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INFLUENCE OF *SALVIA OFFICINALIS* L. HAIRY ROOTS DERIVED PHENOLIC ACIDS ON THE GROWTH OF *CHAETOMIUM GLOBOSUM* AND *TRICHODERMA VIRIDE*

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Abstract:

The influence of Salvia officinalis L. hairy roots derived phenolic acids on the growth of two typical cellulose degrading moulds, Chaetomium globosum and Trichoderma viride, was examined. For this, 26 hairy root lines of S. officinalis were induced and investigated with regard to the production of phenolic acids. Pure, commercial phenolic acids, analogues to that biosynthesised in the hairy root

cultures, were solved in 96% ethanol and applied to malt extract agar plates. The fungal cultures were transferred to the agar plates, and fungal growth was observed 5 and 12 days after inoculation. The hairy root line A produced the highest amount of total phenolic acids (1582.0µg/g), while line B produced the highest amount of sinapic acid (262.8µg/g). Trans-cinnamic and salicylic acid showed antifungal activity to the growth of C. globosum. The growth of this strain was inhibited by vanillic, p-coumaric and sinapic acid. Moreover, it was observed that sinapic acid prevented the spore formation of C. globosum. Protocatechuic, sinapic, and salicylic acid inhibited the growth of T. viride minimal. Furthermore, only sinapic acid inhibited the spore formation of this mould. The investigations have shown antifungal activity of six S. officinalis hairy root derived phenolic acids on malt extract agar plates.

***Keywords**: Salvia officinalis L.; hairy root cultures; phenolic acids; mould fungi; antifungal activity.*

INTRODUCTION

Lignocellulosic materials are hygroscopic and they consist of attractive nutrients for mould fungi. For specific applications they need to be protected against microbial degradation. For products made of wood and paper, chemicals like boric, fluoride, copper or triazole compounds are used as fungicides. The European Biocidal Product Regulation (EP 2012) regulates the market of such fungicide active substances. A lot of them are dangerous for human health and the environment, especially in higher doses. In particular, the use of boric compounds was discussed with much controversy in recent years. For future there are challenges to establish sustainable fungicides, which are not dangerous for human health and the environment. Manufacturers of products made of renewable raw materials searching for bio-active substances as alternative to conventional fungicides.

Secondary metabolites of plants are used for example as flavours or pigments. Furthermore they could have antimicrobial, and especially antifungal activities (Angelini *et al.* 2006). Plants are able to use the secondary metabolites to defend natural enemies like fungi, bacteria, viruses and animals. Sage (*Salvia officinalis* L.*)* is used in cuisine, but also well-known in medicine for its healing ability. Sage contains different agents, like flavonoids, terpenes or phenolics that exhibit antibacterial, antiviral and antifungal activities (Blumenthal *et al.* 1998, Gruenwald *et al.* 2000, Badiee *et al.* 2012, Martins *et al.* 2015).

Extracts or ingredients of plants are traditional produced by agricultural cultivation. Dependent on the location and period of the production, the extractives obtained differs in quality and quantity. For a continuous, sustainable production of plant ingredients, the use of biotechnological processes is required. One possibility is the cultivation of hairy roots (Mehrotra *et al.* 2015), the extraction and purification, as well as the formulation of the desired extracts respectively ingredients.

OBJECTIVE

The subject of this work was to investigate the antifungal activity of metabolites, derived of *Salvia officinalis* L. hairy root cultures. The influence of 9 phenolic acids on the growth of the moulds *C. globosum* and *T. viride* was examined.

MATERIAL, METHOD, EQUIPMENT

Investigation of *Salvia officinalis* hairy root cultures

Hairy root cultures of *Salvia officinalis* L. were induced using the two-phase method as previously described (Marchev *et al.* 2011). For this purpose, young leaves of *S. officinalis* plants were used, their origin and sterilisation conditions were described elsewhere (Haas *et al.* 2014). Hairy roots were cultivated on solid Murashige and Skoog media supplemented with 30g/l sucrose (all chemicals were ordered from *Duchefa*), and sub-cultivated every 3 weeks. After a 3-week cultivation period hairy roots were harvested, rinsed with distilled water, and freeze dried prior extraction and analysis of phenolic acids. The production of the phenolic acids listed in Table 1 by the different hairy root lines was analysed by high performance liquid chromatography (HPLC) as described by Marchev *et al.* (2011).

