoid contamination, protocols for spore cultures include slight surface disinfection before plating. Germination of teliospores is typically low, with the highest rates observed in media supplemented with extracts of some part of the susceptible hosts.

This fact, plus the long time required to form colonies (usually one month), limit the value of microbiological cultures for detecting smut fungal infection or contamination. Supplementation of common culture media with gynophore extracts improved the sprouting of *T. frezii* teliospores, although the improvement was modest (Astiz Gassó and Marinelli 2013). A similar behavior was reported in *T. solani*, and the low germination rate was attributed to different degrees of maturity in a teliospore population, whereas only those that were fully mature (found in sori showing a dusty aspect when transecting a gall) were able to germinate (Andrade et al. 2004). The *T. frezii* spores germinate and mycelium develops in Murashige and Skoog medium when supplemented with peanut peg extracts (Figure 7). Fungal subcultures usually have fewer nutritional requirements and can use conventional culture media without supplements, although slow growth rate remains a limitation.

Several surfaces, including those of peanut seeds and agricultural machinery, may carry *T. frezii* teliospores, and thereby increase their inoculum in soils. Fungal spore adsorption to surfaces can be investigated by means of a "washing test". This is a rapid and cheap assay, based in the microscopic identification of spores in sediments from surface lavages with water and detergents. Marraro Acuña et al. (2012) evaluated the washing test protocol, described in the quarantine procedures manual of the European and Mediterranean Plant Protection Organization (EPPO), and observed consistency and repeatability of the results when it was applied for *T. frezii* spore detection in peanuts. A similar procedure, but replacing centrifugation with natural sedimentation, was performed to detect soil contamination with teliospores (Marinelli et al. 2008). The results of such studies help to predict disease severity in hypothetical scenarios where disease-favorable weather conditions converge.

Sediments of washing solutions were also employed to assess nucleic acids. Cazón et al. (2016a) designed a PCR protocol for detection of *T. frezii* using two specific primers designed from the ITS region. The PCR amplified nucleic acids with high specificity, but not from other microorganisms frequently found in peanut seeds. A detection limit of 10 teliospores from a sample of 400 kernels was reported for this protocol. Sensitivity was improved to up to a single spore by performing a quantitative PCR (qPCR) with the same primers and using SYBR-Green for real-time detection of amplicons (Cazón et al. 2018).

Molecular biology tools were also applied for characterization of *T. solani* (Andrade et al. 2004). DNA fingerprints generated by PCR products, and the partial sequencing and phylogenetic analysis of the large subunit (LSU) rDNA region, led to confirmation of the first successful *in vitro* germination and culture of *T. solani* teliospores in 2001, as well as its classification within the *Thecaphora* genus. In addition, a method based on the amplification by PCR of a DNA sequence from the fungal nuclear ribosomal region was developed for detection of *T. solani* in soil, plants, and tubers (Andrade and Muñoz 2009).

Peanut smut management

Many tactics for managing peanut smut have been recommended or evaluated including crop rotation, application of fungicides in the soil (once peanuts begin to peg), and cultivation of the crop in fields free or with low density of *T. frezii* teliospores (Rago et al. 2017). These strategies should be part of an integrated management of the disease, particularly in conjunction with resistant or tolerant cultivars when possible. Currently, most of commercial varieties of peanut that are widely cultivated are susceptible. There are, however, a few resistant cultivars, including EC-191RC from Criadero El Carmen, and Hispano Ascasubi from the Argentinian National Institute

of Agricultural Technology (INTA) (CABI 2019), although they are not widely used due to their agronomic characteristics.

In addition, genetic variability in cultivated peanut is limited. Recently, Argentinian researchers identified high levels of resistance in wild species (De Blas et al. 2019) and landraces introduced from Bolivia (Bressano et al. 2019) to peanut smut. The aim of these projects was to develop new cultivars with a broad genetic base. To accomplish this, they transferred resistance from wild species and landraces in crossing programs to commercial cultivars. Among the biggest challenges were the ploidy differences among commercial and wild species. Whereas cultivated peanut is allotetraploid ($2n = 2x = 40$), most of the wild species of section *Arachis* are diploid ($2n = 2x = 20$, or $2n = 2x = 18$). For that reason, the wild species *A.rachis correntina*, *A. cardenasii*, and *A. batizocoi* were hybridized and the chromosome number was doubled to be compatible with *A. hypogaea* (De Blas et al. 2019). Conversely, utilizing landrace germplasm has the advantage that they can be immediately transferred to commercial varieties. After 3 years of phenotyping the accessions of wild relatives and landraces in field trials under high inoculum pressure, transfer of resistance to peanut smut was achieved with relatively high frequency (De Blas et al. 2019). In addition, genetic transfer was supported through the use of molecular tools, such as simple-sequence repeats (SSRs) and Insertion/Deletion (InDel) marker genotyping, to follow resistant traits in introgressed lines (Bressano et al. 2019; De Blas et al. 2019). These materials can be used for breeders in peanut improvement programs to obtain commercial cultivars resistant to peanut smut.

