Histochemical and chromatographic characterization of fungal disease related defense structures in perennial *Helianthus* species

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Summary

The basic objective of the present study was to investigate the interrelation between the presence of high-lignified cell walls and several phenolic compound with the tolerance reaction. Histochemical analysis was done on 9 wild *Helianthus* species using Neu's reagent (2-amino-ethyldiphenyl-borinate) by *in situ* detection of the phenolic constituents. The identification and characterization of different phenolic constituents was confirmed by HPTLC. Results showed a high genetic variation among the tested species. In order to allow an easier interpretation they were classified in tolerant, intermediary tolerant and susceptible species. We found that the different tolerance level was related to the lignification of the cortical parenchyma. These lignified cells can not be degraded by *S. sclerotiorum* and so represent a defensive protection. Another resistance factor that appear to be essential for the tolerance in the tested wild *Helianthus* species was the accumulation of phenolic compounds at post-infectional state. These results suggest their involvement in the process of resistance to pertotrophic pathogens. The identification of several wild *Helianthus* species with variable responses to the infection by *S. sclerotiorum* might be of major interest for geneticist and for breeding purposes by using direct gene flow or through biotechnological techniques.

Keywords:
Wild sunflower, resistance, *Sclerotinia sclerotiorum*, lignification, phenylpropanoids

Introduction

Sunflower (*Helianthus annuus*) is the fourth most important oilseed crop of the word. The narrow genetic variability make them susceptible for innumerous fungal and insect pests. Particularly the two pertotrophic pathogens *Botrytis cinera* and *Sclerotinia sclerotiorum* are world wide the major biotic limiting factors in sunflower cultivation (Seiler 1992). In general the cultivated varieties has a low level of resistance to this pathogens. In the temperate areas of Europe, capitulum and particularly mid-stalk infection induced by airborne ascospores between flowering and maturity are more prevalent. Under favorable conditions, in high *S. sclerotiorum* infested sunflower fields, yield losses can reach up to 100% (Masirevic & Gulya 1992; Rashid 1993). In contrast to the cultivated sunflower, in wild *Helianthus* species a large spectrum of resistance has been detected. In this sense, breeders are searching for resistance sources, especially for resistance to *S. sclerotiorum* (Skoric 1987; Seiler 1992, Tourvieille et al. 1996, Köhler 1997).
Several investigations in the past have shown that phenolic compounds are involved in the resistance reaction of the genera *Helianthus* to *S.sclerotiorum* infection (Avila 1984; Bazzalo 1985; Mondolot-Cosson & Andary 1994; Tourvieille et al. 1997; Hemery-Tardin et al. 1998). Accumulation of polymers such as melanin, lignin and low molecular phenolics like chlorogenic acid derivates are particularly active as fungistatics.
The basic objective of this study was to investigate the interrelation between some morphological and physiological characteristics of perennial *Helianthus* species with different levels of resistance to *S. sclerotiorum.* Special focus was made to determine whether either preformed and induced specific morphological characteristics or phenolic compounds could be used as resistance markers to *S. sclerotiorum* mid-stalk rot. Thus, such markers could be applied through biotechnological techniques (Henn et al. 1998, Binsfeld 1999), using perennial *Helianthus* species as new sources of resistance against *S. sclerotiorum*.

Material and methods

Plant and fungal material

A set of 21 perennial *Helianthus* species of diverse origin where first evaluated in field trials for resistance to *S. sclerotiorum* mid-stalk rot. From this set, 9 wild species with different level of resistance where chosen for subsequent microscopic, histochemic and chromatographic analysis. Based on their reaction, they where classified in a) tolerant, b) intermediary tolerant and c) susceptible species. The used *Sclerotinia sclerotiorum* isolate was collected from naturally infected sunflowers and kindly provided from Prof. Friedt (University of Giessen, Germany). The sclerotia were cultured at 20°C on a 1,5% agar medium containing 40% V 8 vegetable juice, in which after two days mycelial germination occurs could be observed.

Stem infection method

Stem inoculation was conducted as previously described by Henn et al. (1997). Mycelial disks of 0,6 cm (∅) were cut from the *Sclerotinia* mycelial culture and placed between the 4th – 5th internodal stem-section of field growing plants. The mycelium was placed directly in contact with the epidermis of the stems. The mycelial discs were fixed with Parafilm and covered with a transparent plastic bag in order to avoid the inoculum dehydration.

Histochemical analysis

Fresh samples (15-25 mm thickness) were prepared from healthy and infected stems sections with a freezing-stage microtome (Leitz-Kryostat 1720, Wetzlar; Germany). Stems were sampled 3-4 days after inoculation. For the analysis, the sections were treated with histochemical reagents during 2 to 7 min. After histochemical reaction the samples were mounted in glycerol:water (1:1) and observed under light or epifluorescence microscopy (Nikon, Eclipse TE 300-DV).

