Rôle of fungal ascospores in the infection of orchardgrass (Dactylis glomerata) by Epichloe typhina agent of choke disease

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ROLE OF FUNGAL ASCOSPORES IN THE INFECTION OF ORCHARDGRASS (*DACTYLIS GLOMERATA*) BY *EPICHLOË TYPHINA* AGENT OF CHOKE DISEASE

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SUMMARY

Choke disease due to *Epichloë typhina* causes economic losses in fields of seed- producing orchardgrass, also known as cocksfoot (*Dactylis glomerata*). There are currently no means to prevent outbreaks and spread of the disease. In this study, we identified several key events of the biological cycle of the fungus, in particular how it enters plant tissues. Study of seeds of partially choked heads ruled out the vertical transmission of *E. typhina* in *D. glomerata*. The fungal mycelium that had colonised the seeds appeared not to be viable: it was not isolated from 360 seeds analysed that had 49% infection rate. Moreover, 280 seedlings and 488 young plants grown from these seeds were free from *E. typhina*. Ascospores, considered as propagules in horizontal transmission, were previously presumed to infect orchardgrass through cut stems at harvest or through stigmas of florets. *E. typhina* was not detected (by microscopic or histological analyses) in 332 cut stems that had been inoculated with ascospores. Our research indicates that ascospores (or conidia produced by ascospores) are more likely to infect young vegetative tillers. We assume that after ejection, ascospores fall on seed lemma and palea and enter seedling tissues after germination.

Key words: Seed production, forage grasses, disease spread, primary inoculum, orchardgrass, cocksfoot, *Epichloe typhina.*

INTRODUCTION

Epichloë spp. (Ascomycota, Clavicipitaceae) are fungi widespread in temperate regions. They infect numerous grass species in the subfamily *Pooideae* (White, 1987; Leyronas and Raynal, 2001). *Epichloë* spp. produce mycelial stromata that develop on emerging inflorescences, preventing seed production. The disease is commonly known as choke (Kohlmeyer and Kohlmeyer, 1974).

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In orchardgrass (*Dactylis glomerata*), an important forage grass, *E. typhina* (Pers.) Tul. et Tul. can cause significant seed yield loss. Choke disease can appear in the first year of production and increase from one year to another, often reaching 30% choked tillers in the fourth year of production (Raynal, 1991a). In France, orchardgrass fields with choke disease can loose profitability after one or two years. In Oregon, where nearly all the certified orchardgrass seed of USA is produced, losses from choke were estimated at $ 820,000 in 2004 (Pfender and Alderman, 2006).

Currently, there are no efficient means to prevent choke disease outbreaks and spread in the field. Fungicides and prophylactic trials have proven unsatisfactory (Fermaud, 1986; Pfender and Alderman, 2003). Thus, there is a need to know more about the life cycle of the causal agent, *E. typhina*, in order to develop new ways of control.

When *E. typhina* infects a plant, it remains infected for the rest of its life. *E. typhina* is closely related to strictly endophytic and seed-transmitted symbionts belonging to the genus *Neotyphodium* (formerly *Acremonium* section *albo-lanosa*; Glenn *et al*., 1996), well known for alkaloid mycotoxin production in grasses (Siegel *et al.*, 1987). Some of these alkaloids are toxic for cattle fed with ryegrass (*Lolium perenne*) or tall fescue (*Festuca arundinacea*) containing endophytes. Ryegrass staggers and fescue toxicosis caused by some endophyte alkaloids are responsible for economic losses in the USA and New Zealand (Fribourg *et al*., 1991; Raynal 1991a, 1991b). Nevertheless, strictly endophytic *Neotyphodium* can enhance plant resistance to biotic and abiotic stresses. Phylogenetic studies have shown that many of these non-choke-inducing symbiotic fungi are hybrids of *Epichloë* species which have lost the ability for sexual reproduction (Schardl *et al*., 1994). Strictly choke-inducing *Epichloë* are different from symbiotic *Neotyphodium* in two important ways: they do not produce mycotoxins, or at very low levels (Leuchtmann *et al.*, 2000), and they do not seem to be vertically transmitted (Western and Cavett, 1959) through clonal growth of mycelium into host ovules and seeds.

