

Fusarium moniliforme Sheld. Association with Species of Orchids

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Abstract. The fungal species Fusarium moniliforme Sheld. was repeatedly isolated in nature from orchid species Cypripedium calceolus L. and C. reginae Walt. A symbiotic relationship was found between the fungus and orchids native to Michigan. Induced fungal pathogenicity was obtained with F. moniliforme in the orchid Zygopetalum machaerii.

Introduction

Over 50 orchid species are found native to Michigan (11,17). Occasionally good populations of selected species occur in undisturbed habitats. From the Michigan species, Cypripedium reginae Walt and C. calceolus L. were selected for mycological investigations. The habitat of C. calceolus is cosmopolitan and it can be found in tamarack - black spruce bogs, in heavily wooded sphagnum swamps, in thickets along streams, in bushy meadows, on sandy swampy lake shores, and in damp meadows, fields and roadsides. Cypripedium reginae is slightly more restricted to such areas as bogs, heavily wooded swamps, swampy lake shores, and in areas where sphagnum grows well.

Initial investigations examined selected orchid plants for the presence of fungi. Repeated isolations of Fusarium moniliforme Sheld. were obtained from various parts of mature orchid plants growing in nature. Orchids are known to possess chemical and morphological qualities which control or hinder fungal growth. Phytoalexins keep invading fungal species under control, creating symbiotic, commensalistic or slightly parasitic relationships (1,3,4).

Various inoculations of Fusarium conidia, originally isolated from field material, were made on domestic orchids. The fungal associations with the orchids created by the inoculations were then examined. Studies were designed to identify the relationship of Fusarium sp. to domestic and native orchids of Michigan.

Literature Review

Regardless of differences in morphological, anatomical, or physiological characteristics, all orchids at some phase in their life cycle are associated with certain fungi. Hyphal filaments serve as absorbing organs within cortical cells of orchid roots (31, 32,33). This orchid - fungal association may be advantageous, neutral, or disadvantageous and parasitic (2). Some species of fungi are found throughout the orchid plant, but mycorrhizae create a symbiotic relationship in the host root system (10,34,38,41). In a mycorrhizal relationship, reciprocal movements of substances between the orchid and the fungus occurs (3,4,35). Little evidence either supporting or discounting the specificity of the host - endophytic relationship has been established, but it appears that certain fungal species seem to

be more effective with select taxonomic groups of orchids (29,31,34).

According to Marx (38), the micorrhizae of orchids have an endomycorrhizal association. Hyphae of the symbiont penetrate the cells and establish close contact with the cytoplasm. Endomycorrhizal fungi have the ability to degrade cellulosic cell walls, but ectomycorrhizal fungi do not have this ability and are limited to utilizing a few simple carbohydrates (41). Apparently seedlings require association with specific fungi soon after seed germination. Fungi infect orchid embryos in the early protocorm stage. Without this infection, embryonic growth continues only by an artificial supply of sugars, vitamins, as well as mineral nutrients (19,42,47,48). Nutrients are first absorbed by the fungal hyphae and then released into the host tissue. Fungal hyphae are digested in the host cells, which is the transfer mechanism of nutrients from fungus to host (15,28,33).

The orchid host has many methods of controlling the fungal symbiont. It is clear that the host - endophyte complex must maintain factors or conditions that bring enzyme activity of the fungus under control. If this control is not attained, a pathogenic association could result. The controls of the enzyme activity of the fungus are due to the interaction of metabolic processes of the endophyte and the host. The fungus is able to break down soil cellulose into nutritive compounds that can be absorbed by the fungus and then in turn by the host (47,48). The absence of invading hyphae in various parts of terrestrial orchids has suggested that orchids possess a mechanism of resistance to, or a substance that is toxic to, mycorrhizal fungi (32). This fungistatic control compound is secreted only by living cells due to the activity of the fungus upon them. Structurally, the chemical is a benzene soluble fungistatic compound with a molecular weight of 256. The chemical was named Orchinol (25,26). It was the first of several compounds to be discovered that were produced by orchids after infection by fungi and which were classified as phytoalexins (1,3,13,14,21,22,24,31,40,49). Phytoalexins are described as plant antibiotics which are inhibitory to microorganisms attacking plants. The compounds arise from metabolic interactions of hosts and parasites (43). Other compounds discovered following studies on phytoalexins were Hircinol and Lorglossol which had a wide spectrum of activity on fungi, however, their modes of action were not specific against only mycorrhizal fungi (20,21).

