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Stenocarpella maydis and diplodiosis: The last major mycotoxicosis caused by an unknown mycotoxin

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Stenocarpella maydis (Berk.) Sutton (=*Diplodia maydis*) is a necrotrophic fungus, member of the Diaporthales family (Ascomycetes, Sordariomycetes), that causes diplodia ear rot, an economically important disease of maize (Burrill and Barrett, 1909). Other fungal pathogens, namely *Aspergillus flavus*, *Fusarium graminearum*, and *Trichoderma viride*, also cause ear rot of maize, but *S. maydis* distinguishes from them in symptomology. *Stenocarpella maydis* typically starts infecting corn ears at their base, then infection progresses toward the tips, resulting in ears completely rotted with visible greyish mycelia on and between kernels (Fig 1A and Fig 1B) (Clayton, 1927). Heavily infected ears become mummified and have a dramatic reduction in mass, as *S. maydis* feeds on the kernels. This unique symptomology led the disease to be initially known as 'dry rot'. Diplodia ear rot predominates among major ear rot diseases in geographically diverse areas where maize is grown, including the Americas (MacDonald and Chapman, 1997; Mario et al., 2017; Rossouw et al., 2009), Africa (Mukanga et al., 2010), and Asia (MacDonald and Chapman, 1997). In particular, during the period from 2012 to 2015, maize yield losses caused by diplodia ear rot were estimated to exceed 167,000,000 bushels in the U.S. and Ontario, Canada (Mueller et al., 2016).

A distinguished feature of *S. maydis* is the production of heavily melanized pycnidia on kernels and husks (Fig 1C). Spores are two-celled, divided by a septum, and typically germinate in a bipolar manner (Fig 1D) (Murphy et al., 1976). Spore wall and plasma membrane are composed of two electron-dense layers separated by an electron-transparent layer. Growing hyphae average 1.5-2.0 μm in diameter, and can be divided into apical, subapical, and vacuolated zones (Fig 1E) (Murphy et al., 1980). As in other fungi, apical zone is abundant in vesicles, but predominantly void of other common cytoplasmatic components, such as nuclei, mitochondria, and ribosomes, which are found in more abundance in the subapical zone. Small vacuoles coalesce to form larger ones, characterizing the beginning of the vacuolated zone (Murphy et al., 1980).