The effectiveness of using commercial fungicides to control peanut smut is quite variable, at best reaching 50-60% control (Astiz Gassó and Wojszko 2011; Cazón et al. 2013; Paredes et al. 2015a). Chemical treatment is constrained by the fact that fungi may develop resistance against

fungicides (Ma and Michailides 2005; Sierotzki and Scalliet 2013). To control peanut smut, the use of carboxamides, strobilurins, and azoles is common. Carboxamides and strobilurins target mitochondrial proteins related to fungal energy metabolism. Azoles target the membrane ergosterol biosynthesis pathway but also can inhibit the enzyme subunit of mitochondrial complex I, among other mechanisms recently described (Bromley et al. 2016, Arias et al. 2019). Arias et al. (2019) indicated potential development of resistance of the pathogen to carboxamide and strobilurin fungicides, and provided a basis to investigate triazole resistance in *T. frezii,* after sequencing the mitogenome and other fungicide targets outside the mitochondria. Therefore, monitoring mutations of fungicide targets in field samples is key to implement changes in fungicide treatments and to determine whether resistance by the pathogen is the cause where the disease is not controlled (Brent and Hollomon 2007).

Research priorities

Is teliospore germination triggered by chemical mediators released by peanut pegs?

Current knowledge of peanut smut recognizes the existence of a two-way chemical communication between the anatomical sites of entry into the plant (pegs) and the adjacent teliospores in the soil. As previously mentioned, *in vitro* spore germination increased by adding peg extracts to the culture medium, and it was enhanced when extracts coming from plants growing in soils contaminated with *T. frezii* were used. These results suggest that in nature, pegs could release biomolecules that enhance teliospore germination. Future research should focus on elucidating the biochemistry involved in this phenomenon, which in turn could lead to development of new strategies to suppress smut. For example, peanut genotypes producing lesser amounts of germination-inducing substances could be selected. Additionally, given that a susceptible host is essential for *T. frezii*

sporulation, chemicals could be applied to reduce soil inoculum by inducing spore germination between growing seasons. A similar strategy of decontamination has already been explored for bacterial spores under laboratory conditions (Kohler et al, 2017). This would reduce teliospore formation and mycelia should die due to the absence of developing peanut pegs in the ground.

Can all *T. frezii* teliospores germinate and produce a dikaryotic infective mycelium?

Soil contamination with *T. frezii* increases with subsequent peanut culture seasons, and at least some spores maintain their infectivity for at least 4 years (Cazón et al. 2018). The incidence and severity of smut is correlated with the extent of soil contamination with this fungus (Rago et al. 2017). But it is unclear if all the teliospores can germinate and produce dikaryotic infective mycelium. Moreover, the low germination rates found in *in vitro* cultures could be partially due to high proportions of naturally occurring immature or non-viable spores. The latter might be increased by surface disinfection protocols applied before plating. This hypothesis has been proposed by Andrade (2005) in an attempt to explain the low germination scores of *T. solani* spores in *in vitro* cultures. The lack of data concerning the proportion of viable teliospores in a population makes it difficult to link low *in vitro* germination rates to suboptimal culture conditions. Furthermore, this data gap represents a significant barrier for the identification of new molecules with sporicidal activity. This target should not be discarded when keeping in mind the low efficacy of antifungals applied to aerial parts or soil. Hence, a better characterization of teliospore physiological status within a population should be considered as a priority.

Can *T. frezii* weaken local and systemic defense mechanisms in peanut plants?

In plants, there are two contrasting phenotypes of defense responses controlled by different signaling pathways. Both are associated with transcriptional changes and the synthesis of

phytohormones. The most effective for the control of biotrophic pathogens is characterized by the activation of the SA pathway. In contrast, necrotrophic microorganisms are more efficiently controlled by mechanisms involving JA and ET. Since *T. frezii* has a biotrophic lifestyle, defense responses mediated by SA would be more competent for its control. We found a direct correlation between susceptibility to *T. frezii* and JA levels in pegs (Mary et al. 2017; Mary et al. 2019). Furthermore, jasmonate levels increased with fungal presence in the soil, suggesting a strategy to evade plant immunity. The infection of some but not all pegs in each plant, and the absence of the fungus in asymptomatic above- and belowground tissues, could indicate tissue-specific defense mechanisms, although systemic effects should not be ruled out.

