Potentially dangerous fusarioid microorganisms associated with rot of hop (*Humulus lupulus* L.) plants in field culture

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ABSTRACT

Several fusarioid microorganisms were isolated as potential pathogens of hop (*Humulus lupulus* L.) but their virulence was not proved in inoculation trials in field conditions. Molecular search for other possible pathogens was then performed. Using terminal restriction fragment length polymorphism (TRFLP), *Gibberella pulicaris* (anamorph: *Fusarium sambucinum*) was identified as a probable cause of the hop wilting. The primary cause of the disease is wounding of hop crowns by feeding of rosy rustic moth (*Hydraecia micacea*) caterpillars or by defect pruning and other unfavourable circumstances. The specific primer HLf1 was designed that can be used to detect the pathogen in soil and in damaged plant tissues.

Keywords: *Humulus lupulus* L.; bine wilt; rootstock; DNA; artificial infection; fusarium canker; soil microflora

Bine wilting and death of hop plants occur in spring period after training, most often during May and June, in all hop-growing areas of the Czech Republic. The incidence of hop canker in the field is usually sporadic, not every bine on hill is affected. Sometimes, however, there is an extensive occurrence of symptoms and the whole plants can be damaged. The disease is described as "fusarium canker", fungus *Fusarium sambucinum* being usually considered to be a probable cause of it (Neve 1990). This is not a specific problem of Czech hop cultures and information describing disease symptoms come also from Germany (Pichlmaier and Zinkernagel 1992), Poland (Solarska 1994) and England (Neve 1990).

The fungus usually invades the plant through mechanically damaged tissue during spring cutting of hop hills. It seems to be a critical point in disease occurrence. In gardens that are cultivated there is usually some mounding of soil around the hop plant. The commonest form of damage is at the base of the bine, which is girdled so that only the innermost core is left attached. This constriction causes the bine to wilt and the reason for this is readily identified by the way the narrow neck can be broken by a gentle pull. Normally, no special control measures are required other than good hygiene, and any diseased material being cut off and removed. Royle and Liyanage (1976), on the basis of information on hop canker from Germany, suggested that dips or drenches with maneb or benomyl might be useful. Trials were carried out to attempt chemical control of the problem and there were encouraging results from basal sprays with a formulation of thiabendazole (Darby 1984). Field observations have suggested that the onset of disease appears to be more severe under wet conditions, including growing seasons following flooding during wet winters. Hops grown in areas where the water table is high or where the drainage is poor showed a higher incidence of canker.

The reason of the problem and disease mechanism is still not fully elucidated. For example, we are still not sure about the identity of soil microorganism(s) that cause the disease. Phalip et al. (2004) report as potential pathogens filamentous fungi of the genera *Ascochyta, Fusarium, Phoma* and *Alternaria*, isolated from diseased hop. These

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microorganisms may accumulate in soil during prolonged monoculture (Čatská 1993), which is the case of hop fields. Soil microbial community thus becomes disbalanced and small plant wounds may then result in the disease development.

The goal of our work was to evaluate the occurrence of potentially pathogenic microorganisms in diseased hop fields in the Žatec county, as a representative region of Czech hop production, and compare the results with already available data. The works were performed in 2004–2007 vegetation periods in cooperation between the Hop Research Institute, Žatec and the Institute of Microbiology AS CR, v.v.i., Prague.

MATERIAL AND METHODS

Isolation and identification of microbial strains and testing their virulence

Soil and plant samples were collected from hop cultures near villages Stekník, Strkovice and Dobroměřice, county Žatec, Czech Republic. Fungal strains were isolated from soil surrounding decaying hops tissues and from tissues showing symptoms of wounding and beginning necrosis probably caused by microbial infection. Water soil suspension (1 g soil per 100 ml) was further diluted to reach dilutions $1:10^2$ and $1:10^3$, pipetted (0.5 ml) into 90 mm plastic Petri dishes and mixed with 20 ml solidifying modified Smith-Dawson agar (Gryndler et al. 2003). In total, 40 Petri dishes were used for isolation of fungi from soil samples.

Isolations of fungi from plant tissues were performed by explanting small pieces of the tissue and locating them onto the surface of solid Smith-Dawson medium in 40 Petri dishes.

After 5–10 days of incubation the growth character and microscopic features of colonies were evaluated and fusarioid colonies were reinoculated on brackish seawater nutrient agar, potato-dextrose agar and oat-meal agar suitable for morphology-based determination (Gerlach and Nirenberg 1982). Using this method, six different *Fusarium* morphotypes (F1, F2, F3, F4, F5 and F6) were isolated from soil and one from plant tissues (ST, Table 1). Isolates were further maintained on potato-dextrose agar and identified using the work of Gerlach and Nirenberg (1982).