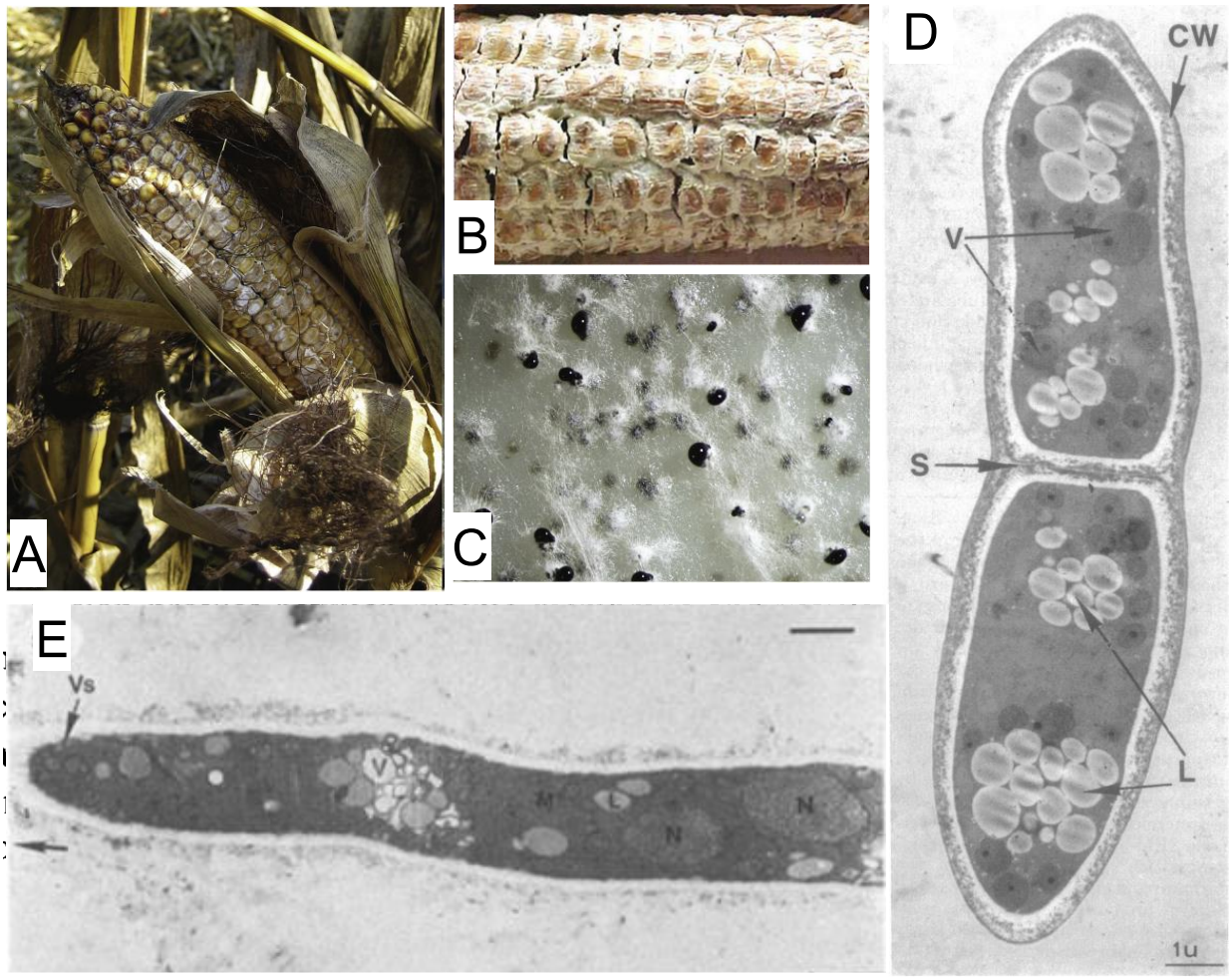

Fig 1: Diplodia ear rot and morphological characteristics of *S. maydis*. (A and B) infected corn ears with Stenocarpella maydis. Abundant fungal growth is visible on and between kernels, with symptoms of mummification. (C) heavily melanized *S. maydis* pycnidia on an infected corn ear. (D) Transmission electron microscope image of a typical *S. maydis* two-celled spore. (E) Growing hyphal tip of *S. maydis*. Scale line = 1 micrometer. CW: cell wall, V: vacuoles, S: septum, Vs: vesicle, L: lipid, M: mitochondria, N: nucleus. Panels A and C from (Zaccaron et al., 2017), panel B from (Riet-Correa et al., 2013), panel D from (Murphy et al., 1976), and panel E from (Murphy et al., 1980).

Stenocarpella maydis is one of the major pathogens that cause ear rot of maize and is associated with substantial yield losses worldwide. But *S. maydis* is also known to be the causative agent of diplodiosis, a neuromycotoxicosis of livestock that feed on maize infected with this pathogen (Kellerman et al., 2005; Riet-Correa et al., 2013). Although *S. maydis* is commonly found wherever maize is grown, diplodiosis has been of particular concern in South Africa, where it is predominantly reported. However, sporadic cases have also been reported in Australia (Darvall, 1964) and South America (Riet-Correa et al., 2013). In field conditions, symptoms of diplodiosis in

animals become visible from one to two weeks after exposure (Kellerman et al., 2005), when affected animals typically have their back arched, and show abnormal salivation and lacrimation. At later stages, affected animals display symptoms of muscular weakness, ataxia, and eventually complete paralysis (Kellerman et al., 2005). Although complete paralysis can subsequently lead to death, complete recovery is often achieved by preventing exposure of the animals to *S. maydis*, combined with supportive treatment (Kellerman et al., 2005). Diplodiosis can severely affect adult animals, and it also poses high risks to the offspring of affected dams. This was demonstrated in a study that induced diplodiosis in ewes by directly feeding the animals with pure *S. maydis* cultures (Kellerman et al., 1991). The study reported high levels of stillbirth or neonatal losses of 66% and 87% from ewes exposed to *S. maydis* in the second and third trimester of pregnancy, respectively. This same study reported visible lesions in the central nervous system of all lambs affected with diplodiosis. These lesions corresponded to spongy degeneration of the white matter of the brain (Fig 2), resulted from expansion of extracellular spaces (Kellerman et al., 1991).