The role of fungal effectors in such antagonistic manipulation of phytohormones during pathogen-host interaction is still an enigma for most smut fungi. So far, the mechanisms of only six effectors of *U. maydis* have been described (Zuo et al. 2019). Concerning the *Thecaphora* clade, the DNA and RNA sequencing of *T. thlaspeos* helped to identify promising effector candidates, providing a first approach to elucidating the possible role of some of them at functional level (Courville et al. 2019). Genome sequences of *T. frezii* and *T. solani* still need to be produced. It will be interesting to determine whether broadly conserved effectors in smut fungi, and the typically dicot-specific effector NLp1, are likely to play important roles in *T. frezii* and *T. solani* virulence. Moreover, the identification and participation of specific mitochondrial proteins as virulence/pathogenic determinants is currently an under-explored field.

In this context, future studies should be focused on defense mechanisms associated with susceptibility and resistance to *T. frezii*, also considering probable fungal modulatory effects. These data will allow the design of new strategies to induce resistant phenotypes that prevent peanut smut.

Which management strategies can be improved to reduce peanut smut?

Growing resistant varieties seems to be the best way to control peanut smut. Critical progress has been achieved recently in development of pre-breeding materials, diversifying the germplasm of commercial cultivars by crossing them with landraces and wild relatives. The development of new tools, such as molecular markers, is needed for germplasm characterization, such us the identification of genomic regions involved in peanut smut resistance and other stresses. This knowledge will boost breeding programs' development of resistant cultivars.

Fungicides continue to be one of the complementary tools for managing peanut smut. Currently, it is clear that determinants of fungicide efficacy, in addition to the active ingredients and development of resistance to fungicides, include application timing and delivery systems. Seed treatments (seeds coated with fungicides) do not control the disease in the field because *T. frezii* infects during the pegging process (Rago et al. 2017). However, since contaminated seed is a long-distance dispersal agent, seed treatments are important to prevent inoculum increases in the soil (Cazón et al, 2018). In the field, strategies using slow-release fungicide formulations, or spraying fungicides at night because plant leaflets are closed, seem to be more effective with greater penetration of fungicide droplets to pegs reported (Augusto et al. 2010; Paredes et al. 2015b; Paredes et al. 2014). Overall, these technologies and practices, in combination with the use of nanoparticles as carriers of fungicides, may eventually lead to enhanced efficiency of fungicide formulations (Camiletti et al. 2020).

Does ingestion of peanuts contaminated with *T. frezii* represent a hazard for human health?

Smut fungi spores are sources of a broad spectrum of metabolites that may cause toxicity when humans and animals ingest them (Pepeljnjak et al. 2005). Peanuts entering the food chain may be contaminated with *T. frezii* teliospores, mycotoxins, and chemical residues. There are very

few studies that address toxicological aspects associated with the consumption of these fungal structures (Mary et al. 2014a, b; Mary et al. 2015); however, their results suggest that it would not represent a hazard to human health. The question if components of smut spores could modify, through toxicodynamic interactions (synergism, antagonism or additive effects), the toxicity of mycotoxins and agrochemicals that contaminate peanuts has not been explored. Therefore, future research must include studies with different toxicological approaches for a better estimation of the impact of *T. frezii* on human health.

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FIGURE CAPTIONS

Figure 1: World's top ten peanut producing countries and Argentinean contribution during the last decade. A) Top ten ranking of peanut producing countries in 2019/20 (USDA, World Agricultural Production report October 2020 (https://apps.fas.usda.gov/psdonline/circulars/production.pdf). Values between brackets indicate peanut production, in million metric tons. B) Geographic location of Córdoba province (dark gray), the main peanut-producing area in the central-north region of Argentina. C) Contribution of Córdoba province to the total peanut production of Argentina in the last decade. (Data source: Ministry of Agriculture, Livestock and Fisheries of Argentina. http://datosestimaciones.magyp.gob.ar/reportes.php?reporte=Estimaciones)

Figure 2: Geographical distribution of *Thecaphora* species responsible for peanut and potato smuts. Although peanut smut was reported only in commercial species in Argentina, *T. solani* was found in Bolivia, Chile, Colombia, Ecuador, Mexico, Panama, Peru and Venezuela.

Figure 3: General life cycle of a typical smut fungus (Ustilaginomycetes).

Under favorable conditions, smut fungi teliospores germinate and give rise to a short promycelium with transverse septa, where, after meiosis, terminal and lateral monokaryotic basidiospores or sporidia are formed (a). Sporidia can grow indefinitely as a budding yeast phase or give small primary mycelia (b). When two compatible partners are found, plasmogamy occurs with development of a secondary dikaryotic mycelium that can invade host tissues (c, d). Later, immature dikaryotic teliospores are formed from the hyphal mass by simple fragmentation and acquisition of a resistant wall (e). Karyogamy produces diploid teliospores, which spread in the environment and infect susceptible hosts (f). The red inner circle represents the nuclear cycle of smut fungus with mitosis (M), plasmogamy (P) and karyogamy (K) preceding the haploid (slender solid line), dikaryotic (double line), and diploid (heavy solid line) phases, respectively. Saprophytic (dark grey) and parasitic (light grey) fungal stages are also depicted. The diffuse white line marks the beginning of fungal cycle, and progression is shown clockwise.