In flowering tillers, the endophytic mycelium of *E. typhina* grows and proliferates within and among inflores-

cence tissues, binding the growing apex and leaf sheath in a stroma. Typically, seeds do not develop. In rare cases, inflorescences can partially escape from the choke and a few seeds are produced (Kirby, 1961).

At first, stromata choking inflorescences are white (*Neotyphodium typhinum* Morgan-Jones and Gams, ex *Sphacelia typhina* Sacc.) and produce small reniform conidiospores. The spores function as spermatia and are transported from stroma to stroma by dipteran Anthomyiidae of the genus *Botanophila*, feeding and ovipositing on stromata (Bultman *et al.*, 1995; Schardl *et al.*, 2004). *Epichloë* species are heterothallic: when spermatia are deposited on a stroma from an opposite mating type, sexual reproduction begins. Orange perithecia develop in the thickened mycelium of fertile stromas; ascospores (about 100 µm long and 2 µm wide) are ejected from mid-June to mid-July, during nocturnal peaks of humidity (Raynal 1991a). Ascospores, by iterative germination, produce a new generation of spores, morphologically identical to spermatia (Bacon and Hinton, 1988), but lacking the same germination ability: spermatia give rise to two germ tubes, while conidia from ascospores give rise to a germ tube and a typical conidiophore forming new conidia (Leyronas, 2005).

The infection phase of *E. typhina* life cycle is not well understood. There are basically two hypotheses for which there is experimental evidence, based on horizontal transmission, i.e. ascospores are believed to be propagules for contagious spread. A proposal is that infection occurs through vegetative plant tissues and that fungal hyphae reach the meristems. Western and Cavett (1959) reported that ascospores of *E. typhina* germinate and grow down the cut end of stems (stubble) when orchardgrass is harvested in July, allowing proliferation of mycelium into the cut stem. More recently, Brem and Leuchtmann (1999) showed that stem or leaf tissues provide a route of entry for *E. sylvatica* in plants of *Brachypodium sylvaticum*. Healthy plants became infected after one or two years transplanting near a choked population. The other hypothesis supposes that infection occurs through stigmas of florets via ascospores or conidia produced by ascospores. Chung and Schardl (1997) were able to produce infected plants by placing fertile, sporulating choke of ryegrass around healthy ryegrass inflorescences. One percent of the plants obtained from seeds originating from these inflorescences were infected by *E. typhina*. This suggests that *E. typhina* may also infect seed of orchardgrass via ascospores infecting inflorescences. Moreover, phenological observations made on four natural populations of *B. sylvaticum* showed the temporal coincidence of host flowering and availability of ascospores (Brem and Leuchtmann, 1999).

The objectives of our study were: (i) to determine whether seeds are a source of *E. typhina*-infected plants, which could explain how choke disease can appear in the first production year in orchardgrass fields far from other diseased fields, and (ii) to elucidate the mechanism by which the proportion of choked tillers increases from year to year by discovering how healthy adult plants become infected.

MATERIALS AND METHODS

Study of seeds as primary sources of inoculum

Seeds from partially choked inflorescences. Seeds of partially choked inflorescences were collected in orchardgrass fields of cv. Lupré (INRA, France) just before harvest in 2002, 2003 and 2004. Seed germination, mycelium transmission from seeds to young plants and endophytic mycelium viability were checked.

Germination of seeds from healthy seed heads and partially choked inflorescences from the 2004 harvest was evaluated by a standard germination test following ISTA (International Seed Testing Association) methods (ISTA, 2003). For each source of seeds (healthy or choked inflorescence), 20 replicates of 20 seeds were placed on moist paper in the dark at 5°C. After incubation for one week, seeds were transferred to 20°C for 14 days to complete germination. Counts of seedlings were made 7 and 14 days after placing seeds at 20°C.

