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Infection of Barley by *Ramularia collo-cygni*: Scanning Electron Microscopic Investigations

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Abstract *Ramularia collo-cygni* causes leaf spots on barley (*Hordeum vulgare*), a disease of growing economical importance. Scanning electron microscopy was used to study the life cycle of the fungus on barley during the vegetation period and in winter. The infectious stage started with conidium germination on the surface and the penetration into the leaf via the stomatal pore where the hyphae grew within the cells that became necrotic. The conidiophores emerged through the stomatal pore. On older leaves, however, they frequently emerged apart from it and the results suggested a pushing apart of adjacent cell walls of the epidermal cells. An assessment of the amount of conidium formation of one heavily infested barley plant resulted in 4.05×10^6 conidia per plant. For the first time, conidiophores, conidium production and germination of conidia were also observed in winter on barley and on maize leaves.

Keywords *Ramularia collo-cygni* · Barley · Leaf spots · Micromorphology · Conidium production in winter and summer

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Introduction

Ramularia leaf spot (RLS) was discovered about 100 years ago, but scientific interest did not concentrate on it until the late 1980s when unidentified leaf spots on barley in Austria were assigned to *R. collo-cygni* [1, 2]. In recent years, infections have been reported from all over Europe, Mexico, South America and New Zealand, and RLS is now considered a serious threat to barley yields [2, 3]. *R. collo-cygni* has also been observed on other cereals and grasses and has been observed on *Cannabis sativa* too [4].

First symptoms are visible after flowering as small dark brown spots surrounded by a yellowish halo on the leaves [5, 6]. Typical spots measure 0.8–1.4 mm in length and 0.5–0.7 mm in width and are usually bordered by leaf veins [1]. During the further progress of the disease, these spots intensify and eventually merge, leaf sheaths and awns are infected, and finally, the infected structures start to die off prematurely [5]. The infection of seeds has also been reported [3, 7].

The anamorph fungus *R. collo-cygni* is placed close to *Ramularia brunnea* or *Ramularia grevilleana* in the *Mycosphaerella* complex [3, 4, 8, 9]. This classification was confirmed by the recent discovery of rubellins, i.e. toxins, that are involved in the phytopathogenic event [10, 11]. These photo-dynamically activated toxins produce reactive oxygen species and induce fatty acid peroxidation which

ultimately leads to membrane destruction and necrosis [12, 13].

Rubellins are yellow- or red-coloured anthraquinone derivatives so far only described for *Mycosphaerella rubella*, a causal agent of a necrotic spot disease of *Angelica sylvestris* [11, 14]. Uredinorubelline I and II, new members of the rubellin family, as well as the possible precursors helminthosporin and chrysophanol were recently isolated from cultures of *R. collo-cygni* and from mycelium of *Ramularia uredinicola* [15].

The typical RLS symptoms appear on foliage after emergence of the ear [3] though the pathogen can be detected in the leaf before the onset of symptoms using PCR methods [16, 17]. Recent evidence shows that the incidence of RLS is closely connected to the ontogenetic senescence status of barley leaves and that susceptibility is dramatically increased with the age-related breakdown of the antioxidative capacity [18].

Despite the increasing economic importance of RLS, knowledge about pathogen morphology in association with its host tissue is limited. Moreover, no investigations of conidium germination or growth of infection hyphae on leaves in the field under summer and winter conditions have been made. Therefore, the objective of this work was to elucidate these aspects of biology and infection processes of *R. collo-cygni* on barley.

Materials and Methods

Plant Material

All materials originated from the Institute of Organic Farming, Stadl Paura, Austria, and were sampled between 1999 and 2004.

Samples of leaves from field grown spring barley cv. Baccara and winter barley cv. Virgo (*Hordeum vulgare* L.) were collected during the vegetation period at appropriate stages in order to investigate the micromorphology of the pathogen, the growth on and within the plant, the penetration into the leaf and the emergence of the conidiophores (early to late milk stage; BBCH 75-77; spring barley in summer—germination of conidia and conidiophores, winter barley in spring—conidiophores).