The formation of phytoalexin is induced in the plant by diverse fungi. The fungal types include obligate and facultative parasites, specifically adapted pathogens to the plant, and phytopathogenic fungi not infecting the given plant species (40). However, all fungi do not necessarily bring about phytoalexin formation. Orchinol was not formed when common saprophytic and semiparasitic soil fungi were used for the infection of orchids (25,26). Phytoalexin formation occurs in all host plant organs. A plant is resistant to a parasite if the concentration of phytoalexin is high enough to inhibit the growth of a parasite. The orchid becomes a susceptible host if the phytoalexin concentration is insufficient to inhibit the pathogen (40). It is not known if the effect of these substances allows certain mycorrhizal fungi to exist in roots and at the same time inhibit the asso-

ciation of other fungal species. This could explain the selectivity of fungal species to orchid symbiosis (32).

Hyphae invading orchid tissues are surrounded by a membrane that is close to the fungal wall or separated from the host tissue by a distinct layer. This membrane is considered to be of host origin while the metabolic transfer is from fungus to host (13). Intracellular hyphae did not penetrate the plasmalemma of the orchid cell (29).

Not all fungal relationships with orchids are beneficial, and a pathogenic state can exist between the fungus and its host. The difference between a mycorrhizal fungus and a root pathogen may not be great (48). The occurrences of fungal pathogenicity in orchids are few, but there are examples of serious pathogenic associations (9,16,18,39). Certain species of fungi can cause severe root rot in various genera of orchids (2,36,46). Bud rots and leaf stains are other diseases caused by fungi and are characterized by discolored spots or patches on the leaves, stems, and fruit which are followed by death or rotting of these organs (5). The association of F. moniliforme with domestic and wild orchids was examined to determine what relationship was established between the orchids and the fungal isolate.

Materials and Methods

Two orchid species, Cypripedium reginae Walt. and Cypripedium calceolus L. were selected for study. The collection site was Kalkaska County, Michigan. The habitat consisted of a balsam - cedar forest opening onto a bog. Mature plants, soil samples, and orchid leaf litter were obtained at the collection sites then taken to the laboratory.

One inch sections of orchid leaves from living plants were surface sterilized with 70% ETOH 5 seconds and 5% Chlorox bleach 15 minutes before placing in the moist petri chamber. Sterile distilled H₂O was periodically added to the petri plates to prevent drying of the orchid material. Pure cultures of the fungi isolated from the incubated material were maintained on Potato Dextrose Agar (PDA) and identified according to the literature (6,7,8,12,23,24,27,37,45,46).

Serial dilutions of soil suspensions were plated on PDA and incubated at room temperature. Orchid leaf litter collected in nature was placed in moist petri dish chambers and incubated. Fungal isolates were identified and maintained in pure culture.

Mature hybrid orchid plants were obtained from the collections of Ilgenfritz Nurseries, Monroe, and Black River Orchids, Grand Haven. Hybrids selected included Cattleya sp., Cypripedium sp., Cymbidium sp., Dendrobium sp., Diacattleya sp., Epicattleya sp., Epilaeliocattleya sp., Laeliocattleya sp., Oncidium sp., Paphiopedalum sp., Phalaenopsis sp., Sophrolaeliocattleya sp., and Zygopetalum sp.

Hybrid orchid leaves were incubated with fungal colonies on agar by placing the colony surface down on the leaf blade. After 3 weeks incubation leaf sections containing the colony inocula were removed from the plant, fixed in FAA, dehydrated in an alcohol series, and embedded in Tissuemat. Material was sectioned at 8-10 μ then placed on slides controlled with Haupt's adhesive (1 g gelatin, 2 g phenol,

15 ml glycerine to 30 ml distilled H₂O) and flooded with 4% formalin. Slides were drained and air dried one week before staining. Sectioned plant material invaded with fungi was stained with a modified Conants quadruple stain in a xylene alcohol dehydration, safranin in 30% ETOH, orange gold and fast green in clove oil, with a Canada balsam mounting.

Excised Cypripedium roots, leaf sections, and stems were obtained from living plants in nature, surface sterilized then placed on PDA, Knudson's Orchid Agar, and Noble Agar for growth and isolation of fungi associated with the plant tissue.