Solutions of the bio-active substances

The used phenolic acids in the form of pure, commercial compounds and the fungicide control (see Table 1) were solved in 96% ethanol with a concentration of 5.88wt% (6.25 $g_{active\ substance}$ + 100 $g_{solvent}$). Only sinapic acid was solved with a concentration of 2.64wt% (2.71 $g_{active\ substance}$ + 100 $g_{solvent}$) because of its limit of solubility. For sterilization the produced solutions were filtrated by a cellulose acetate membrane with a pore size of 0.2µm.

Table 1

Used phenolic acids appearing in Salvia officinalis hairy roots, reference and controls

Phenolic acid		Control or reference	
Name (*supplier*)	**Abbreviation**	**Name (supplier)**	**Abbreviation**
p-coumaric acid (*ABCR*)	CA	Malt extract agar (*Carl Roth*), reference	REF
Ferulic acid (*Carl Roth*)	FA		
Gallic acid (*ABCR*)	GA	96 % ethanol, solvent control	ETOH
Protocatechuic acid (*ABCR*)	PA	Cyproconazole (*CHEMOS*), fungicide control	CYPR
Salicylic acid (*ABCR*)	SA		
Sinapic acid (*ABCR*)	SIA		
Syringic acid (*ABCR*)	SYA		
trans-cinnamic acid (*ABCR*)	TCA		
Vanillic acid (*ABCR*)	VA		

Fungal cultures

Two different mould species were used: *Chaetomium globosum* Kunze:Fries (DSM No. 1962), and *Trichoderma viride* Persoon:Fries (DSM No. 63065). *C. globosum* causes soft-rot (Takahashi 1978), and discolorations in wood (Schmidt 2006). *T. viride* degrades the cellulose of wood by using several enzymes, like endo- and exoglucanases, as well as β-glucosidases (Eriksson *et al.* 1990). Fungi were cultured on 92mm Petri dishes (*Sarstedt*) with 1.5% malt extract agar at 28°C ± 2°C. Before that, culture medium was autoclaved at 121°C for 20min.

Preparation, inoculation and incubation of agar plates

For the screening 92mm Petri dishes with 1.5% malt extract agar were used. Culture medium was autoclaved at 121°C for 20min. A volume of 200µl of each bio-active substance solution was pipetted to an agar plate, and spread on the agar surface with a sterilized Drigalski spatula. A fungal culture with a diameter of 10mm was outdone of an agar pre-culture, and transferred into the middle of the treated agar plate with tweezers. The samples were incubated at 28°C ± 2°C.

Fungal growth measurement

The fungal growth was documented 5 and 12 days after inoculation. The investigated agar plates were photographed with the same light and focus distance all time. The radius of the fungal growth was measured using the image processing programme *ImageJ*. For this, the maximal growth radius was measured in four directions. The mean value was calculated for all tested bio-active substance solutions.

RESULTS AND DISCUSSION

Production of phenolic acids by *Salvia officinalis* hairy roots

Using the common methods of direct infection or co-cultivation (Pavlov *et al.* 2002) the induction of hairy roots from *S. officinalis* pot-grown plants was not successful. No hairy roots appeared, and the explants started browning after 2-3 weeks. Therefore, the two-phase method was used which based on the application of absorber resin to the induction media, and was developed by Marchev *et al.* (2011) for the hairy root induction with *Salvia tomentosa* Mill. The reason for the unsuccessfully induction of *S. officinalis* hairy roots could be the same as suggested for *S. tomentosa*. The excess of secondary metabolites, in particular phenolic compounds, lead to explant necrosis before the hairy root induction. Analysis of resins used during the induction revealed phenolic acids and flavonoids whereas no or less amounts of these substances were found in the media. The binding of the substances avoided explant death, allowed the formation of hairy roots, and finally 26 lines were obtained. Grzegorczyk *et al.* (2006) induced hairy roots of *S. officinalis* from shoot cultures. Under in vitro conditions a plant or organ could have reduced amounts of secondary metabolites as the environment is fully controlled and free of pathogens that need to be fended. This could be the reason why this approach was working too.

Hairy roots induced in the present study were screened for the above mentioned phenolic acids after one sub-cultivation period to identify applicable lines. Two lines are presented exemplarily

in Table 2. Line A produced the highest amount of phenolic acids, and Line B produced the highest amount of sinapic acid.