The appearance of *R. collo-cygni* in winter was studied on spring barley (cv. Baccara) and on maize. In order to study the effect of frost exposure to fully developed conidiophores of *R. collo-cygni* spring barley was planted in pots in the summer of 2000, where the early planting was necessary to ensure the correct developmental stages of the pathogen for the winter investigations. In order to expose them to the cold, the pots were placed outside under normal field conditions only covered by a clear plastic roof, so to avoid temperature isolating effects of snow coverage. Samples were taken in January (mean temperature: −2°C).

Infected leaves of maize were sampled in the field shortly before harvesting in October 2000 and placed outside on soil so that the rotting process and the further development of *R. collo-cygni* could be studied. Samples for micromorphological investigations were taken in December following a period of high humidity and cold, but not freezing weather with mean temperatures of 5.1°C in November and 4.1°C in December.

Specimen Preparation

Leaf samples were cut into small pieces and chemically fixed (glutaraldehyde 2.5% pH 7.0; ethanol:acetic acid, 3:1 v/v; formalin:ethanol:acetic acid, 9:5:0.5 v/v/v). Results and observations were similarly irrespective of the fixation type [19]. After dehydration (acetone series for samples fixed with glutaraledehyde and ethanol series for the others), samples were critically point dried (CO_2; BALTEC CPD 30) and mounted on aluminium stubs with carbon impregnated double-sided tape. In order to investigate the inner part of a leaf, the dried samples were mounted on stubs—another stub with a sticky tape was placed on top and the stubs were torn apart so that part of the leaf stayed on one stub and the other part was on the other stub. All samples were sputter coated with gold (Agar Sputter Coater) and investigated using a Philips XL30 ESEM (FEI) operating in the high vacuum mode at 20 kV.

Assessment of Conidium Formation

A total amount of conidia were counted on air-dried samples (to avoid the removal of spores during the fixation and dehydration processes) of leaves of spring barley sampled in mid-summer. Samples from upper and lower leaf surfaces, from the base and the tip of different leaves (F, F-1, F-2, F-3) were chosen;

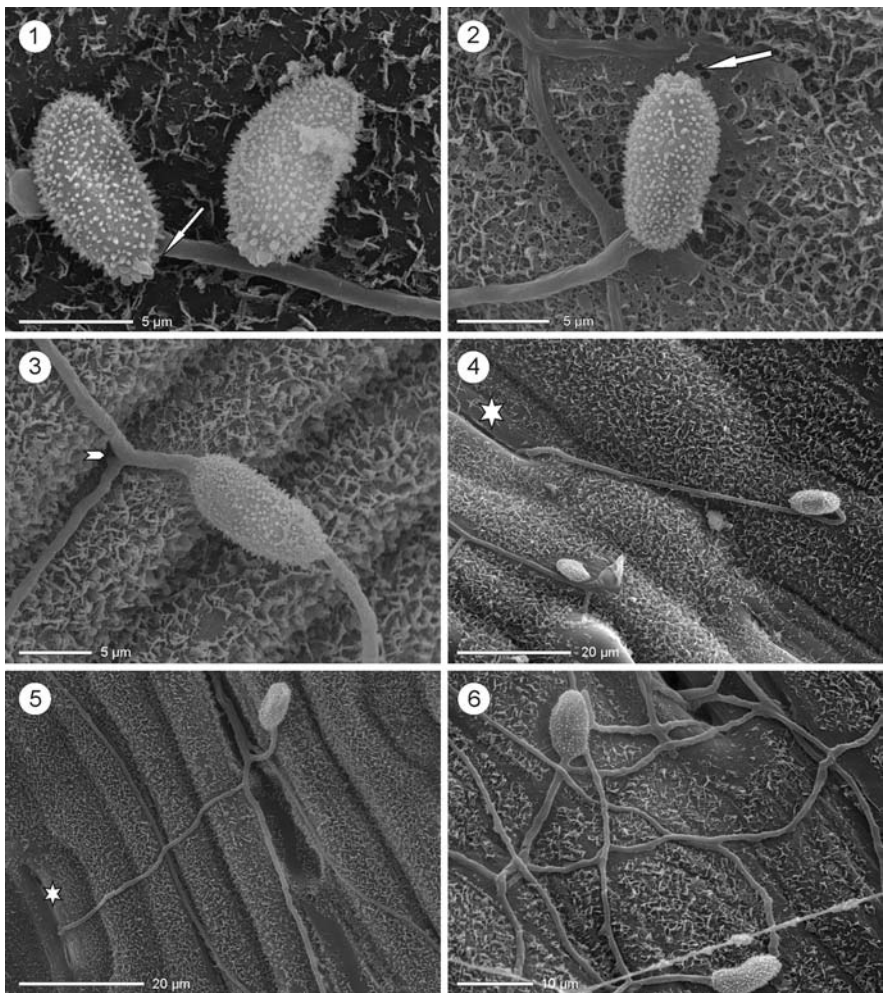
the amount of conidia was assessed on 60 randomly chosen areas and extrapolated to the total leaf area. Statistics were performed using Mann–Whitney *U*-test (Statistica 6.0).

