POTENTIALLY DANGEROUS FUSARIOID MICROORGANISMS ASSOCIATED WITH ROT OF HOP (*Humulus lupulus* L.) PLANTS IN FIELD CULTURE

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INTRODUCTION

Occasions of bines wilting and death of hop plant occur in spring period after training, the most frequently during May and June in all hop growing areas of Czech Republic. The incidence of hop canker in the field is usually sporadic, not every bine on hill is affected. But sometime there is an extensive occurrence 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, Poland and England. The fungus usually invades the plant through mechanically damaged tissue during spring cutting of hop hills. Field observations have suggested that the onset of disease appears to be more severe under wet conditions. 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 are the cause of the disease. Phalip et al. (2004) report filamentous fungi of the genera Ascochyta, Fusarium, Phoma and Alternaria, isolated from diseased hop, as potential pathogens. The goal of our work was to evaluate the occurrence of potentially pathogenic microorganisms in diseased hop field in Žatec county, as a representative region of Czech hop production, and correlate the data with already known facts.

MATERIALS AND METHODS

Isolation and identification of microbial strains and testing their virulence

Soil and plant samples were collected from hops cultures localized 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 microbial infection 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). Six different Fusarium morphotypes (F1, F2, F3, F4, F5 and F6) were isolated from soil and one from plant tissues (ST, Table 1). DNA was extracted from mycelia further amplified in PCR using fungus-specific primers ITS4S and ITS5. Amplified fragments were purified and sequenced using the primer ITS4S on capillary sequencer ABI Prism 3100Genetic Analyzer. The obtained sequences were comparedto 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 and introduced into freshly deeply (2mm) wounded hops footstalks. Woundings were then covered by polyethylene tape to avoid excessive leak of water. Five hops plants were this way inoculated at the beginning of 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, homogenized with DNA extraction buffer 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. The restriction fragments were desalted by Post-Reaction Clean-Up Column (Sigma) and dried up under vacuum. A molecular weight standard was added to each sample before analysis.

Search for occurrence of damage hop plant associated organism in soil

An amount of approx. 100 mg soil from 4 different localities near Stekník village was extracted using UltraClean Soil DNA Kit (MoBio). A soil subsample taken from surface soil layer and a subsample from the depth 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.

RESULTS AND DISCUSSION

Results of isolation and virulence of microbial isolates

Potentially phytopathogenic isolates of fungi were obtained from damaged plants: Fusarium oxysporum, Fusarium equiseti, Fusarium redolens and Fusarium tabacinum (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 fragment gave only very weak signal.

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).

Table 1. Fusarioid fungal isolates used in inoculation experiment

Isolate	Origin	Identity [2]
F1	Soil from hop field	Fusarium oxysporum
F2	Soil from hop field	Fusarium tabacinum
F3	Soil from hop field	Fusarium tabacinum
F4	Soil from hop field	Fusarium redolens
F5	Soil from hop field	Fusarium oxysporum
F6	Soil from hop field	Fusarium equiseti
ST	Infected hop 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 experimental field of Institute of Plant Production, Prague-Ruzyně. This field is not used for hops 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.

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

GenBank accession No.	Identity	% 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)

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 No					
	1	2	3	4	5	6
Intact	None	74, 76, 96, 100, 125, 143, 158, 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

HLf1-ITS4 primer pair provided an amplified product only in one of the 4 studied soil samples and, more over, only in 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.

In June 2005 multiple wilt of hop plants was observed on farm of Hop Research Institute in Stekník. The death was observed mainly on 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 hop plants death that can survive there due to 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. 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 (see Fig. 1-3).

At the beginning of May 2006 fungal isolates were applied to hop plants in the form of mycelia suspension in water shortly after pruning. There were expectation how direct inoculation of hop plants with pathogenic microorganisms at the time of pruning influence the helth state of hop plants during next vegetation reason. No growth defects, bine wilting or even plant death were observed till the end of current and subsequent (2007) vegetation seasons. It shows that neither direct inoculation of pruned hop plants by pathogenic soil microorganisms 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 here, whenever the isolates of the said organisms will be available.

However, 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, avoiding crashing which produce large surface of the wounded tissue to soil microorganisms.

CONCLUSION

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, avoiding crashing which produce large surface of the wounded tissue to soil microorganisms.

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Fig. 1 Catepillars and cocoons of rosy rustic moth (Hydraecia micacea)

Fig. 2 Couch grass (Elytrigia repens L.) and wilted hop bines

Fig. 3 Detailed view of damaged hop bines by rosy rustic moth and fusarium fungi

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