Results

Leaf litter and soil obtained adjacent to Cypripedium species in nature contained numerous fungi and bacteria. Fusarium moniliforme was the dominant fungal species most frequently found in each collection. Repeatedly F. moniliforme was isolated from living Cypripedium leaves that were surface sterilized and placed in incubation chambers. A suspension of F. moniliforme conidia inoculated into various hybrid orchids caused blackening of the host tissue at the inoculation site with no further fungal involvement.

One orchid, Zygopetalum machaerii served as a suitable host plant for fungal invasion from agar block transfers of F. moniliforme cultures to the orchid leaves. Other orchids that proved negative with this method included Cymbidium baltis, Dendrobium rididum, Dia-cattleya sp., Epicattleya sp., Epilaeliocattleya sp., Laeliocattleya sp., Oncidium ampliatus, Paphiopetalum callosum, Phalaenopsis sp., and Sopholaeliocattleya sp. With Zygopetalum sp., a darkened area appeared similar to the inoculations on the other plants, and in addition, Fusarium established a vigorous colony on the leaf surface independent of the agar block.

Sectioned Z. machaerii leaf tissue demonstrated a definite fungal association. Sclerotia were found in abundance in the leaf tissue. Most sclerotia were round or kidney shaped with a defined dark colored outer rind enclosing a medulla of densely packed hyphae lacking any consistent orientation. Some microsclerotia were observed containing few cells without an outer rind. In one case the whole end of the leaf had been taken over by a sclerotial cap. The sclerotial rind contained coalesced hyphal segments with thick, agglutinated, dark colored walls. Agglutinated segments were also found within the medulla interior. Internal consolidation consisted of intercalary expansion and septation associated with hyphal anastomoses. Reserve materials accumulate as the wall thickened.

The palisade and mesophyll areas of the leaf were heavily invaded with mycelia. The upper and lower epidermis appeared desiccated and completely replaced by hyphae. Macroconidia were common near a sclerotium. Aerial hyphae on the abaxial sides of the leaves were abundant, and remnants of the leaf cells could be seen surrounding the rind of the sclerotium on the adaxial side.

In several leaf sections of Z. machaerii, many stromatic pustules were found. These pustules served as perithecial initials or immature sporodochia. Hyphae were dominant throughout the entire leaf section, and the epidermis on both adaxial and abaxial sides was lifted or sloughed off near the stromatic pustules (Fig.1). Pustules

found directly over a vascular bundle had a tendency to invade the vascular bundle but apparently did not disrupt the function of the tissue. Macroconidia were very abundant and aerial hyphae were again found in large amounts. Hyphal penetration appeared to be both intercellular and intracellular and some cells were completely packed with hyphal strands. The pustules appeared heavily packed with pseudoparenchymatous material with small amounts of agglutinated cells as found in the sclerotium.

Sectioned Cypripedium roots appeared heavily infected with F. moniliforme. The epidermis and inner cortical cells were dense with hyphae and conidia (Fig.2). Inner cortical cells also contained loosely packed hyphal strands with macroconidial formation on phialides. Large stroma were also observed which appeared dark in areas and contained large isodiametric cells with slightly thickened walls. Aerial hyphae were found in abundance with macroconidia.

Cypripedium sp. seeds collected in nature were surface sterilized and shaken vigorously in sodium hypochlorite solution and inoculated on orchid agar plates. The seeds prior to inoculation were stored 6 months at room temperature and 6 months in a freezer to induce the overwinter cycle. Soon after incubation the seeds supported fungal growth. The organism was identified as pure cultures of F. moniliforme.

Cypripedium roots were obtained from potted plants transported to the laboratory from the original collecting site. Surface sterilized roots placed on PDA, orchid agar, or nutrient agar supported abundant mycelial growth within 5 to 7 days incubation at room temperature. The predominant fungal species again was F. moniliforme.

Fusarium moniliforme form microconidia in chains or on polyphialides, spindle to ovoid in shape. The macroconidia are slender with thin walls, commonly three septate, having an appearance of quarter moons. The cultures range in coloration from brownish-white to orange-cinnamon with a stroma white to deep violet. No chlamydospores are formed, however, large sclerotial beds occur over the host or substrate. Growth on PDA is initially filmy, colorless, and rapid. The reverse colony surface becomes typically deep violet but sometimes lilac or cream. Aerial mycelium is generally dense with a felt texture.