Results

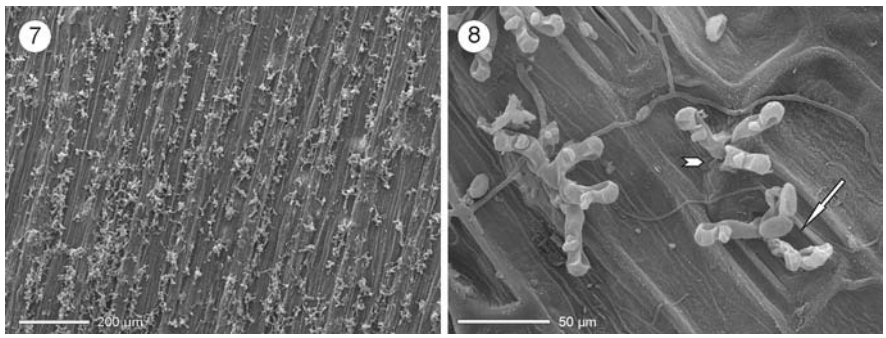
The infection of barley by *R. collo-cygni* started with deposition and adhesion of conidia on the leaves. On spring barley, this was observed in early summer where the highest amount of germinating spores were found on necrotic and chlorotic leaves and at a lower amount on green leaves too. No conidiophores of *R. collo-cygni* were detectable at this phase. The typical conidia of *R. collo-cygni* were obovoid to ellipsoid, revealed a verrucose surface, had an eccentrically located basal hilum and were 7–11 µm long and 3–6 µm wide (Fig. 1).

The germ tube usually emerged at either the tip or the base of the conidium (Figs. 1, 2), and sometimes the formation of another germ tube on one conidium could be observed (Fig. 3). On the whole, the epicuticular wax plates were not affected but the incidental dissolving of wax structures in the surrounding areas of the spores and hyphae could be seen (data not shown). The hyphae found their way into the plant via the stomatal pore. Rarely, they did enter the leaf directly after germination (Fig. 4). Usually, the penetration occurred after branching (Fig. 5) and forming a dense network of hyphae on the leaf surface (Fig. 6).

Later on in the development of the plant and the infection (mid summer for spring barley, spring for



Figs. 1–6 (**1**) Conidia on the epidermis; the epidermis is covered by wax plates; the conidia are ovoid, warty and show a slightly eccentrically located scar near the base (*arrow*); here the infection hyphae emerges at the base. (**2**) The germ tube emerges at the tip of the conidium (arrow—base of the conidium). (**3**) Conidium with two germ tubes emerging at the tip and at the base and branching (*arrowhead*). (**4**) The hypha is growing directly into the stoma (*asterisk*). (**5**) After germination the hypha is branching (*arrows*) and one branch is growing into the stoma (*asterisk*). (**6**) Germinating conidia branch and form a dense network of hyphae on the leaf surface. Scanning electron microscopic micrographs of *R. collo-cygni*. Spring barley, sampled in June



Figs. 7–8 (**7**) Caespituli on the leaf surface are arranged in lines. (**8**) The clusters of conidiophores reminiscent of a swan's neck emerge through the stomal pore (*arrow*) and apart from it (*arrowhead*). Scanning electron microscopic micrographs of *R. collo-cygni*. Spring barley, sampled in July

winter barley), the typical RLSs were visible macroscopically on chlorotic and necrotic leaves. The caespituli, visible in weak magnification as small white spots, were arranged in lines between the veins (Fig. 7). They appeared in small fascicles, first on the lower leaf surface and with increasing development on the adaxial side as well, and were characterised by the typical swan's neck-like apexes (Fig. 8) that allowed the unequivocal identification of *R. collo-cygni*.