The technique of observing *E. typhina* in seeds was the same as that used to detect seed-transmitted *Neotyphodium* (Clark *et al.*, 1983), i.e. seeds were soaked for 48 h in a 3% sodium hydroxide solution, rinsed with tap water, then stained with aniline blue (1g in 600 ml of lactic acid). Before mounting on a microscope slide, each seed was dissected to remove starch, which could obscure observation of fungal hyphae. *E. typhina* mycelium looked like that of the strictly seed-transmitted *Neotyphodium*; it was unbranched, convoluted and located between aleurone cells (Siegel e*t al.*, 1987).

To evaluate if *E. typhina* mycelium in seeds gave rise to infected plants, seeds of partially choked inflorescences were sown in 2002, 2003 and 2004 in a mixed substrate (2/5 topsoil + 2/5 clay soil + 1/5 sand), placed in a greenhouse and grown at about 20°C, with a 12 h photoperiod. Four hundred and eighty eight 2 to 6 month-old plants were analysed by microscopy and by isolation of the fungus on potato dextrose agar (PDA) plus 100 ppm streptomycin to check for the presence of *E. typhina*. Moreover, in 2004, one hundred and ninety 14-day-old and ninety 21-day-old seedlings were analysed in the same way.

Viability of mycelium in seeds was evaluated in 360 seeds collected in 2004. The seeds were soaked in sterile water for 24 h. Lemma, palea and embryos were removed before disinfection with 10% commercial bleach (0.38% sodium hypochlorite) for 1 min. Seeds were plated on PDA plus streptomycin and incubated at 20°C for 10 days. Removing the embryos forced the mycelium to grow on the medium (Naffaa, 1998).

*Artificial infection of florets and seeds***.** The cut end of orchardgrass stems bearing choked inflorescences were placed in tubes containing water. Chokes became turgescent and ejected numerous ascospores of *E. typhina*. These sporulating stromata were put 10 cm away from inflorescences (at anthesis stage) of healthy orchardgrass cv. Greenly (RAGT, France) grown in pots. Stromata and healthy inflorescences were maintained in a moist atmosphere in plastic bags for 3 days at 20°C. Florets received numerous ascospores. Plants were then kept outside for seed maturation, at the end of June. When mature, seeds were sown and plants were analysed 12 to 24 weeks later.

In a separate experiment, disinfected mature seeds with and without palea and lemma were placed under sporulating fertile stroma of *E. typhina* for 2 h. Treated seeds were placed in a watch glass at 20°C in sealed Petri dishes containing wet blotting paper. Seeds had no direct contact with water. Seedlings were planted in the above mentioned mixed substrate and grown in a greenhouse. They were analysed by histological and microscopic observations when 12 to 16-week-old.

Mechanism of disease spread.

Healthy and choked plants. All the healthy and choked orchardgrass plants used for artificial infection were of cv. Greenly. Diseased plants were collected each year in fields grown for seed production and planted outdoor near the laboratory. Choked tillers with mature stromata were collected in the morning to obtain abundant fresh and viable ascospores for experiments on the same day.

Ascospore inoculum. The cut ends of choked orchardgrass stems were immersed in water. Ten minutes later, the mature orange stromata started ejecting ascospores. The sporulating stromata were placed above sterile water or PDA medium (Potato Dextrose Agar) to collect ejected ascospores. Spores in water were counted with a Malassez-cell and suspensions were adjusted to the desired concentration.

Artificial inoculation of cut flower stems. At the end of June, flower stems of potted orchardgrass plants were cut about 20 cm above ground in order to simulate harvest cut. A few centimetres of the stem were left above the last node. Ascospore suspensions were applied in two ways to wounded stems: 100 ml suspension of 10^6 spores ml^{-1} was either sprayed on 100 stems with a miniaturized sprayer (Ecospray, France), or a few droplets of a suspension containing 2.5×10^5 spores per ml were placed directly on the cut ends of 140 stems with a hypodermic needle. In a third method, ascospores were collected on PDA medium and plugs of the substrate were cut with a cork borer. Agar plugs bearing several hundred ascospores per mm^2 were applied directly on the cut ends of 92 stems and removed 7 days later. All the inoculated plants were placed in plastic bags, in a humid atmosphere (90% relative humidity), with a 12 h photoperiod at 20°C.