Table 2

Contents of phenolic acids of Salvia officinalis hairy root cultures

Phenolic acid	Content [µg/g dry weight]	
	Line A	Line B
PA	491.6	178.6
SA	330.3	104.9
FA	233.7	130.9
VA	196.8	152.2
SIA	153.5	262.8
CA	65.9	50.1
TCA	64.2	0.0
SYA	46.0	0.0
GA	0.0	0.0
Total	*1582.0*	*879.5*

Growth of *Chaetomium globosum*

The growth radiuses of *C. globosum* on agar plates treated with different phenolic acids are displayed in Fig. 1. After 12 days of incubation the bio-active substances SA and TCA showed the same antifungal activity, like the fungicide control CYPR. SIA and CA inhibited the growth of *C. globosum* slightly, and VA moderately. GA, SYA, FA and PA had no impact to the fungal growth. Furthermore it was observed, that SIA prevented the spore formation of this fungal species. The solvent control ETOH nearly halved the growth compared to the reference.

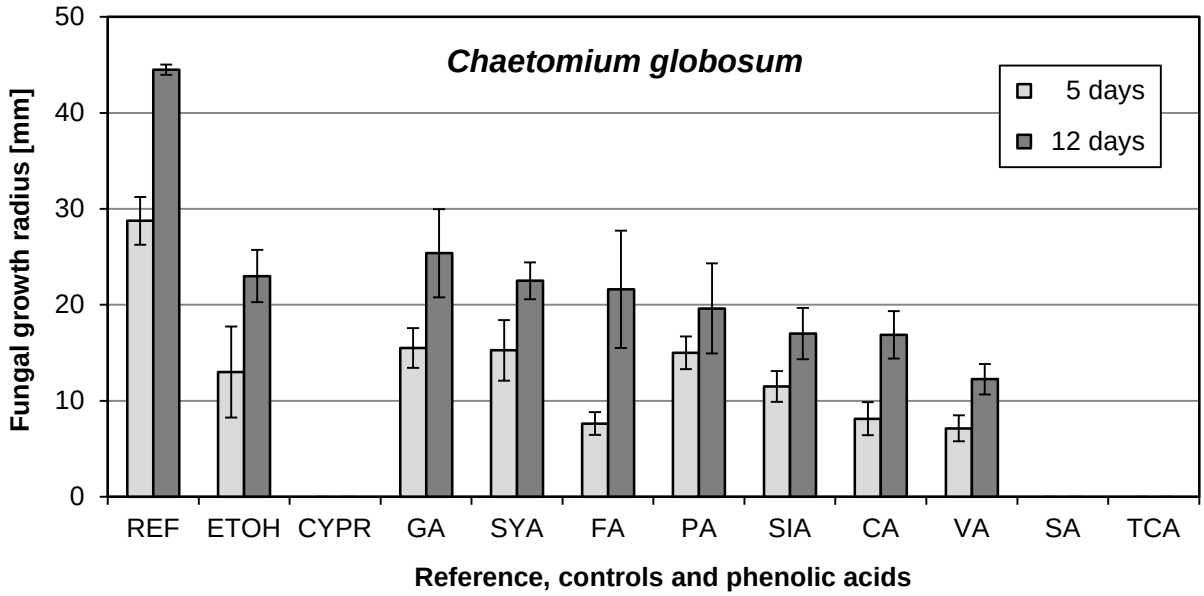

Fig. 1.
Growth radius in mm (n = 8) of Chaetomium globosum after 5 and 12 days of incubation for the reference, the controls and phenolic acids, appearing in the investigated Salvia officinalis hairy root lines

In Fig. 1 is also visible that FA and VA showed nearly the same slight inhibition after 5 days, but after 12 days of incubation FA showed no inhibition, in contrast to VA.

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The influence of ETOH, CYPR and TCA on the growth of *C. globosum* is depicted in Fig. 2. Besides the reduced growth of the fungus on the agar plate with ETOH (b), the spore formation was reduced as well, in comparison to the reference (a). This effect was observed for all agar plates treated with phenolic acids. As can be seen, a total inhibition of the fungal growth was caused not only by the standard fungicide CYPR (c), but also by the bio-active substance TCA (d).