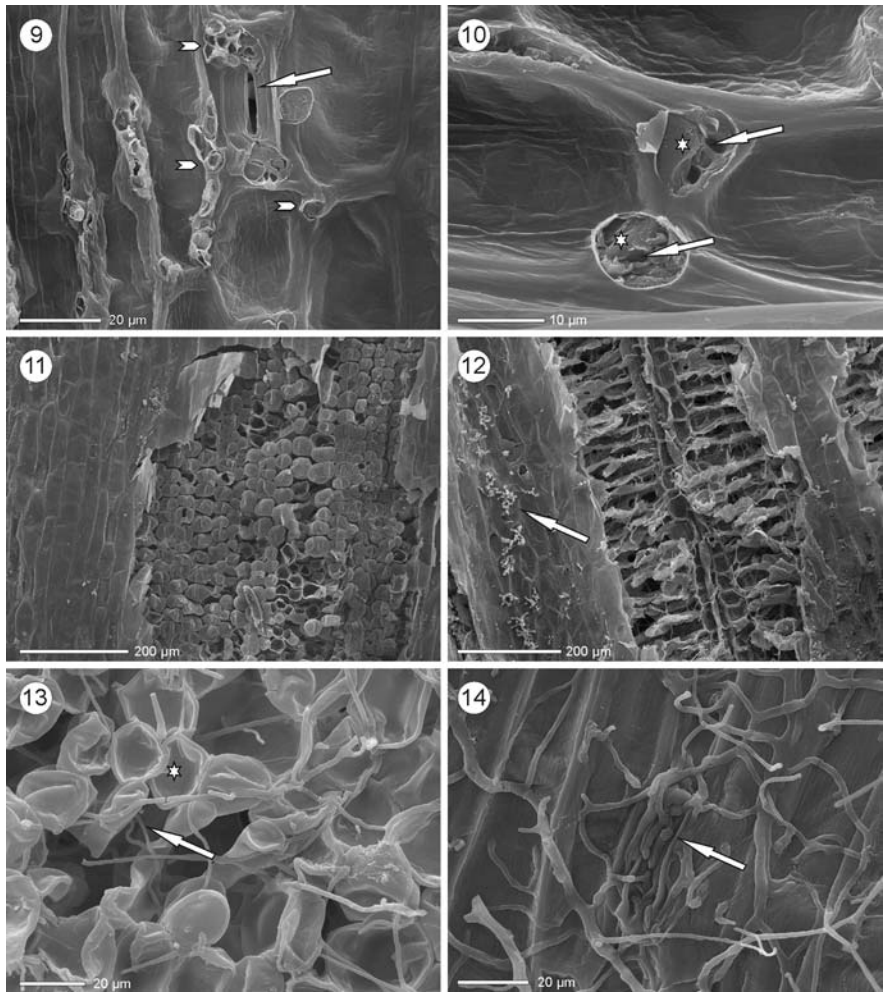
Conidiophores emerged through the stomatal pore (Fig. 8) thus explaining the typical arrangement in lines. However, they also emerged through the epidermis near the stoma or even some distance from it (Fig. 8). This was principally observed on older leaves.

In order to investigate this emerging characteristic, conidiophores protruding over the epidermal surface were removed. The fungal structures were only observed among the adjacent epidermal cells; they clearly followed the epidermal cell borders (Fig. 9). Observations at higher magnification identified that the hyphae grew in the region of the middle lamella (Fig. 10).

After removing the epidermal cells, a look at the mesophyll showed intact cells in those areas where no *R. collo-cygni* conidiophores were observed (Fig. 11). In contrast, the cells were collapsed in areas with many of these typical conidiophores (Fig. 12). A closer view showed collapsed cells and hyphae growing intercellularly forming a dense network between the cells (Fig. 13). The inner surface of the epidermis was covered by a dense network of hyphae (Fig. 14).