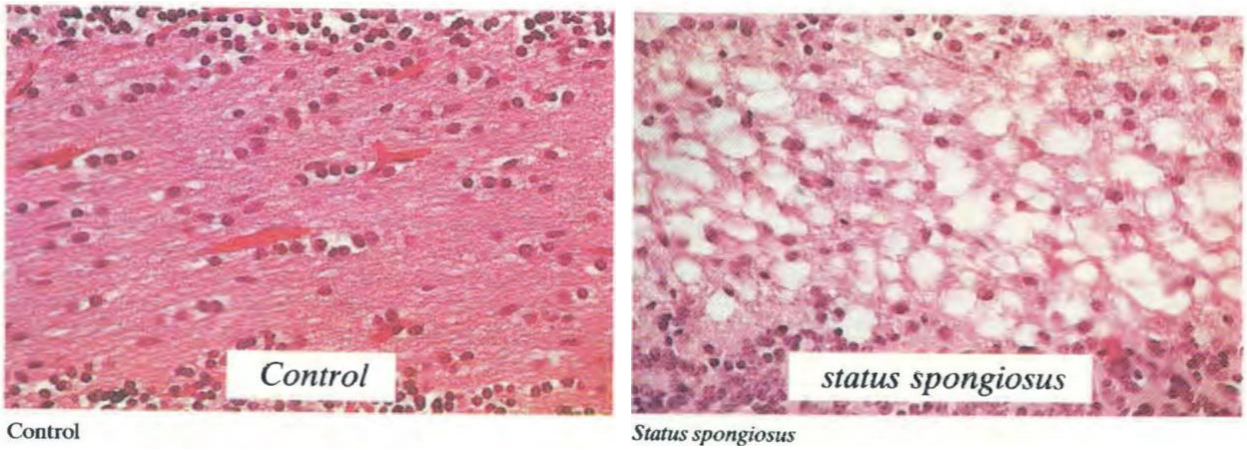

Fig 2: Spongy degeneration (*status spongiousus*) of the cerebellar white matter of lamb affected by diplodiosis. Figure adapted from (Kellerman et al., 1991).

The first reports of diplodiosis dates back to 1914 in South Africa (van der Bijl and Pole-Evans, 1914). Around the same time, the constant reports by farmers of harmful results after cattle have consumed maize in moldy conditions, led to the first experiment that successfully reproduced diplodiosis by feeding cattle with maize naturally infected with *S. maydis* (Mitchell, 1918). For over 100 years there is a clear consensus that diplodiosis is caused by *S. maydis*. However, to this day the specific mycotoxin that induces diplodiosis is currently unknown. Several metabolites have already been isolated from *S. maydis*, including diplodiatoxin (Steyn et al., 1972),

diplonine (Snyman et al., 2011), diplosporin (Wicklow et al., 2011), dipmatol (Ackerman et al., 1995), and chaetoglobosins K, L, M and O (Rogers et al., 2014; Wicklow et al., 2011). But none of these metabolites have conclusively been responsible to cause diplodiosis. In part, this is due to a lack of studies aiming to induce the disease by administering these purified metabolites to cattle and sheep. As a result, diplodiosis is considered the only major mycotoxicosis for which the responsible metabolite(s) is still elusive (Marasas et al., 2012).

Among the metabolites produced by *S. maydis*, diplodiatoxin has been shown to induce liver degeneration in chickens at doses of 265 mg/kg body wight (BW) (Louw, 1969). Subsequent studies treated rats with 5.7 mg/kg BW oral or 0.27 mg/kg BW sub-acute doses of diplodiatoxin (Rahman et al., 2002). Treated rats had a considerable reduction in body weight, and showed symptoms that included tremors, dullness, and convulsions. More recent studies showed cytotoxicity effects of diplodiatoxin and dipmatol in cell cultures of mouse neuroblastoma, Chinese hamster ovary, and Madin–Darby bovine kidney (Masango et al., 2015). Exposure to 750 µM of diplodiatoxin induced necrotic lesions in all three cell lines, and triggered caspase-dependent apoptosis within 72 hours of exposure (Fig 3). Diplodiatoxin also caused mitochondrial damage and affected profoundly cell morphology, as cytoplasmatic vacuolation and nuclear fragmentation were observed (Fig 3) (Masango et al., 2015). Considering the cytotoxic effects of diplodiatoxin, it is reasonable to consider this metabolite as a candidate responsible for diplodiosis.