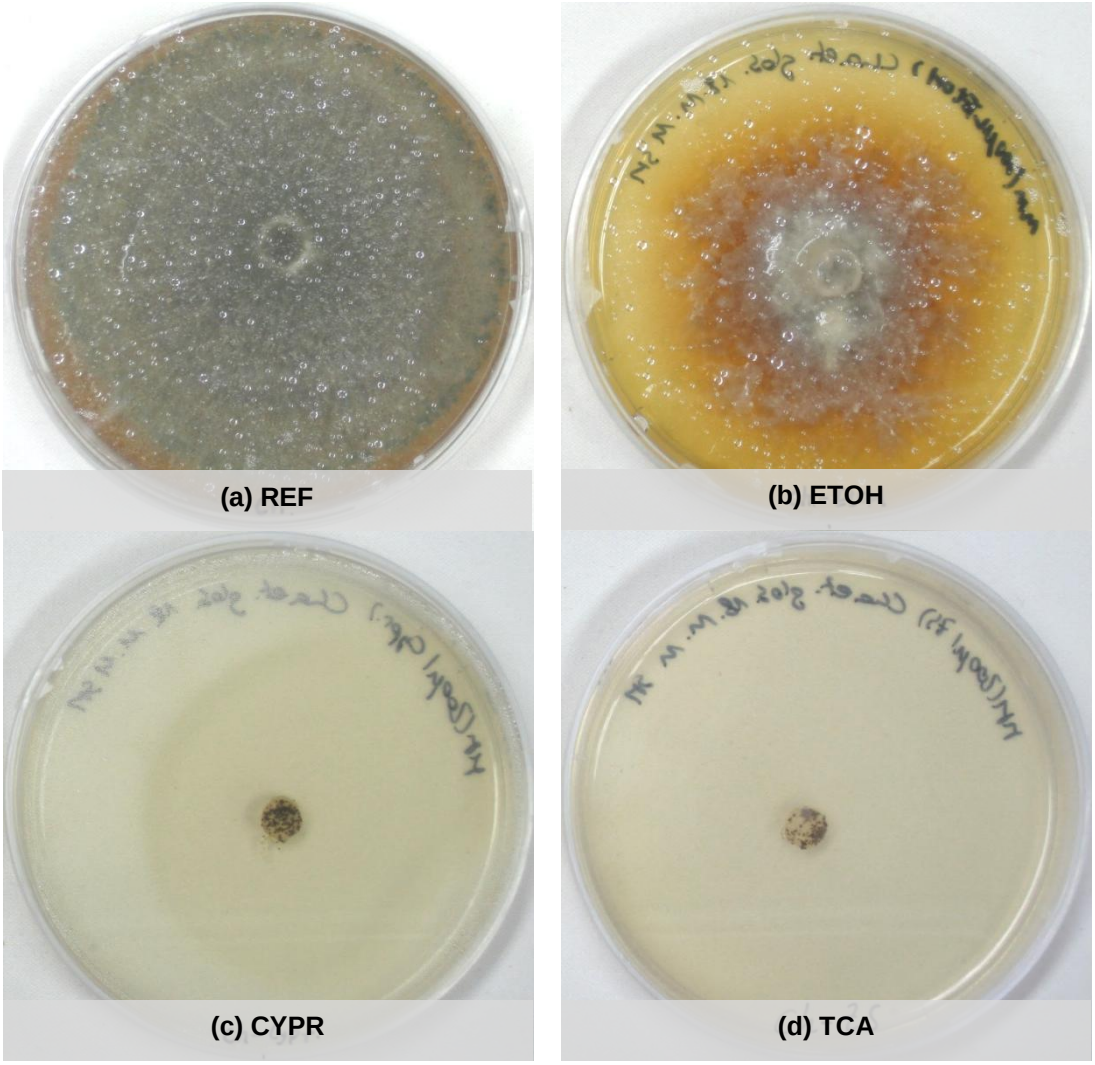

Fig. 2.
Influence of ETOH (b), CYPR (c) and TCA (d) on the growth of Chaetomium globosum after 12 days of incubation

Within the regulation of biocidal products in the European Union (EP 2012) CYPR had been approved as an active substance for use in biocidal products for wood preservatives (EC 2014). The chemical fungicide CYPR is harmful to human health and dangerous to the environment. In contrast, TCA is only irritant, but not dangerous for human health and the environment.