DNA was extracted from mycelia of all 7 mentioned isolates using DNeasy Plant Kit (Qiagen). DNA was further amplified in PCR using fungus-specific primers ITS4S and ITS5 amplifying ITS region of the rDNA cassette. Reaction mixture contained 10 ng DNA template, 20 pmol primers, 0.2 mmol dNTP's and 1 unit DynaZyme DNA polymerase with buffer provided by the supplier. Thermocycler programme comprised initial incubation for 150 s at 95°C, followed by 32 cycles of 30 s at 95°C, 30 s at 55°C and 60 s at 72°C. Finally, the mixture was incubated for 9 min at 72°C.

Amplified fragments were purified using JetQuick kit (Genomed) and sequenced using the primer ITS4S on capillary sequencer ABI Prism 3100Genetic Analyzer.

The obtained sequences were compared to the sequences published in GenBank using NCBI BLAST searching tool to verify the identity of isolates.

In order to check the virulence, all the isolates obtained and 5 reference *Fusarium* isolates (Table 1) were cultivated for 10 days at 25°C on solid PDA medium. Resulting aerial mycelium was harvested using fine forceps and introduced into freshly deeply (2 mm) wounded hops footstalks. Wounds were then covered by polyethylene tape to avoid excessive leak of water. Five hops plants were inoculated this way at the beginning of the vegetation period by each isolate. Plants were regularly controlled for possible symptoms of infection till senescence.

Analysis of microbial communities of infected and intact underground plant tissues under field conditions

The samples of intact and infected hops rhizomes were taken at 6 different locations near the village Stekník. A piece of tissue was superficially explanted from each root sample (previously washed with sterile water), homogenized with DNA extraction buffer using sterile mortar and pestle and extracted using UltraClean Soil DNA Kit (MoBio). ITS region of rDNA cassette was then amplified in PCR using primers ITS1 and ITS4 (amplifying eukaryotic DNA), ITS1F and ITS4 (amplifying fungal DNA) as well as 16Seu27f and 783r amplifying eubacterial DNA.

PCR mixture contained 1 µl of DNA template in water, 13 µl REDTaq^{TM} ReadyMix^{TM} PCR Reaction Mix with $MgCl_2$ (Sigma), 0.5 µl of each primer and 10 µl water.

Eukaryotic DNA was amplified from 10 ng of extracted template DNA. After initial incubation for 5 min at 94°C, the mixture was subjected to 34 PCR cycles of 60 s at 94°C, 60 s at 52°C and

120 s at 72°C. Finally, the sample was incubated for 10 min at 72°C.

Eubacterial DNA was amplified from the same amount of extracted template DNA. After initial incubation for 5 min at 95°C, the mixture was subjected to 34 PCR cycles of 45 s at 94°C, 45 s at 57°C and 90 s at 72°C. Finally, the sample was incubated for 5 min at 72°C.

Amplified DNA was further analyzed using terminal restriction fragment length polymorphism (TRFLP). Briefly, approx. 200 ng of amplified fragments purified using UltraClean PCR Cleanup DNA Purification Kit (MoBio) was cleaved using 2 units of restriction endonuclease Taq I (Sigma) in buffer SB (Sigma) containing 0.17% BSA (total volume 25 µl) for 1 h at 65°C.

The restriction fragments were desalted by Post-Reaction Clean-Up Column (Sigma) and dried up under vacuum. A molecular weight standard (22 DNA fragments, 60–550 bp) was added to each sample before analysis. Terminal restriction fragments with lengths close to the lengths of standard fragments (approx. 16% of electropherograms) were excluded from analysis because of possible interference.

Electrophoretic analysis was performed on MegaBACE 1000 genetic analyzer (GE Biosciences, Piscataway, NJ) equipped with 60 cm long fused silica capillaries (40 cm effective length) with internal diameter 75 µm containing denaturing matrix GMC-MTRX and buffer GMC-MBBUFF01 (both from Genomac International, Prague, CZ). Samples were loaded electrokinetically for 3 s at 8 kV, time of analysis was typically up to 90 min at 50°C under 10 kV separation voltage. Detection was performed using laser-induced fluorescence detector (488 nm excitation and 520 nm emission wavelengths).

Search for occurrence of organisms associated with the damage of hop plant in soil

An amount of approx. 100 mg soil from 4 different localities near Stekník village was extracted using UltraClean Soil DNA Kit (MoBio) according to the supplier's recommendations. A soil subsample taken from surface soil layer and a subsample from the depth of 50 cm were always extracted per each soil sample. A part of ITS region of rDNA cassette was then amplified in PCR using primers HLf1 (see below for nucleotide sequence) and ITS4 under the same conditions as indicated above. The occurrence of amplification products was checked using routine agarose electrophoresis in TBE buffer with ethidium bromide staining.