Similar to other mycotoxins, diplodiatoxin is likely produced by a secondary metabolite gene cluster. However, such gene cluster in the genome of *S. maydis* has not yet been identified. Interestingly, the molecular structure of diplodiatoxin is similar to that of betaenone B (Fig 4C), produced by *Phoma betae* (Ichihara et al., 1983), a fungal pathogen that causes leaf spot and root rot diseases in sugar beet. The gene cluster for betaenone B biosynthesis (*bet*) contains eight genes (*bet1-4*, *ORF1-4*), one of which (*bet1*) encodes a highly-reducing polyketide synthase (HR-PKS) as the key enzyme for betaenone B biosynthesis (Ugai et al., 2015). The similarity of the molecular structure of Diplodiatoxin and betaenone B raises the possibility that they are produced by homologous gene clusters. Indeed, a recent study (Zaccaron et al., 2017) identified a gene cluster homologous to the *bet* cluster in the genome of *S. maydis* (Fig 4A), and pointed out that this gene cluster is likely involved in diplodiatoxin biosynthesis. This candidate gene cluster for diplodiatoxin biosynthesis contains eight genes, one of which (Sm_02669; homolog of *bet1*) encodes a HR-PKS

that, similar to *bet1*, is the backbone of the polyketide chain. This gene contains a domain architecture identical to that of *bet1* (Fig 4B), composed of a ketoacyl synthase (KS), an acyl transferase (AT), a PKS dehydratase (DH), a methyltransferase (MT), a keto reductase (KR), an acyl carrier protein (ACP), and a NAD-binding terminal domain (TD) commonly found in fatty acyl-coenzyme A reductases in higher Eukaryotes, but atypical in fungal PKSs. The seven genes around the *S. maydis* HR-PKS homologous to *bet1* encode putative tailoring enzymes that might participate in the biosynthesis and transport of diplodiatoxin outside the cell. These genes encode cytochrome P450s (Sm_02668 and Sm_02675), a major facilitator superfamily transporter (Sm_02670), an aldo/keto reductase (Sm_02671), a short-chain dehydrogenase (Sm_02672; homolog of *bet4*), an FAD-binding protein (Sm_02673; homolog of *ORF1*), and an enoyl-reductase (ER; Sm_02674; homolog of *bet3*). ER domains are typically found within HR-PKSs, however, this is not always the case, as ER-encoding genes can contribute to the construction of the polyketide chain in *trans*. This *trans*-ER behavior has been reported for the biosynthesis of betaenone B in *P. betae* (Ugai et al., 2015), and therefore it also likely takes place during diplodiatoxin biosynthesis. Finally, similar to the *bet* cluster, no gene encoding a transcription factor was present within the putative diplodiatoxin cluster, indicating that its biosynthesis is likely regulated by a global transcription factor located elsewhere in the genome.

To test the hypothesis that diplodiatoxin induces diplodiosis, a recent study administered purified diplodiatoxin in three juvenile goats (Botha et al., 2020). However, at doses of 2 and 4 mg/kg BW intravenously for five days, and 2 mg/kg BW orally for three days, no symptoms of diplodiosis were observed. These results suggest that diplodiatoxin alone is unlikely to induce diplodiosis, and a different *S. maydis* mycotoxin, or a combination of them, could be responsible for this disease. Indeed, a different study (Snyman et al., 2011) suggested that diplonine is the mycotoxin that induces diplodiosis. This study reported diplodiosis symptoms in guinea pigs upon dosing methanol extracts from *S. maydis* cultures and purified diplonine. However, doses of methanol extracts equivalent to 200 g culture/kg BW, and 1.5 g/kg BW of pure diplonine were administered for the symptoms to appear. Such high doses are physiologically incompatible with livestock, as the equivalent amount of cob and stover that cattle and sheep have to ingest to achieve such high concentrations of diplonine far exceeds typical livestock food intake in field conditions. Although it has not been experimentally tested, a counter argument is that guinea pigs could be less

sensitive to diplonine than cattle and sheep. Taken together, these studies show inconclusive results for the *S. maydis* mycotoxin responsible for diplodiosis in livestock.