Figure 4: Peanut smut symptoms caused by *Thecaphora frezii*.

A) Infected seed tissues are replaced by a powdery mass of reddish-brown teliospores. B) Correlation between external symptoms of disease and tissue destruction in pods with one (middle two), and both affected seeds (right). An uninfected pod is shown on the left for comparison. C) Grains not infected (left) and partially damaged by *Thecaphora frezii*.

Figure 5: Peanut smut by *T. frezii*: Biological features in comparison with those from other agronomically important smuts.

Disease cycle of *T. frezii* (A), *T. solani* (B), and *Ustilago tritici/ U. nuda* (C). Soil-contaminating teliospores cause peanut infection and smut development, whereas infected seed tubers and infected seeds transmit potato and wheat/barley smuts, respectively. Mating of compatible sporidia (*T. frezii* and *T. solani*) or hyphae (*U. tritici* and *U. nuda*) produces the infective dikaryotic mycelia. Infection and gall formation are limited to the underground plant parts of peanut (gynophores or pegs) and potato (potentially all growing stem parts but not roots), while aerial organs are affected in wheat and barley. Symptoms of infection are evident at harvest (peanut and potato), or immediately before spike emergence in wheat and barley.

The red inner circle represents the nuclear cycle of each smut fungus with mitosis (M), plasmogamy (P) and karyogamy (K) preceding the haploid (thin solid line), dikaryotic (double line), and diploid (thick solid line) phases, respectively. Saprophytic (dark grey) and parasitic (light grey) fungal stages are also depicted. The diffuse white lines mark the beginning of fungal cycles, and progression is shown clockwise.

Figure 6: Functionally characterized effectors of *Ustilago maydis* and *Thecaphora thlaspeos*.

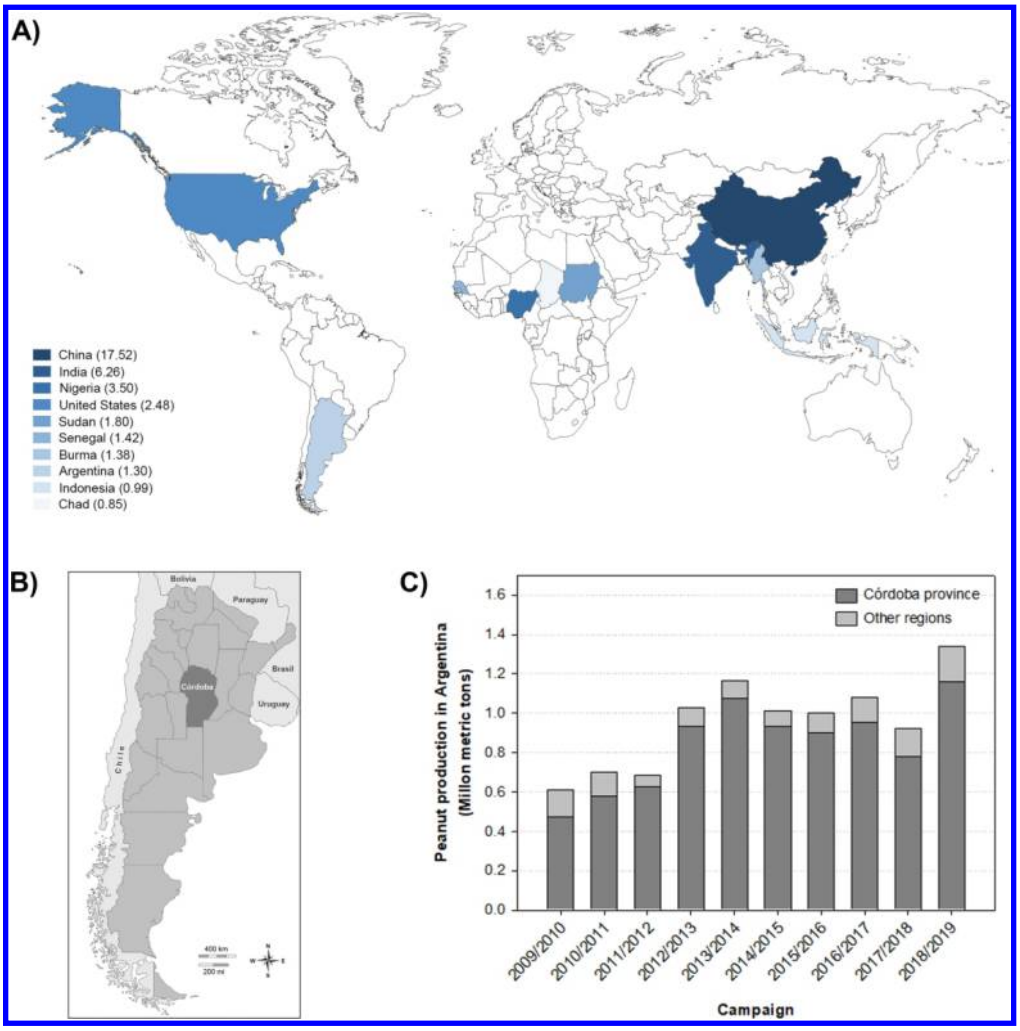
Upper: Plant defense manipulation of *U. maydis* by the apoplastic Pep1 (a Protein essential during penetration 1) and the cytoplasmic Cm1 (Chorismate mutase 1), Tin2 (Tumor inducing 2) and See1 (Seedling-efficient effector 1) effectors. *U. maydis* can grow both intra- and intercellularly in host plants. Conversely, many other smut grasses as well as *T. thlaspeos,* and *U. maydis* infect locally rather than systemically and form large tumors on all aerial tissues. Several effectors are required to induce tumors.