The upper leaf surface was generally not as heavily covered by condiophores as the abaxial one, and no other species of fungi could be observed in these affected areas. The amount of conidia produced on the surface of one barley plant was estimated by counting the conidia on different leaf levels (flag leaf and the three following ones) (Table 1). Despite high variability, a distinctly higher production of conidia on the lower leaf surface in comparison to the upper surface was observed. The oldest leaves (F-3) showed the lowest amount on both leaf sides, whereas there were no differences between the other age classes on the upper side. On the lower leaf side, the highest conidium formation was observed on F-2. From this data, a total amount of 4.05×10^6 conidia produced on these four leaves was extrapolated (Table 1).

Infested leaves producing conidiophores and conidia were not only found during the vegetation period, but were also observed on frost-exposed material sampled in winter. Plants (spring barley) were potted in summer and placed outside. Samples taken in January showed the typical distribution of the conidiophores on the epidermis (Fig. 15) as well as the typical emergence out of the stomatal pore and also apart from it (Fig. 16). High quantities of conidia were produced, and the germination of conidia as well as the penetration of hyphae into the leaves via the stoma could be observed (Fig. 17). On barley leaves, *R. collo-cygni* conidiophores were more or less unrivalled; no other fungal structures were detected on these areas. With maize the situation was different. Infected leaves of maize were placed outside on soil after harvesting in October, and then samples were taken in December. Conidiophores of *R. collo-cygni* were detectable on the surface (Fig. 19). In contrast to barley samples, however, these conidiophores grew in the immediate vicinity of other fungal structures (Fig. 18). To a great extent, conidium formation and germination could be observed (Figs. 19, 20).



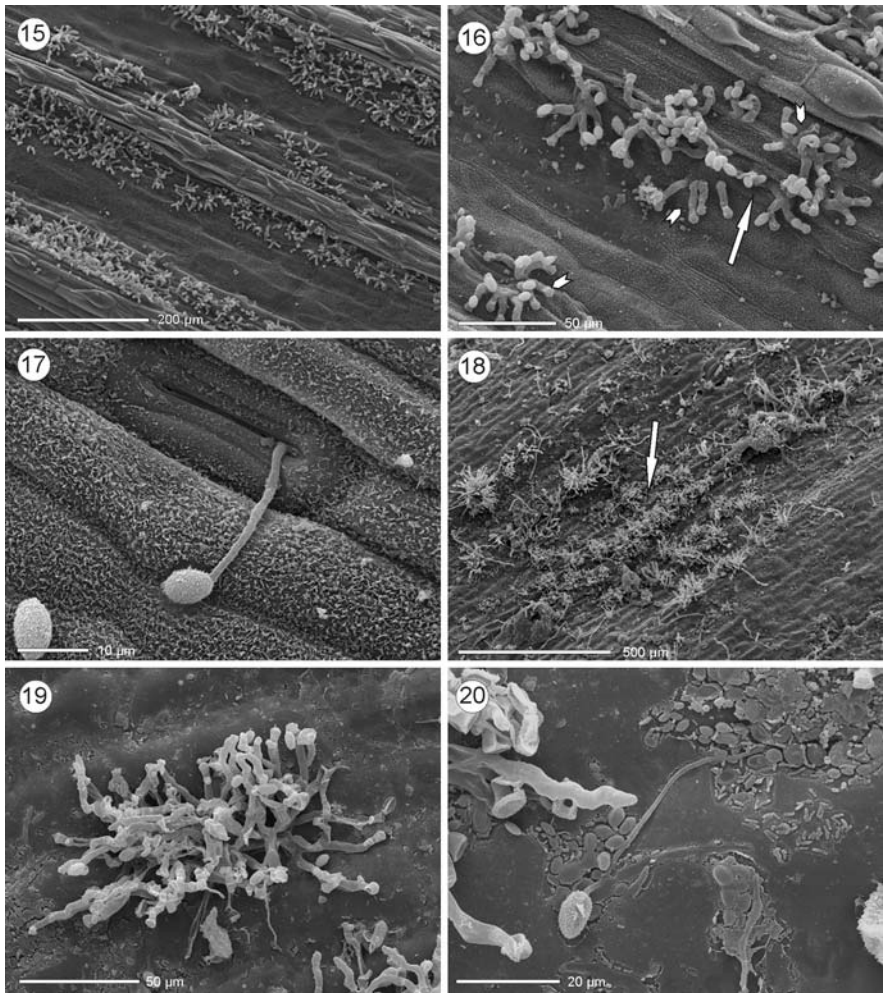
Figs. 9–14 (**9**) The stomatal pore (*arrow*) is free of conidophores; the cell borders of the epidermal cells are visible with conidiophores (*arrowheads*) emerging between adjacent cells. (**10**) Four epidermal cells; the conidiophores emerge in the region of the middle lamella (*arrow*) in between the cell walls (*asterisk*) of the adjacent cells. (**11**) No conidophores are visible on the epidermis; the mesophyll cells are turgescent. (**12**) Conidiophores of *R. collo-cygni* on the epidermis (*arrow*); the mesophyll cells are collapsed. (**13**) Hyphae (*arrows*) grow intercellularly, the mesophyll cells (*asterisk*) are collapsed. (**14**) Inner side of the epidermis; hyphae grow on the inner epidermis forming a dense network and concentrate in the stomatal pore (*arrow*). Scanning electron microscopic micrographs of *R. collo-cygni*. Micrographs resulting from the removing of the supraepidermal parts of the conidiophores (**9–10**). View on the mesophyll cells after removing the epidermal layer (**11–14**). Winter barley, sampled in spring