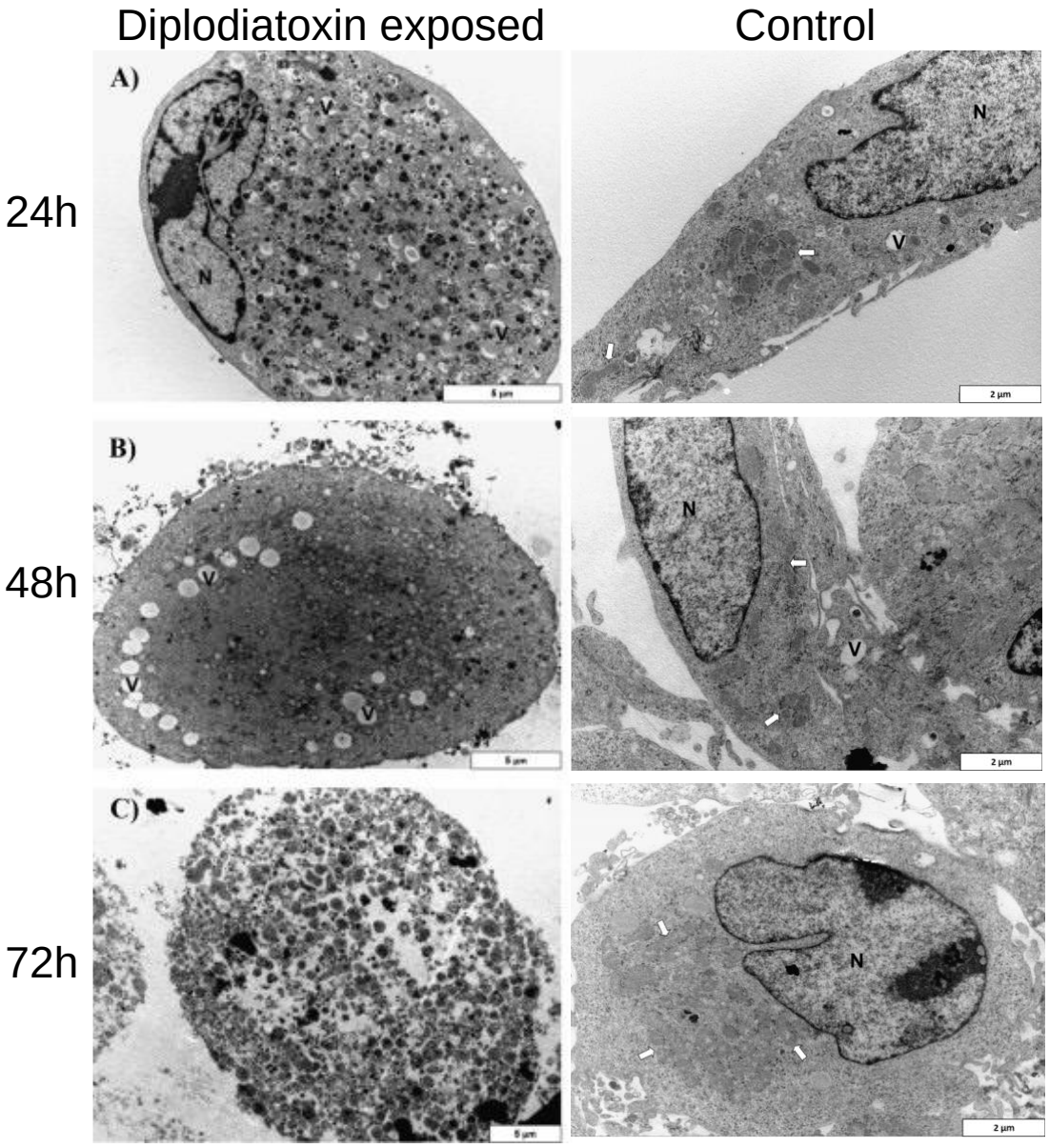

Fig 3: Electron micrographs showing mouse neuroblastoma (Neuro-2a) cells exposed to 750 µM of diplodiatoxin for 24, 48, and 72 hours (A, B, and C, respectively). Control Neuro-2a cells exposed to media are shown on the right-hand side. N = nucleus; V = vacuoles; white arrows = mitochondria. At 24h of exposure to diplodiatoxin, nuclear and plasma membranes are intact, while mitochondria are not visible. At 48h of exposure, mitochondria and nuclei were absent, and numerous electron-lucent vacuoles were present. At 72h of exposure, disruption of the cell and loss of plasma membrane were observed. Figure adapted from (Masango et al., 2015).