RESULTS AND DISCUSSION

Results of isolation and virulence of microbial isolates

Potentially phytopathogenic isolates of fungi were obtained from damaged plants: *Fusarium oxysporum* Schlecht., *Fusarium equiseti* (Corda)

Table 1. Fusarioid fungal isolates used in inoculation experiment checking their virulence

Isolate	Origin	Identity[2]
F1	soil from hops field	*Fusarium oxysporum*
F2	soil from hops field	*Fusarium tabacinum*
F3	soil from hops field	*Fusarium tabacinum*
F4	soil from hops field	*Fusarium redolens*
F5	soil from hops field	*Fusarium oxysporum*
F6	soil from hops field	*Fusarium equiseti*
ST	infected hops tissue	*Fusarium equiseti*
RF186	tilled reference soil[1]	*Fusarium redolens*
RF285	tilled reference soil	*Fusarium redolens*
SF131	tilled reference soil	*Fusarium* sp.

[1]soil from an experimental field of the Institute of Plant Production, Prague-Ruzyně. This field is not used for hop production and annual crops are cultivated there instead

[2]identity is based on morphological determination verified using comparison of sequences of ITS region with GenBank data. ITS sequence of isolates F2 and F3 show 99% homology with sequences published for *Monographella cucumerina* (Lindf.) Arx (in GenBank under syn. *Plectosphaerella cucumerina)*, imperfect stage of *Fusarium tabacinum* (Arx 1984)

Sacc., *Fusarium redolens* Wollenw. and *Fusarium tabacianum* (J.F.H Beyma) W. Gams (Table 1). Their virulence was examined together with three reference isolates (RF186, RF285 and SF131). With these isolates, we never succeeded to obtain the symptoms of pathogenicity in hop plants and even negligible marks of necrosis were not induced on the inoculated place of wounding.

Identification of an organism associated with infected plant tissues using TRFLP

Fragment (237 bp) tending to associate with damaged plants was found in 5 out of 6 infected tissue samples and in only 1 intact sample (Table 2) and was a major fungal fragment in one of the samples which enabled us direct amplification and sequencing of its bearer's DNA. The obtained sequence was compared with GenBank data and a region of very high sequence variability was chosen as the basis for designing the primer, which would recognize the target sequence of the organism associated with damaged plant tissues. The one intact sample with positive detection of 237 bp fragments gave only a very weak signal.

The origin of this region is localized at the distance of –121 bp from TaqI restriction site (thymine) and +116 bp from the origin of the ITS1F priming site and is localized in ITS1 region. The forward primer corresponding to this sequence was named HLf1 and its sequence is 5'-CGGATCAGCCCGTTCCTCG-3'. When used together with reverse primer ITS4, it gives a product of approximate length 560 bp.

Whole sequenced region is 491 bp long and its sequence corresponds to 13 GenBank sequences showing 100% homology of HLf1 site. These GenBank sequences belong to fungi *Gibberella pulicaris* (Fr.) Sacc., *Fusarium sambucinum* Fuckel and *Fusarium tumidum* Sherb. (Table 3).

Search for occurrence of organisms associated with the damage of hop plant in soil

HLf1-ITS4 primer pair provided an amplified product only in one of the 4 studied soil samples and, moreover, only in the depth of 50 cm, indicating that the bearer of HLf1 priming site is relatively infrequently present in the soil, but is not associated only with surface soil layers.

Our work was directed to detect possible pathogenic fungi causing the rot of underground hop tissues primarily damaged by cutting maintenance. We isolated several fusarioid fungi but did not succeed to induce the infection using these organisms; it indicates that the used microorganisms are probably not the cause of the disease and that pathogenic agens cannot be easily isolated.

Genetic analysis of microorganisms colonizing intact and infected plant tissues revealed that an uncultured fungal organism tending to associate with infected plant tissue could be recognized. When the DNA fragment characteristic for this organism was sequenced, a homology was found with a set of GenBank sequence records belonging to *Gibberella pulicaris*, *Fusarium sambucinum* and *Fusarium tumidum*. *Fusarium sambucinum* var. *sambucinum* is an anamorph of *Gibberella pulicaris* (Saccardo 1877). *Fusarium tumidum* and *Fusarium sambucinum* belong to the same section of Discolor/Fusarium (Gerlach a Nirenberg 1982). The taxonomic position of all the three taxa is thus concordant.