Discussion

Ramularia collo-cygni is a pathogen of increasing economical importance [2, 3]. The scanning electron microscopic investigations presented here provide an insight into the appearance of the fungus on the epidermis of its main host, barley, during the vegetation period and the winter season.

The general habitus of *R. collo-cygni* with the typical conidiophores reminiscent of a swan's neck and the ornamented conidia is consistent with other reports [1, 4, 20]. It causes the typical dark brown spots on barley leaves on which the conidiophores develop later on [2, 6].

Some conidia of fungi, also infecting barley, release exudates that alter surface morphology and surface chemistry, changing it from a hydrophobic to a hydrophilic state (*Erysiphe graminis* [21]; *Blumeria graminis* [22, 23]). However, this does not seem to be the case for *R. collo-cygni*. With a few exceptions,



Figs. 15–20 (**15**) Lines of caespituli on the epidermis. (**16**) Conidiophores emerging through the stomatal pore (*arrow*) and apart from it (*arrowhead*). (**17**) The germination tube of the conidium grows straight to the stomatal pore and infests the leaf. (**18**) Cluster of conidiophores (*arrow*) on the surface that is covered by lots of different fungal structures. (**19**) Typical conidiophores and conidia. (**20**) Conidium with germ tube; note lots of bacteria on the devastated surface. Scanning electron microscopic micrographs of *R. collo-cygni*. Barley, sampled in winter (**15–17**). Maize, sampled in winter (**18–20**)

Table 1 Amount of conidia produced on the upper and lower leaf surface of four different leaf levels (F—flag leaves, leaves F-1, F-2, F-3) of one spring barley plant in midsummer; mean number of conidia per mm^2, standard deviation (SD; $n = 60$) and total number of conidia extrapolated to total leaf area; different letters mean significant differences ($P < 0.001$)

Leaf	Mean (conidia mm^{-2})	SD	Conidia on total leaf area
Upper epidermis			
F	393[a]	504	0.19×10^6
F-1	375[a]	516	0.35×10^6
F-2	359[a]	431	0.27×10^6
F-3	198[b]	391	0.11×10^6
Lower epidermis			
F	1095[a]	935	0.53×10^6
F-1	1157[a]	1075	1.03×10^6
F-2	1750[b]	1397	1.33×10^6
F-3	442[c]	480	0.25×10^6

no morphological changes of the leaf surface directly surrounding the conidium, and therefore, indicating exudation products were observed.