Lower: *T. thlaspeos*: manipulation of plant defenses by Pep1, likely localized in the apoplast, and two likely cytoplasmic Nlp1 (Necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins) and Tue1 (*Thecaphora* unique effector 1) effectors.

Note that germinated teliospores of *T. thlaspeos* can directly infect the host plant. The development of *T. thlapeos*' dikaryotic hyphae (dashed lines) may be possible. Plant signals are required by *T. thlaspeos* in order to germinate. *T. thlaspeos* grows intercellularly. Later, the infection is systemic and symptomless, like endophytes, until it sporulates in the siliques replacing or covering the seeds.

Figure 7: *Thecaphora frezii* teliospore morphology and *in vitro* growth.

Before seeding, spores grouped in the form of glomeruli, composed of 2 to 7 teliospores (A), are superficially disinfected with 1.5% sodium hypochlorite. Germination takes place after 5-7 days in Murashige and Skoog medium supplemented with peg extracts (B), and sporidia are seen after 15 days of incubation (C). White or sparsely pigmented colonies are observed in 1-month-old subcultures in potato dextrose agar (D).



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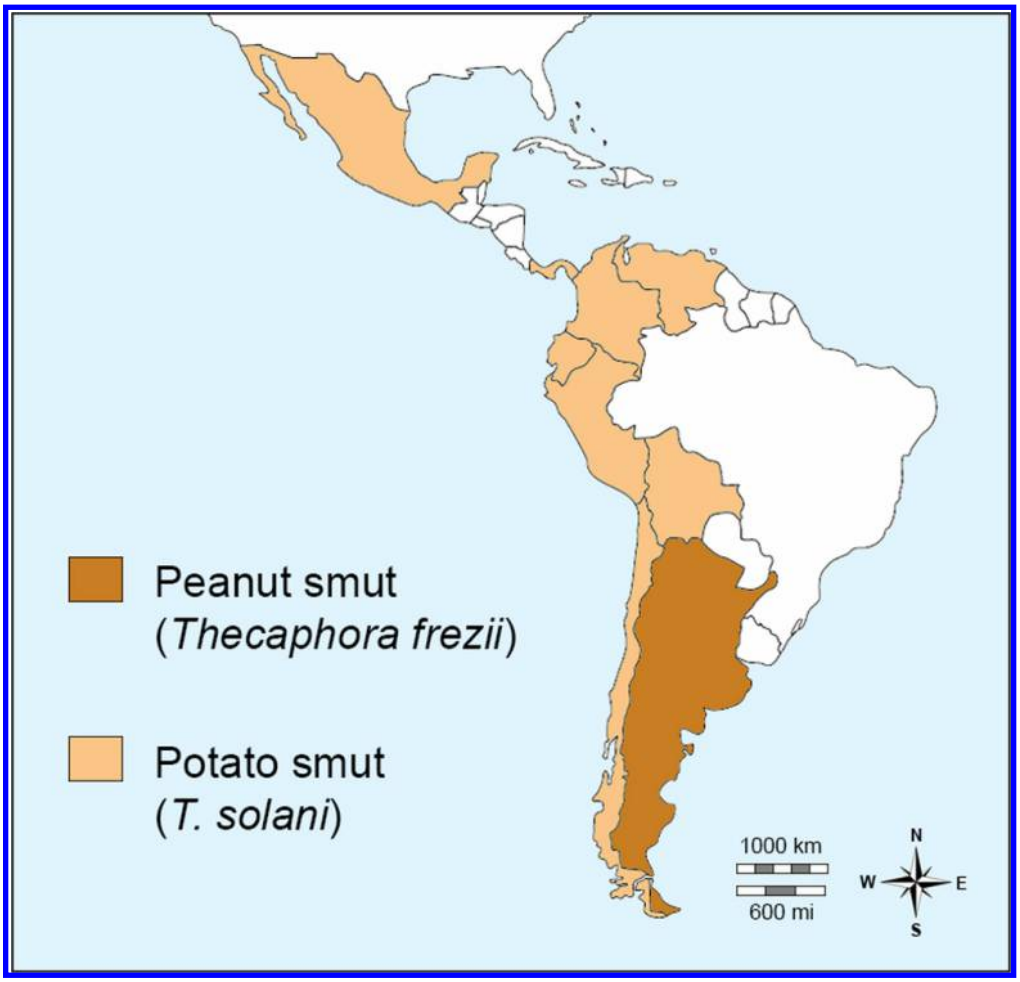