Artificial inoculation of vegetative parts and meristems. Non-choked flowering orchardgrass plants were removed from pots, and vegetative tillers were separated from each other. For each tiller, leaves were cut down to vegetative tiller bud and roots were kept intact. Tiller buds were then placed beneath turgescent choked stems for 8 h. Roots were protected from ejected ascospores by a piece of plastic placed over them. After exposure, the treated tillers were put in a moist atmosphere for one week. Then they were potted and maintained in a growth chamber with a 12 h photoperiod at 20°C. New tillers were analysed for infection 10 weeks later by plating tissues on PDA medium.

In a separate experiment vegetative tillers were cut down to 1 cm above the ground. One or two cm of leaf sheath remained above tiller buds. Droplets of ascospore suspensions (1.2×10^5 ascospores ml^{-1}) were deposited in the exposed centre of the tillers. Plants were maintained in a growth chamber with a 12 h photoperiod at 20°C and examined microscopically 3 to 24 weeks later.

Histological and microscopic observations. Microscopic detection of *E. typhina* in plants was done by removing an internal piece of leaf sheath tissue or stem pith, placing it on a microscope slide and staining with aniline blue. Slides were heated for a few seconds to accelerate staining. *E. typhina* mycelium appeared straight and unbranched, running between plant cells, sometimes with a few convolutions.

Fungal isolation was also used to check for artificial infection. Pieces of plant tissue were placed for 1 min in 70% alcohol, 2 min in 5% bleach (0.15% sodium hypochlorite), then rinsed three times in sterile water. Pieces were plated on PDA plus 100 ppm streptomycin and incubated at about 24°C for 10 days. If *E. typhina* was present in plant tissues, white slow-growing colonies developed. They were examined at 200x magnification to detect the typical reniform conidia.

Statistical analysis. Statistical analyses were performed with StatView (version 5, SAS Institute). Statistical inferences were made at the 5% level of significance, unless otherwise indicated.

RESULTS

Seeds of partially choked inflorescences. Microscopic analyses showed that 78% in 2002 to 42% in 2003 of the seeds from partially choked heads were infected by the endophytic mycelium of *E. typhina* (Table 1). Seed colonisation was not as homogeneous as in seeds infect-

Table 1. Presence of *E. typhina* mycelium in seeds, seedlings and plants from partially choked inflorescences harvested in 2002, 2003 and 2004.

	Seeds		Seedlings		Plants	
Harvest year	Total analysed	Infected by *E. typhina* (%)	Total analysed	Infected by *E. typhina* (%)	Total analysed	Infected by *E. typhina* (%)
2002	106	78	/	/	130	0
2003	135	42	/	/	108	0
2004	200	49	280	0	250	0

ed by a strictly endophytic fungus like *N. lolii* in ryegrass seeds. Sometimes, *E. typhina* hyphae were not localised in the aleurone layer but only in the tegument. This heterogeneity seems to be linked to the distance between seeds and mycelial stroma. We speculate that seeds close to the stroma were more accessible to the fungus than seeds more distant from the stroma.

During the three years of study, 280 seedlings and 488 2- to 6-month-old plants were grown from seeds of partially choked inflorescences and analysed. *E. typhina* was never detected in these plants (Table 1). This result leads to two hypotheses: either seeds with the endophyte do not germinate, or endophytic mycelium was no longer viable at the time of germination in these particular seeds and consequently it was not transmitted to plants.

Germination rates of seeds from partially choked heads at 7 or 14 days were 60% and 67%, respectively and were not significantly different from those of seeds from healthy heads (Table 2). This suggests that endophyte infection does not prevent seeds from germinating, thus the first hypothesis was dismissed.

Viability tests were made on the 2004 seed sample that had 49% infection rate (Table 1). None of the 360 seeds analysed gave *E. typhina* colonies.

Artificial inoculation of seeds. None of the 240 analysed plants grown from seeds from inoculated inflorescences were infected by *E. typhina* (Table 3).

None of the 110 plants grown from inoculated seeds with palea and lemma became infected, but plants from 20 out of 80 seeds without palea and lemma (25%) became infected. This suggests that germinating ascospores or conidia that had access to the seed surface were able to enter seedling tissues.