Discussion

It was determined by this study that an association does exist between Cypripedium reginae, C. calceolus and Fusarium moniliforme. The complete nature of this association remains unknown. It is highly probable that a symbiotic relationship developed between the opportunistic fungus F. moniliforme and the Cypripedium species, and that a mycorrhizal situation exists within the roots of these orchids.

The greatest Fusarium growth was associated with the roots of the Cypripedium species. This is the area in the orchid plant where the concentration of phytoalexins would be at the lowest level therefore accounting for the profuseness of Fusarium. When pure cultures of Fusarium moniliforme were reintroduced onto leaves, stems, or other tissues of living Cypripedium orchids there was no response and the fungus eventually died after the small agar block on which

it was growing was depleted. When various commercial orchids were used for inoculation purposes, in all but one example, Zygopetalum machaerii var. Lee, there was no outward response noted. Only a browning effect caused by the apparent scar tissue produced by the orchid in response to the scalpel cuts. Microscopic examination of embedded tissue of this brown area revealed no fungal hyphae or fungal remnants, and the underlying orchid cells appeared normal. A lesion appeared on Zygopetalum after Fusarium inoculation. The Fusarium eventually grew entirely through the leaf and appeared healthy and vigorous. Stromatic pustule lesions were observed on both the adaxial and abaxial sides of the leaf. These lesions may be perithecial initials or they may develop into true sporodochia (12). Scattered lesions on the Zygopetalum leaves appeared craterous with the centers sunken and containing a large amount of hyphal material situated around the outside of these pustule craters. Macroconidia were apparent on the surface around the pustules, and agglutinated hyphae were also present. Host leaf cells were partially filled with hyphae. No vascular tissue of the Zygopetalum orchid plants was affected by the fusarial growth. Even in cases where the fungus was growing adjacent to the vascular bundles there was no evidence of plugging or any disarray of xylem vessels. In most fusarial infections of other plants the vascular system is attacked and the fungal presence is observed. Cells immediately beneath the stromatic pustule appear to be normal without the appearance of being crushed or mutilated even though many cells are filled with fungal hyphae. The infected area was localized to the section of leaf where initial infection had taken place. There was no evidence of wilt as if the vascular system was being invaded and only the local lesions were apparent. Sclerotia formed on the ends and sides of the Zygopetalum leaves and apparently caused malformation of host cells. Epidermal areas as well as cortical cells were completely dominated by the sclerotia.

In the Cypripedium roots no stromatic pustules were formed or detected upon examination of the infected tissue. The emerging Fusarium fungus was allowed to completely envelop the root in the incubation chambers before killing-fixing. When sectioned, infected roots were found to be free of any outward breaks in the root epidermis caused by the fungus, however, the roots were completely infiltrated with Fusarium. It was quite evident that the fusarial growth developed within the root before it emerged at the surface. The epidermal cells were packed with the fungus, but there was little disruption of cell walls.

The relationship of fungi with orchids whether pathogenic, saprophytic, opportunistic or symbiotic is of a highly complex nature. Hardy orchid species survive by the establishment of an association with the fungal invader. Cypripedium species are similar to other orchid genera in that associations exist with a fungus which are necessary for the survival of the orchid. Fusarium moniliforme was determined by this study to have a definite association with Cypripedium reginae and C. calceolus. No lesions occurred in Cypripedium in spite of the dominance of F. moniliforme in healthy plant material, however, a commercial orchid developed lesions by the invading fungus. Fusarium moniliforme and Cypripedium species in Michigan appear to be

symbiotic while F. moniliforme with Zygopetalum machaeii is parasitic.

Summary

Fusarium moniliforme Sheld. was isolated repeatedly in nature from Cypripedium reginae and Cypripedium calceolus, orchids native to Michigan. Fusarium moniliforme introduced onto various cultured orchid species produced no pathogenicity in all but one species. Stromatic pustules developed on upper and lower leaf surfaces of Zygopetalum machaeii, and macroconidia were found in abundance. Sclerotia formed on Zypopetalum leaf tissue and they were composed of well developed outer rinds surrounding the medullae of agglutinated hyphae. The leaf was also desiccated and malformed by the fungus. Fusarium moniliforme was symbiotic to C. reginae and C. calceolus, and pathogenic to Z. machaeii.