At the same time that the search for the mycotoxin responsible for diplodiosis continues, numerous other metabolites produced by *S. maydis* remain to be discovered. This was highlighted in the first genomic analysis of the *S. maydis* genome, which revealed that this pathogen harbors one of the largest arsenal of genes involved in secondary metabolism among fungi (Zaccaron et al., 2017). It contained a total of 87 genes encoding key enzymes for secondary metabolite biosynthesis, classified into 49 PKSs, 5 PKSs-like, 8 nonribosomal peptide synthetases (NRPSs), 12 NRPSs-like, 3 PKS-NRPS hybrids, 4 dimethylallyl tryptophan synthases (DMATs), and 8 terpene synthases (TS). In comparison, among 1,986 fungal genomes currently available (04/2021) at JGI MycoCosm (Grigoriev et al., 2014), only three had more PKS-encoding genes than *S. maydis* (i.e., *Lophodermium piceae* with 51 PKSs, *Nemania abortiva* with 56 PKSs, and Diaporthaceae sp. PMI_573 with 62 PKSs). These conspicuous numbers show that *S. maydis* stands out among other pathogens, for its capability to produce an unusual large number of secondary metabolites, particularly PKS-derived mycotoxins.

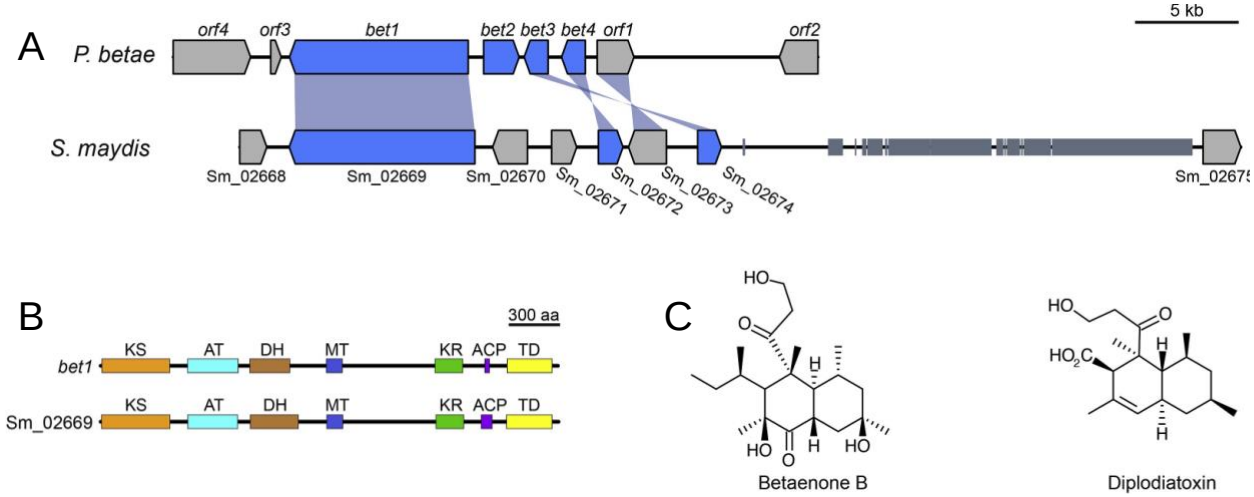

Fig 4: Putative *Stenocarpella maydis* secondary metabolite gene cluster involved in diplodiatoxin biosynthesis. (A) Secondary metabolite gene cluster from *Phoma betae* involved in betaenone B biosynthesis and a homologous gene cluster in *Stenocarpella maydis*. (B) Domain architecture of the highly reducing polyketide synthase *bet1* involved in betaenone B biosynthesis and its homolog in *S. maydis*. Small dark grey boxes indicate repetitive DNA regions in *S. maydis* gene cluster. (C) Molecular structure of betaenone B (Ichihara et al., 1983) and diplodiatoxin (Steyn et al., 1972). KS: ketoacyl synthase; AT: acyl transferase; DH: PKS dehydratase; MT: methyltransferase; KR: keto reductase; ACP: acyl carrier protein; TD: terminal domain. Figure was adapted from (Zaccaron et al., 2017).

It is surprising that so little is known about the metabolites and activity of enzymes encoded by *S. maydis* genes, particularly considering that it stands together with the much better studied fungi *A. flavus* and *F. graminearum* as major ear rot pathogens of maize. The unusually large number of *S. maydis* genes involved in secondary metabolite biosynthesis is a clear indicative of its potential to produce an extraordinary number of mycotoxins. Yet, only a few have been discovered thus far, and even fewer had their cytotoxicity evaluated. In particular, the mycotoxin(s) that causes diplodiosis, considered a major mycotoxicosis in South Africa, remains elusive. Perhaps future studies will raise *S. maydis* from the status of an understudied pathogen, and shed light into the mechanisms of biosynthesis and action of its metabolites.

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