Figure 2: Geographical distribution of Thecaphora species responsible for peanut and potato smuts. While peanut smut was reported only in commercial species in Argentina, T. solani was found in Bolivia, Chile, Colombia, Ecuador, Mexico, Panama, Peru and Venezuela.

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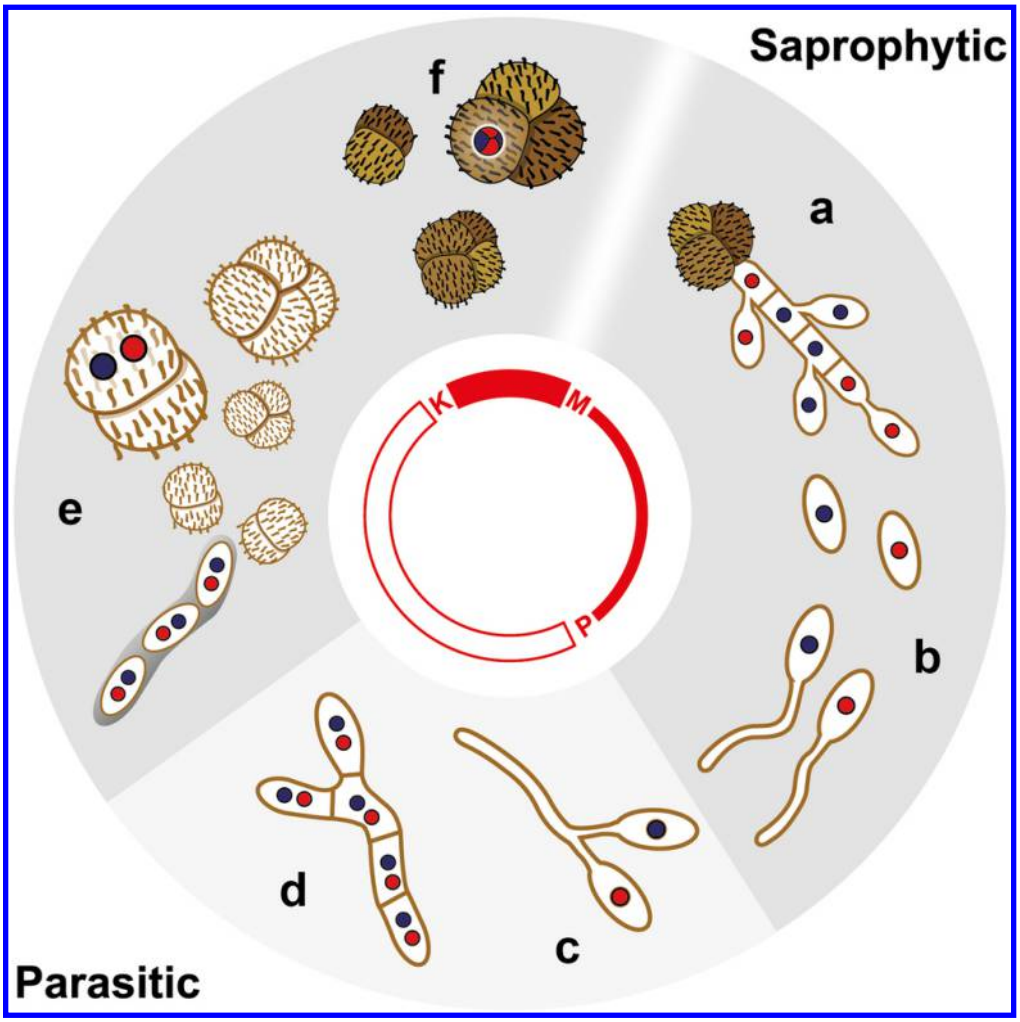