Artificial inoculation of cut flower stems. *E. typhina* mycelium was not detected (by microcopic or histological analyses) in the 332 stems inoculated with ascospores deposited on their cut ends, regardless of the method with which the ascospores were applied (sprayed, in droplets, on agar plugs) (Table 3).

Table 2. Germination of seeds of healthy heads and partially choked heads harvested in 2004.

	Germinated seeds (%)		
Germination after 7 and 14 days at 20°C	Healthy heads (%)	Partially choked heads (%)	Statistical significance
7 days	60.9	59.9	NS (PFisher =0.8283)
14 days	70	67.6	NS (PFisher =0.4924)

Table 3. Results of inoculation of orchardgrass organs with ascospores.

Inoculation objective	Organs inoculated with ascospores	Presence of *E. typhina* after inoculation
Determination of seed infection	Seeds formed on inflorescence	0/240 plants (with flowering glumes)
	Mature seeds	0/110 plants (without flowering glumes)
		20/80 plants
Explaining mechanism of choke disease spread	Cut stems	0/332 stems
	Meristems	5/19 plants (= 9/79 tillers)
	Cut vegetative tillers	6/282 tillers

Artificial inoculation of vegetative parts and meristems. After 10 weeks, the 19 meristems inoculated with ascospores produced 79 tillers in total. Nine of these tillers (11.3%), emerging from 5 different plants, developed *E. typhina* colonies after isolation (Table 3). Thus, *E. typhina* infected an average of 26% artificially inoculated meristems. In three plants, all the tillers were infected. In the two other plants, only 1 out of 6 and 1 out of 3 tillers were infected.

In the inoculated cut vegetative tillers, a total of 282 were analysed. In the first harvest after 4 weeks, 6 out of 95 were shown to be infected by *E. typhina*. But in the second harvest after 10 to 24 weeks none of the remaining 187 tillers proved to be infected (Table 3).

DISCUSSION

Seedlings and plants grown from seeds of partially choked heads of orchardgrass were found free of *E. typhina* despite a high percentage of seeds colonised by the fungus. This result agrees with those of Chung and Schardl (1997) who did not detect *E. typhina* in 288 seedlings from seeds of partially choked heads of ryegrass infected by *E. typhina*. This differs from the almost 100% transmission of *N. lolii* and *N. coenophialum* in ryegrass and tall fescue seeds, respectively (Bacon and Siegel, 1988). In our study, all seed samples were stored at 5°C from harvest until their use in the experiments. If *E. typhina* had been viable in orchardgrass seeds and able to infect the plant embryo or growing seedling, it would have been expected to be transmitted to seedlings, at least in low proportion. Moreover, attempts to isolate *E. typhina* from seeds were not successful, suggesting that *E. typhina* was no longer viable. Symbiotic *Neotyphodium* and some other *Epichloë* spp. (e.g. *E. festucae*) can be isolated from seed (Latch *et al*., 1984; Naffaa, 1998). It is well known that viability of seed-transmitted *Neotyphodium* decreases rapidly when seeds are stored at ambient temperature (Siegel *et al*., 1984; Rolston *et al*., 1986). It is possible that choke-forming *Epichloë* that are not adapted to seed transmission may die even more rapidly. We can conclude that *E. typhina* is not vertically transmitted in *D. glomerata*, possibly due to low viability of *E. typhina* in *D. glomerata* seeds.

Artificial inoculation of inflorescences with ascospores resulted in either no infection or a level of infection below the level of detection. Indeed, Chung and Schardl (1997) found only 13 infected out of 1030 plants. Seedlings from ascospore-treated seeds whose lemma and palea had been removed became infected at a rate of 25% but none of the seedlings from seeds covered by flowering glumes. Ascospores (or conidia produced by ascospores) apparently can germinate and infect young seedlings, but palea and lemma seem to provide a physical barrier to infection. If more plants from seed with lemma and palea had been analysed, perhaps a lower percentage of infection could have been detected. Inoculation of coleoptiles of 7-day-old orchardgrass with *E. typhina* hyphae gave 27% of infected plants (Leyronas, 2005). Thus, we can assume that *E. typhina* enters seedlings in a way similar to *Tilletia* spp., the agent of wheat bunt. *Tilletia* spores present on the seed surface enter coleoptiles of seedlings just after seed germination in the soil (Raynal, 1997). To confirm this hypothesis, orchardgrass seeds should be inoculated with *E. typhina* ascospores and conidia and a much larger number of plants analysed.