Table 2. Occurrence of fungal terminal restriction fragments (lengths are given in bp) in intact and infected plant tissues collected at 6 different sampling locations

Plant tissue	Location number					
	1	2	3	4	5	6
Intact	none	74, 76, 96, 100, 125, 143, 58, 235, 239, 254, 287	73, 99, 123	99, 130, 158, 166, 224, 234, 239, 244, 241, 247, 265, 272, 481, 482, 484, 489	158, 164, 239, 242, 251, 258, 275, 293	158, 164, 234, 237, 240, 248
Infected	96, 131, 234, 237	237, 296	158, 209, 234, 241, 244, 254, 258	131,158, 164, 225, 237, 240, 246, 255, 268, 277	234, 237, 240, 247, 249, 254, 255, 257, 266, 291	124, 133, 158, 163, 172, 220, 237, 241, 249, 258, 260, 270, 272, 286, 287, 297, 339

Table 3. GenBank sequences showing 100% homology of HLf1 priming site. If the Genbank sequence shows deletions or insertions, it is indicated in the rightmost column by remarks (del) or (ins), respectively

GenBank accession number	Identity	Percentage of whole sequence homology	Length of alignment (bp)
AY305372	*Fusarium* sp. 02TRU712 from *Humulus lupulus*	99	489
AY147372	*Gibberella pulicaris* isolate IBT8158	100	491
AY147371	*Gibberella pulicaris* isolate IBT2365	100	491
AY147370	*Gibberella pulicaris* isolate IBT1744	100	491
X65481	*Fusarium.sambucinum* (NRRL 13700, 13504)	99	491/489 (del)
X65478	*Fusarium sambucinum* (NRRL 20444, 20663)	99	491/489 (del)
AF111062	*Gibberella pulicaris* strain BBA67721	100	419
U85542	*Fusarium tumidum*	99	491/492 (ins)
U85540	*Gibberella pulicaris*	100	491
AF006349	*Fusarium tumidum* NRRL 22240	100	491
AF006346	*Gibberella pulicaris* NRRL 22203	99	491
U34579	*Fusarium sambucinum* NRRL 13708	100	491
AY188921	*Gibberella pulicaris*	99	491/490 (del)

The homology of the sequence of our 237 bp terminal restriction fragment with *Fusarium* sp. 02TRU712 isolated from rotting hop by Phalip et al. (2004) is interesting. This indicates that our results are consistent with the results of other authors and that organisms taxonomically close to *Fusarium sambucinum* may be commonly associated with rotting of mechanically damaged hop tissues. It is thus a possible pathogenic agens contributing to the rotting process. This agrees with the results of Royle and Liyanage (1976) and Neve (1990).

Unfortunately, we were unable to isolate the organism from rotting tissues; hence, we could not study directly its ability to cause the disease. It probably attacks mechanically damaged hop tissues together with other components of soil microflora and must be thus considered as potentially dangerous phytopathogen.

Fusarium tumidum, another possible candidate to be the disease-causing agens, is a phytopathogenic fungus that may be used as a bioherbicide against *Ulex europeus* and *Cytisus scoparius* (Morin et al. 2000). If this fungus or other closely taxonomically related species are used as bioherbicides in areas where hop is cultivated, it may represent a menace for plant culture and its compatibility with hop should be carefully assayed.

In June 2005 multiple wilt of hop plants was observed on the farm of Hop Research Institute in Stekník. The death was observed mainly on the margins of hop gardens, which are usually weedier than internal rows. The presence of rosy rustic moth (*Hydraecia micacea*) was determined as primary reason of death of hop plants; it can survive there due to the presence of couch grass (*Elytrigia repens* L.). In early spring, cutworms of rosy rustic moth start to eat young hop shoots, draw down to the crown and continue flattening of root system. Detrimental action of rosy rustic moth in the season terminates by turning to cocoon. Cocoons survive in soil and the process repeats the next spring. Eating of root system creates entrance gateway for action of pathogenic soil microorganisms. Damaged plants really showed typical "fusarium canker" symptoms. The affected bine wilted rapidly. They could be detached readily from the crown with a gentle tug. The point of bine attachment to the crown was tapered and rounded off so that only a few vascular elements connected the bine to the crown. In this situation, the isolation of several isolates of saprotrophic fungi was attempted. It could be rightfully supposed that isolates are associated with the damage of hop root system.

At the beginning of May 2006 fungal isolates were applied to hop plants in the form of mycelia suspension in water shortly after pruning. The aim was to examine how direct inoculation of hop plants with pathogenic microorganisms at the time of pruning influence the health state

of hop plants during next vegetation season. No growth defects, bine wilting or even plant death were observed till the end of current and following (2007) vegetation seasons. It shows that direct inoculation of pruned hop plants by pathogenic soil microorganisms does not cause the fatal damage of hop plants. The whole problem seems to be much more difficult. Further research is necessary to evaluate the pathogenicity of the fusarioid organisms discussed in this article, whenever its isolates are available.

Nevertheless, it is possible to minimize the opportunity of microbial attack of hop tissues by careful management (cutting) the plants, keeping hop gardens free of weed as well as by avoiding crashing which produce large surface of the wounded tissue to soil microorganisms.

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