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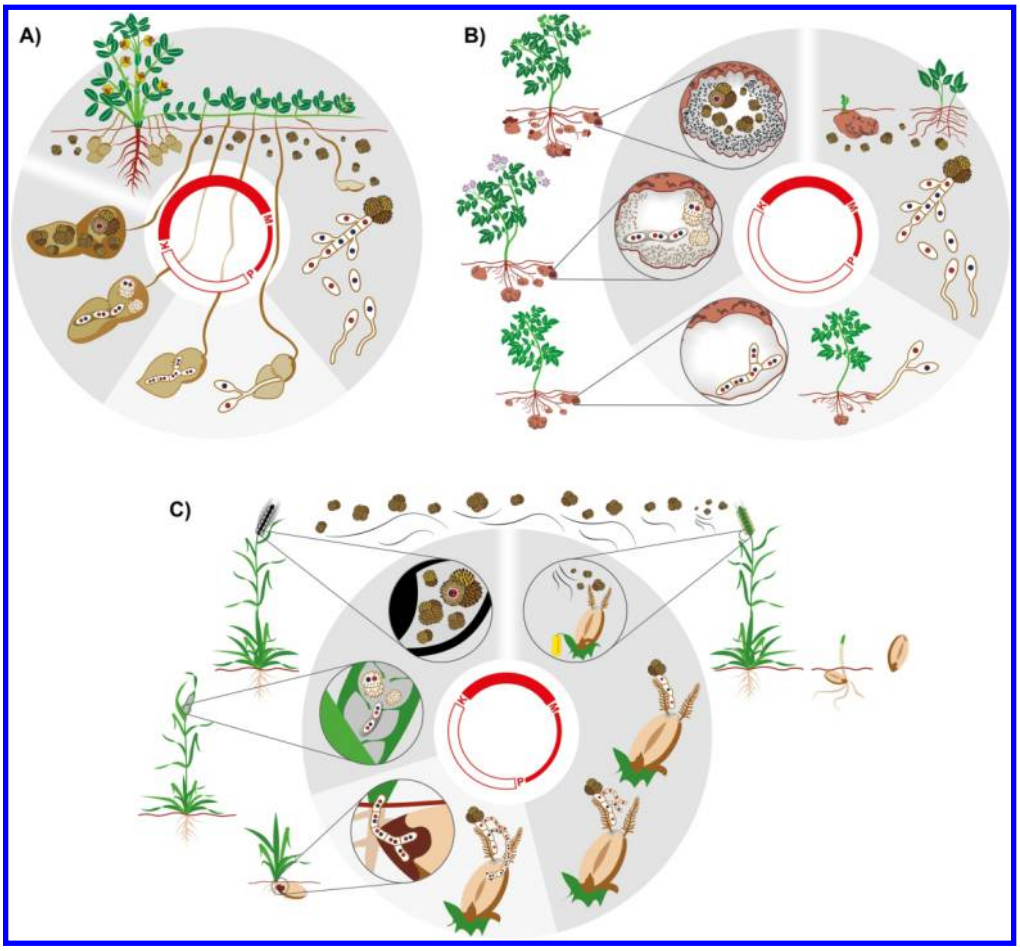

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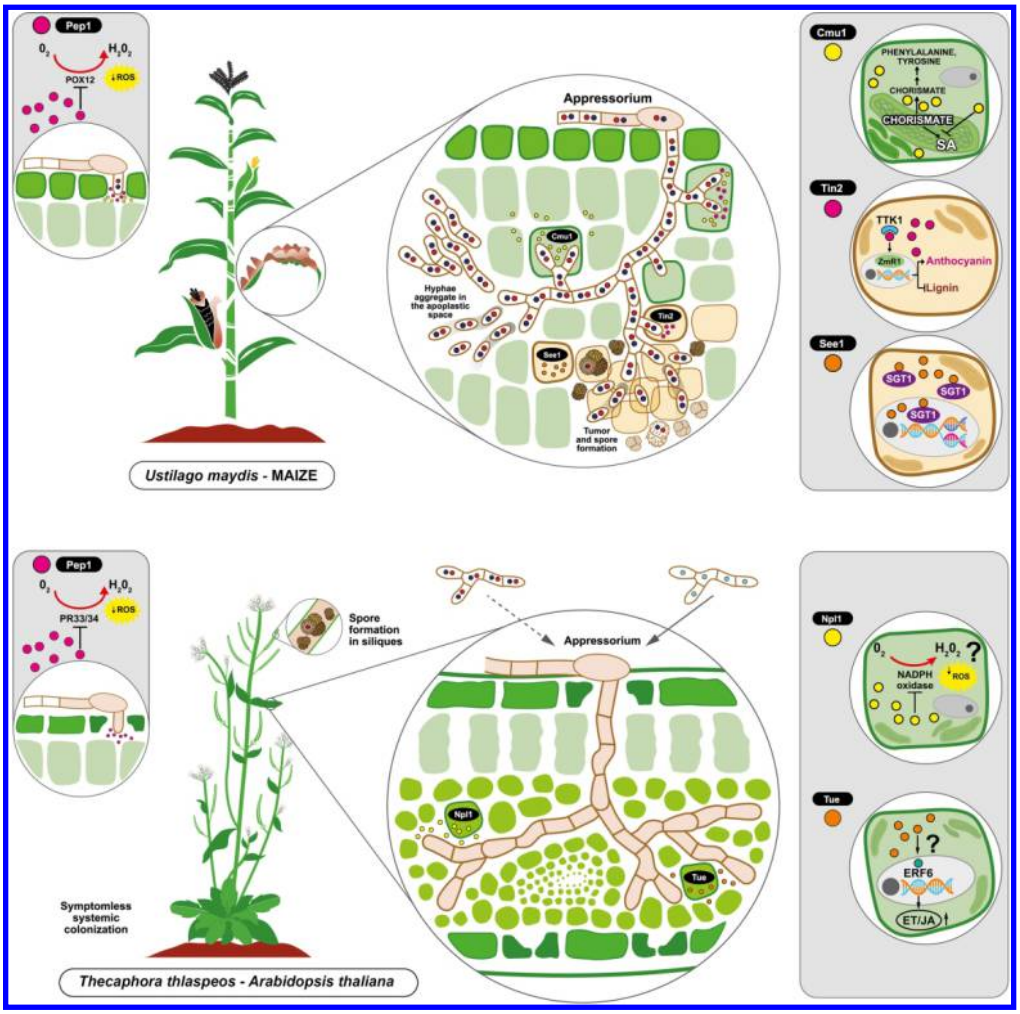