Inoculations of cut stems with ascospores failed to reproduce the results of Western and Cavett (1959). These authors obtained 6 infected plants out of 28 by placing *E. typhina* ascospores on cut stem surfaces. In our experiments, 332 stems were inoculated and microscopic examinations and histological analyses were made three weeks after inoculation but *E. typhina* was never detected. However, we observed considerable growth of saprophytic fungi. *E. typhina* is a relatively slow-growing fungus and it seems improbable that it could compete with rapidly growing saprophytes and be able to colonize stem pith. Moreover, at harvest time, stems are dessicated. In order to stay in a living part of orchardgrass during winter, *E. typhina* should be able to grow in the pith of dried stubble (about 20 cm), pass through the nodes and enter the meristem. Thus, Western and Cavett (1959) finding that *E. typhina* infects stems at harvest could not be confirmed under our experimental conditions. It is more likely that ascospores infect directly orchardgrass parts that survive winter. Indeed, vegetative tillers that are at the base of flowering stems stay alive after harvest, survive, are vernalized and are likely to produce flowering stems in the following year.

Although our experimental conditions were very favourable for ascospore infection (numerous ascospores on each meristem, high humidity) and not completely representative of field conditions, our results of vegetative tiller buds and cut vegetative tillers inoculation suggest that infection of these organs via ascospores (or conidia produced by ascospores) is possible. Horizontal transmission was also shown by Brem and Leuchtmann (1999) on *Brachypodium sylvaticum* with *E. sylvatica*. This mechanism could explain the spread of choke disease in the field. Indeed, several million ascospores are ejected by each stroma during the night in the field (Raynal, 1991a), and some of them could be carried down stems by dew or rain and enter the inner leaf whorls of vegetative tillers to reach plant meristems. This could be facilitated after harvest, when vegetative tillers are shorter and wounded.

Some of our inoculation experiments were positive but the tissues were not as heavily infected as expected. We wonder why infection of *D. glomerata* with ascospores is so difficult to obtain. When placed on or-

chardgrass tissues, ascospores produce conidia (Leyronas, 2005), thus if ascospores were only dissemination propagules, then conidia would be expected to infect orchardgrass tissues. Ascospore-derived conidia are not identical to those produced by the white stroma, for they are able to produce conidiophores and new generations of conidia (Bacon and Hinton, 1991; Leyronas, 2005). However, their ability to infect plant tissues is still to be determined.

Another dispersion route of *E. typhina* could be the conidia produced by the mycelial net observed by Moy *et al.* (2000) on some leaves of endophyte-infected plants, but this sporulating mycelial net does not seem to occur in all associations between *Neotyphodium/ Epichloe* and grasses. Whether this mycelial net exists in orchardgrass and if these conidia can infect plant tissues is still to be determined.

Following Western and Cavett (1959) hypothesis, it was previously recommended to protect cut stems by a fungicide treatment. The results of our stem inoculation experiments show that control methods against *E. typhina* should be revised. Fungicides were ineffective in controlling the endophytic stages of *E. typhina* when applied on infected plants (Fermaud, 1986; Pfender and Alderman, 2003). Hence, a strategy to control *Epichloë* spread would be to target its sexual stage, i.e. ascospores on contaminated seeds, or young, or sporulating stromata. Seed treatments with a suitable chemical, yet to be identified, may prevent ascospore germination or sporulation. Application of fungicides on grasses when chokes are emerging may stop sexual processes and the formation of perithecia. Finally, it may be possible to treat orchardgrass at the time of ascospore ejection with a product that would inhibit ascospore germination or iterative sporulation. These approaches may help decrease disease spread. Fungicides with efficacy towards ascospores *in vitro* were not effective on chokes *in vivo* (Leyronas, 2005). Thus, further research on suitable active ingredients is needed or alternative ways of infection (conidia, animal vectors) have to be considered.

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