Penetration into the leaf unequivocally took place through the stomatal pore. The conidium produced one, or sometimes two, hyphae that could invade the leaf immediately after germination. More often, however, a more or less dense network of hyphae was formed before entering the leaf. Entry into the leaf through open stomata occurred within 24 h after germination and rapid growth of the mycelium [3, 20]. For various pathogens, it is known that topographical and chemical features play an important role in the recognition of the stoma [24–26]. The way *R. collo-cygni* recognises an entrance path into the leaf is still unknown.

Subsequently, conidiophores appeared first on the abaxial leaf surface, then on the adaxial surface, and

then conidium formation started. Germinating and infesting conidia were observed on leaves where abundant conidiophores were already present. A first assessment of the amount of conidia estimated 4.05×10^6 conidia per plant. An estimation of up to 50,000 conidia produced by a heavily infected leaf was reported recently [27]. As a first approximation, this amount demonstrates the enormous pressure host plants are exposed to. It is difficult to compare this data with results from spore traps where in one study 186 conidia per cm^2 per day were reported [28]. A recent study counted only germinating spores of *R. collo-cygni* (others were suppressed by an added fungicide) and gave maximum values of 10,000–12,000 germinating spores per m^2 per day (=1–1.2 spores cm^{-2}) [18].

After entering the leaf, the fungus grew intercellularly. The presented results provide no evidence of intracellular growth.

Symptoms of RLS become visible only after ear emergence [3, 18]. The pathogen, however, can be detected earlier using PCR [16, 17]. The outbreak of RLS is supposed to be triggered by the breakdown of the antioxidative protection system in older leaves [18]. The light regime during plant growth seems to be of importance for symptom expression [29], whilst the detailed role of environmental parameters, however, is still not completely understood [3]. The rubellins produced by *R. collo-cygni* [10, 11] are supposed to have an impact on the antioxidative system. They are activated by light, and reactive intermediates can oxidatively attack pigments and induce fatty acid peroxidation finally leading to membrane destruction, chlorosis and necrosis [12, 13]. Their detailed part in pathogenicity, however, has to be investigated in more detail.

In accordance to their intercellular growth in the leaves, the conidiophores emerged through the stomatal pore. This is comparable to the conidiophore emergence of other *Ramularia* species [4]. On older leaves, conidiophores could also be formed apart from stomatal pores. However, they did not rupture the epidermal layer. Instead, they grew in the intercellular space and pushed apart the adjacent cell walls of the epidermal cells. Based on the presented results, it is not possible to determine whether the hyphae produce pectic enzymes that loosen the middle lamella between host cells or if the growth occurs just by physically and mechanically pushing the cells apart as was reported for other fungi [30].

The first infection occurs on young winter barley leaves in warm autumn days, and the pathogen hibernates on the oldest leaves [2]. At the beginning of the growing season in the following spring, it starts spreading and producing masses of conidia that infect other plants and species. For example, on spring barley *R. collo-cygni* was detected only after it sporulated on winter barley [17]. However, as was shown here, conidium formation and infection were not limited to the vegetation season, but *R. collo-cygni* was also able to sporulate and infest in winter. These observations are in accordance to a recent report where *R. collo-cygni* was detected using direct PCR from the very first sampling in February till June [17]. The pathogen is not only present but is able to grow and produce conidia. The low temperatures in winter do not seem to be a barrier for germination as was also demonstrated in a recent study where even at 4°C more than 70% of the spores germinated [18].

Conidiophores, production and germination of conidia were not only observed on leaves of barley but also on leaves of maize. The maize leaves, harvested in autumn and placed outside, served as a model system for plant residues staying on the fields after harvest. These harvest residues may be a conidium source, thus being an origin of infection for other species. So far, *R. collo-cygni* is known to colonise only dead maize leaves, but recently, the infection of green leaves was reported [31].

The host spectrum of *R. collo-cygni* is not limited to barley but rather covers several Poaceae and *C. sativa* [3, 4]. The occurrence on hemp may be a hint for a broader host spectrum as is currently known. However, molecular examinations are still lacking and should be the purpose of further studies. The ability to remain active in the cold parts of the year, to sporulate on living and dead plant material and its host spectrum makes this fungus independent of its main host—barley. These features will facilitate the further spread of the disease and *R. collo-cygni* will go on as a matter of particular interest for plant pathological and agronomical research.

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