Growth of *Trichoderma viride*

The influence of the tested phenolic acids to the growth of *T. viride* is illustrated in Fig. 3. After 12 days of incubation SA, SIA and PA inhibited the fungal growth slightly. It should be noted that SIA was dissolved in lower concentration. GA, CA, FA, TCA, SYA and VA had no influence to the growth of *T. viride*. The solvent control ETOH showed nearly no inhibitory effect after 12 weeks of incubation, and CYPR as fungicide control just inhibited the fungal growth. CYPR prevented, and SIA inhibited the spore formation of *T. viride*. It can be seen that *T. viride* consists of a lower growth rate as *C. globosum*, because of a smaller fungal growth radius of the reference. In contrast, *T. viride* is more resistant to the bio-active substances SA and TCA as *C. globosum*. In Fig. 3 is visible that SA and

CYPR showed a similar moderate inhibition after 5 days of incubation. After 12 days CYPR inhibits still moderate, but SA only slightly the growth of *T. viride*.

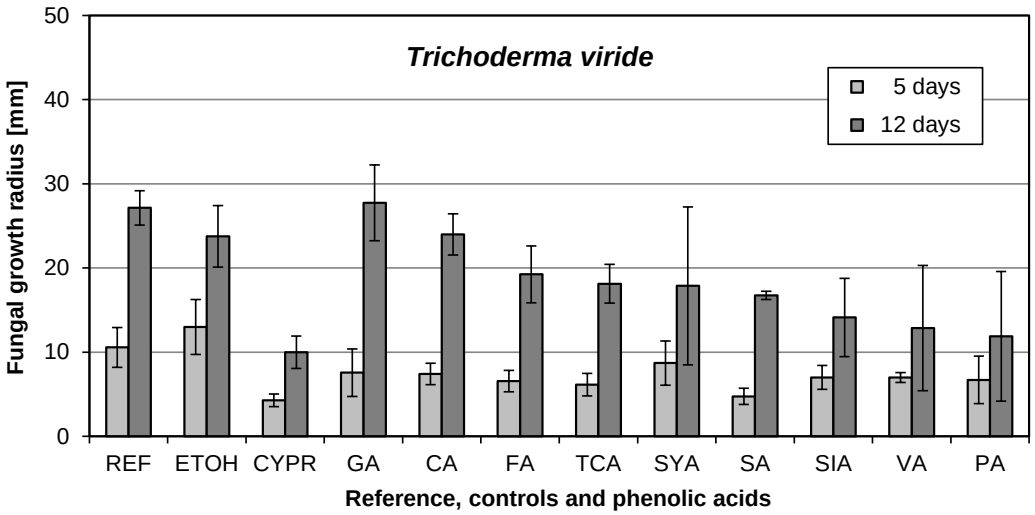

Fig. 3.
Growth radius in mm (n = 8) of Trichoderma viride after 5 and 12 days of incubation for the reference, the controls and phenolic acids, appearing in the investigated Salvia officinalis hairy root lines

CONCLUSIONS

In summary two phenolic acids, *trans*-cinammic acid and salicylic acid, appearing in *S. officinalis* hairy root lines showed the same antifungal activity against *C. globosum*, like cyproconazole. The influence of the tested phenolic acids on the growth of *T. viride* was lower as on the growth of *C. globosum*. Specific attention should be given to sinapic acid which inhibited the growth of both tested fungi, but in a lower concentration. It is remarkable that cyproconazole as fungicide control, just inhibited the growth of *T. viride*.

The determination of minimum inhibitory concentrations of the phenolic acids with inhibitory effects against *C. globosum* and *T. viride* will be examined in future experiments. There is also a need to clarify the influence of phenolic acids on the growth of other wood and paper affecting moulds. For this, it is necessary to increase the number of samples to achieve a better confidence level.

A further interesting study would be to analyse the crude ethanolic extracts from different *S. officinalis* hairy root lines with regard to their antifungal activities. To correlate the antifungal activity of the extracts and to identify the most active phenolic acid would contribute to investigate synergistic and antagonistic effects as reported for other biological activities such as antioxidant activity (Li *et al.* 2011, Zanfini *et al.* 2010). *S. officinalis* hairy root lines are very appropriate for that as they provide various metabolite spectra which can be different from the intact plant too.

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