Suberized tissue: Sections were stained for 15 min in a 70% ethanolic solution of Sudan IV , rinsed with 50% Ethanol and examined by light microscopy.
Lignified fibers: Sections were fixed in absolute ethanol and placed for 3 min in a 10% ethanolic solution of phloroglucinol, washed with concentrated HCl and examined by light microscopy.
Condensed tannins: Sections were immersed in vanillin-HCl solution (10% [w/v] vanillin in a solution containing 1:1 (v/v) Ethanol absolute:concentrated HCl for 5 min. Observations were made by light microscopy.
Phenolic compounds (flavonoids, coumarines, hydroxycinnamates): Sections were immersed for 2-4 min in a 1% methanolic solution of diphenylboric acid-b-aminoethylester [Neu´s reagent (Neu 1956)]. Examination was performed by epifluorescence microscopy.

Chromatographic analysis

Analysis of phenolic derivates was performed by HPTLC (10x10 cm, Silica-Gel 60 F 254, Merck, Darmstadt, Germany). Infected and healthy stems were cut in three parts: a) Necrotic tissue b) one cm above and c) one cm below the lesion-zone. Approximately 0,2-0,5 g stem samples were rapidly extracted with cold 50% MeOH, centrifuged at 10.000 (4°C) and the supernatant used for subsequent analysis. 0,1 – 0,3 ml of this methanolic extract were spotted on HPTLC-Plates and developed in a horizontal TLC chamber (Desaga, Heidelberg, Germany). The solvent system consist of Ethylacetate - Formic Acid – H2O (67:15:20). Phenols were detected under UV light at 254 nm for fluorescence reduction and at 366 nm for autofluorescence. For qualitative determination of phenolic compounds plates were sprayed with a 1% methanolic solution of diphenylboric acid-b-aminoethylester (Neu 1956; Kirchner 1978) detected under UV light (366 nm) and characterized by their fluorescence color and Rf value.

Results and Discussion

The overall results of the histochemistry observations are shown in Tab. 1 and Fig 1. The detection of the lignified fiber content of healthy and infected stems revealed the presence of a high amount of lignified cells in the tolerant species *H. maximiliani* ACM, *H. maximiliani* AC 7 and *H. salicifolius*. In healthy stem sections these sclerenchymatic tissues (xylem vessels and cortical pericycle fibers) display a moderate blue autofluorescence under UV light (Ex. 365 nm) and altered to moderate greenish-white or whitish-blue in the presence of Neu´s reagent. This conversion in fluorescence color induced by a treatment with Neu´s reagent is characteristic to caffeic acid derivates (Steinegger & Hänsel 1992). Three to four days after infection, sclerenchymatic tissue only of the tolerant species were highly impregnated by caffeic acid derivates, giving a intensely whitish-blue fluorescence next to the fungal infection point. This strong fluorescence was not observed in susceptible species which could be indication that: a) fungal penetration and expansion in the tissue is faster than the synthesis of phenolic fungistatic compounds or b) these species are deficient in synthesizing such components.

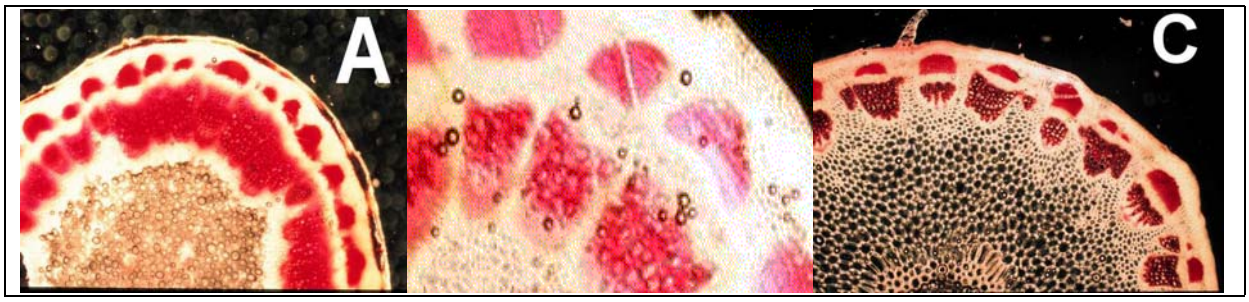

Fig 1. Histochemical staining of sclerenchymatic stem tissue by phloroglucin-HCl. A = *H. maximiliani* ACM (Tolerant); B = *H. rigidus* (Intermediary tolerant); C.=. *H. tuberosus* (Susceptible)

The presence of ester-linked monomeric and dimeric hydroxycinnamates reinforces the plant cell wall and leads to reduced biodegradability by the macerating enzyme set of perthotrophic fungal parasites. In addition, the presence of induced low molecular phenolics next to the side of fungal activity creates a fungistatic environment that can inhibit further mycelial ingression (Nicholson 1992; Dixon & Paiva 1995; Faulds & Williamson 1999).

Table 1. Histochemical response of 9 evaluated perennial *Helianthus* species.