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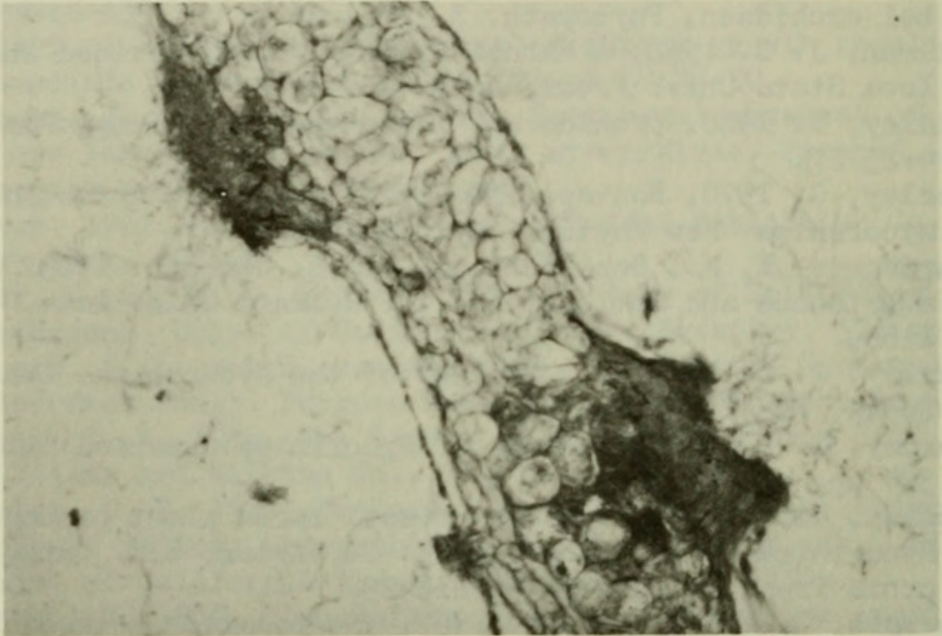


Figure 1. Stromatic pustules of Fusarium moniliforme on the adaxial and abaxial surfaces of Zygopetalum machaerii leaf. x 390.

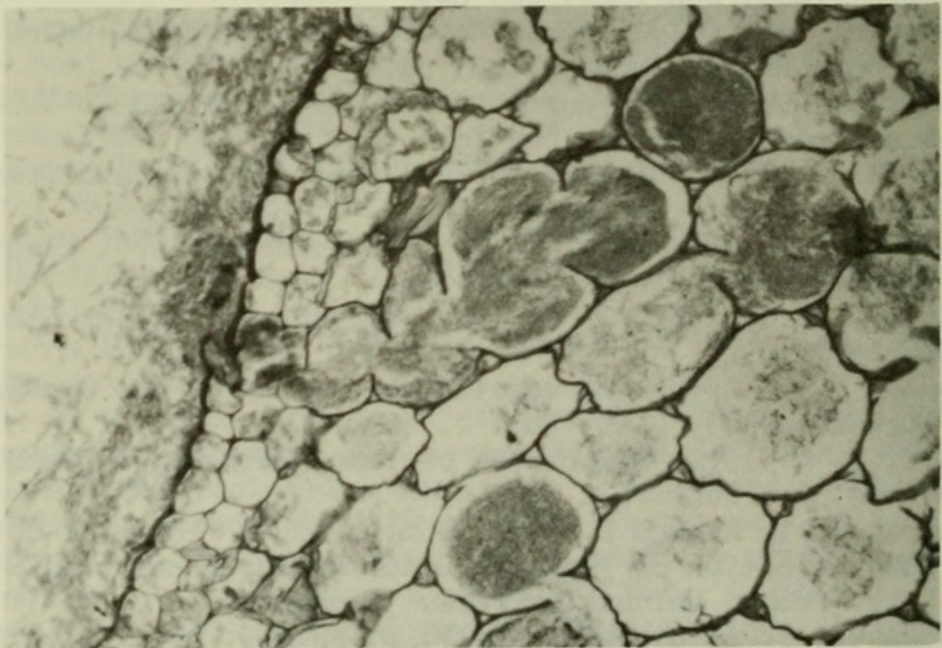


Figure 2. Cypripedium root with a dense lateral development of Fusarium moniliforme in epidermal and cortical cells in addition to loosely organized mycelial formation randomly found in host tissue and a dense growth on the host leaf surface. x 390.



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