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Lower: T. thlaspeos manipulation of the plant defense by Pep1, likely localized in the apoplast, and two likely cytoplasmic Nlp1 (Necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins) and Tue1 (Thecaphora unique effector 1) effectors.
Note that germinated teliospores of T. thlaspeos can directly infect the host plant. The development of T. thlapeos' dikaryotic hyphae (dashed lines) may be possible. Plant signals are required by T. thlaspeos in order to germinate. T. thlaspeos grows intercellularly. Later, the infection is systematic and symptomless, like endophytes, until it sporulates in the siliques replacing or covering the seeds.

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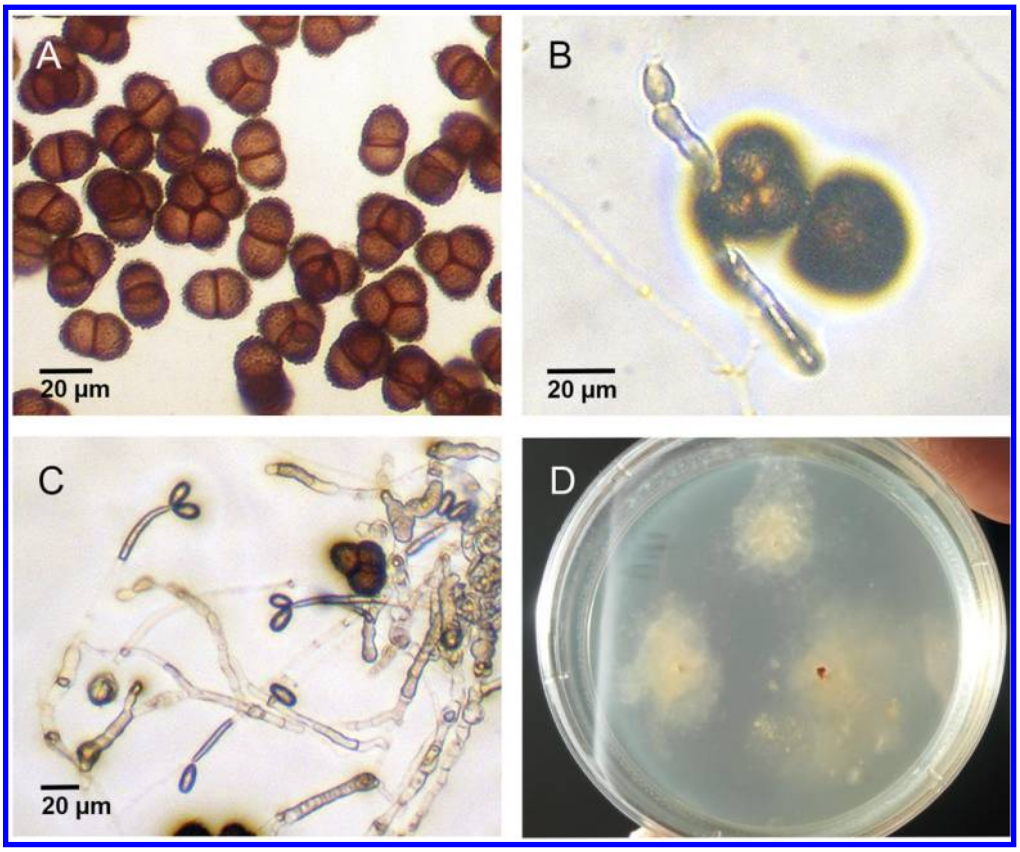

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