Perennial b **Species**	**Staining magnitude** a									
	pre-infectional					**post-infectional (3-4 days)**				
	Sudan IV	Vanillin-HCl	Phlorogl. HCl	Autofluo-rescence	NEU´S reagent	Sudan IV	Vanillin-HCl	Phlorogl. HCl	Autofluo-rescence	NEU´S reagent
H. maximiliani ACM [t]	±	-	+++	+	+	±	-	+++	++	+++
H. maximiliani AC7 [t]	-	-	+++	+	+	-	-	+++	+	+++
H. salicifolius [t]	+	-	+++	+	+	+	-	+++	++	+++
H. rigidus [i]	-	-	++	±	+	-	-	++	+	++
H. strumosus [i]	-	-	++	±	+	-	-	+	+	++
H. pauciflorus [i]	-	-	++	+	+	-	-	+	+	+
H. tuberosus [s]	-	-	+	±	±	-	-	+	±	+
H. grosseserrat [s]	-	+	++	±	+	-	+	++	±	+
H. giganteus [s]	-		++	±	+	-	-	++	±	+

a - = no reaction, ± = weak reaction, + = moderate reaction, ++ = strong reaction, +++ = very strong reaction
b [t] = tolerant reaction, [i] = intermediary reaction, [s] = susceptible reaction to *S. sclerotiorum*.

Chromatographic investigation of healthy stem segments revealed no significant qualitative differences in fluorescent compounds. In all cases the major fluorescent compounds are several caffeic acid derivates (caffeoylquinic acids, chlorogenic acid) and flavonoids. However, in inoculated stem segments, a new compound appeared as diffuse band (Rf ~ 0,9) which is exclusively present by tolerant (necrotic) reaction to *S. sclerotiorum* infection. In watery and softy lesions such compound were absent (Fig. 2). Interestingly, further chromatographic analysis detects this compound not only in necrotic tissues but also 1-2 cm above and below the lesion zone of the two tolerant *H. maximiliani* species, indicating a more systemic response to fungal penetration. Our results are supported by similar chromatographic analysis in *Botrytis cinera* Pers. using inoculated necrotic tissues. Different response was achieved in stem segments naturally infected by biotrophic fungal parasites like *Erisiphe cichoracearum* DC. Ex Meret and *Sphaeroteca fuliginea* Schlecht. Ex feret. This leads to the hypothesis that certain resistance responses in tolerant species, are triggered by the perthotrophic behavior of the mold (e.g. pathotoxins like oxalic acid, polygalacturonases). The chemical nature of this compound is presently not clear and will be subject of subsequent investigations.

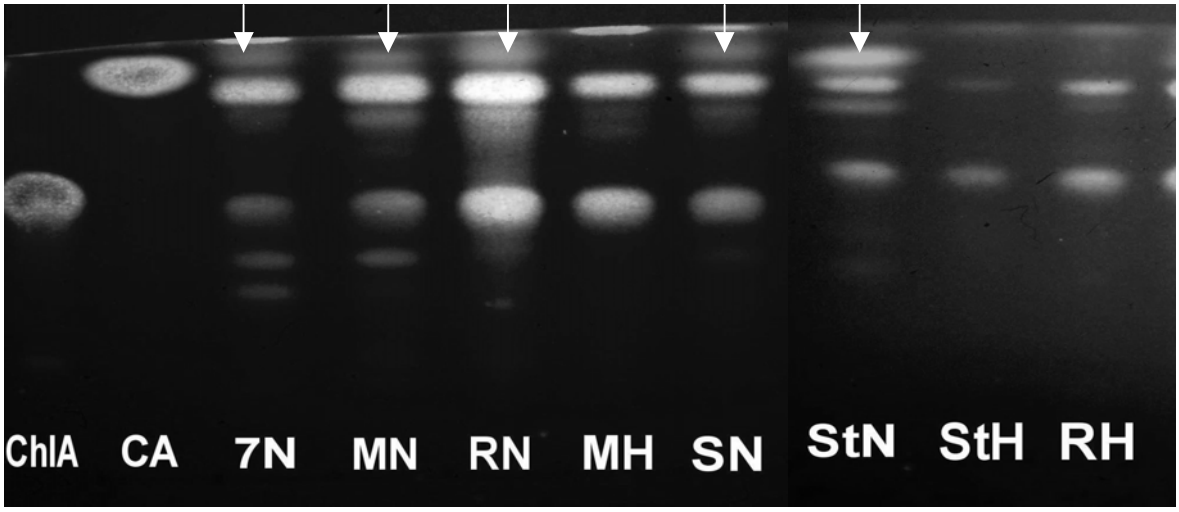

Fig. 2 HPTLC-chromatograms of different healthy and *S. sclerotiorum* inoculated stem tissues. ChlA = chlorogenic acid; CA = caffeic acid. The first letter indicates the species: 7 = *H. max* AC7, M = *H. max* ACM, R = *H. rigidus*, S = *H. salicifolius*, St = *H. strumosus*; the second letter indicates N = necrotic reaction and H= healthy tissue; arrows indicates fluorescent compound related to necrotic resistance reaction.

Conclusion

The results of the present investigation show that the correlation between lignification of the cortical stem tissue as well as the ability to produce low molecular fungistatic phenolic compounds might be closely related to *S. sclerotiorum* resistance. In addition the present results suggest that resistance linked markers can be developed for practical application in future breeding programs using wild *Helianthus* species as source for *Sclerotinia* (mid-stalk) resistance.

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