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STSM TITLE: SCREENING OF BIOLOGICAL ACTIVITY FROM EXTRACTS OF ENDOPHYTIC FUNGI ISOLATED FROM GRAPEVINE

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MOTIVATION FOR THE STSM:

An increasing demand for new bioactive molecules in the pharmaceutical and agrochemical industries is pushing research towards the exploitation of ecological interactions in diverse biological systems. It is thus necessary to investigate the potential of molecules produced by organisms engaged in symbiosis, given that there is an intense chemical welfare among the symbionts, the host and the surroundings. Endophytism is a trait of some microorganisms in which colonization of the host plant is apparently asymptomatic in a process that might benefit the host through the production of bioactive compounds by the endophytes.

From a human standpoint, antibiotics are among the most important types of molecules synthesized by endophytes. These have become the target of research because of their biological activities and specificity, but also because the massive production of such compounds could be possible in a controlled environment in the laboratory. Nonetheless, other types of molecules with a wide range of biological activitiy have been outlined, including analgesics, blood-pressure lowering compounds, antivirals, plant hormones, etc. The disadvantages of these applications, however, are problems regarding the up-scaling for industrial synthesis and the limited understanding of the ecological contexts in which these molecules are produced.

The more we get to know about endophytism from the ecological perspective, the better our skills will become when it comes to commercially producing such molecules. The further advantages of understanding which molecules and why they are produced relate to the evolutionary implications of virulence and how research can tackle virulent organisms by using a more ecological and environmentally friendly approach that would reduce the amount of resistance generated by the current chemotherapeutic agents used in infection control.

GOALS OF THE STSM:

This short term scientific mission aimed at screening extracts from 14 fungal endophytes isolated from Italian grapevine (*Vitis vinifera* L) for biological activity both in mono-culture and in dual culture with bacteria. The selection of the fungi used in this work comes from a previous experiment in which we had tested the virulence potential of the fungal endophytes in different hosts (grapevine, tomato plants and *Arabidopsis thaliana*).
In the STMS, after inoculation of dual cultures with reference strains *Escherichia coli* K12 and *Bacillus megaterium* DSMZ 319, we observed possible antagonism in this setting and proceeded to extract secondary metabolites from the cultures. Finally, bioguided assays in 96-well plates revealed potential candidate extracts that will be further analyzed for different types of biological activities and the responsible secondary metabolites.

DESCRIPTION OF THE WORK CARRIED OUT:

1. **Experimental design**:

   14 strains of endophytic fungi isolated from grapevine were simultaneously inoculated in dual culture with the reference strains *E. coli* K12 and *B. megaterium* DSMZ 319. Each assay was done in triplicate and before extraction the samples belonging to the same treatment were pooled.

2. **Experimental procedures:**

   2.1. Organisms

   A list of the endophytic fungi used in this work is provided below

   Table 1. Endophytic fungi isolated from grapevine and employed in these experiments. Endophytes were selected previously after virulence tests in different host plants. Fields marked in green are strains that are avirulent in *V. vinifera* L. Fields marked in orange are strains that were virulent in *V. vinifera* L and in most of the other hosts (tomato and *A. thaliana*)

ID number	Possible taxonomy and virulence	Host	Isolation place	Year	Molecular marker for taxonomy
2	*Cryptococcus* sp.	Vitis vinifera silvestris	Milano, Italy	2012	ITS 1 - ITS 4
3	*Epicoccum nigrum*	Vitis vinifera silvestris	Milano, Italy	2012	ITS 1 - ITS 4
6	*Phoma herbarum*	Vitis vinifera vinifera	Trentino/Alto Adige, Italy	2011	ITS 1 - ITS 4
10	*Fusarium* sp.	Vitis vinifera vinifera	Trentino/Alto Adige, Italy	2012	ITS 1 - ITS 5
11	*Giberella pulicaris*	Vitis vinifera vinifera	Trentino/Alto Adige, Italy	2013	ITS 1 - ITS 6
14	*Emericella* sp.	Vitis vinifera vinifera	Trentino/Alto Adige, Italy	2011	ITS 1 - ITS 4
15	*Nectria mauritiicola*	Vitis vinifera vinifera	Trentino/Alto Adige, Italy	2011	ITS 1 - ITS 4
16	*Beauveria bassiana*	Vitis vinifera vinifera	Trentino/Alto Adige, Italy	2011	ITS 1 - ITS 4
17	*Paecilomyces variotii*	Vitis vinifera vinifera	Trentino/Alto Adige, Italy	2011	ITS 1 - ITS 4
19	*Truncatella angustata*	Vitis vinifera vinifera	Trentino/Alto Adige, Italy	2011	ITS 1 - ITS 4
21	*Alternaria* sp./*Coniothyrium* sp.	Vitis vinifera vinifera	Trentino/Alto Adige, Italy	2011	ITS 1 - ITS 4
23	*Ampelomyces humali*	Vitis vinifera vinifera	Trentino/Alto Adige, Italy	2011	ITS 1 - ITS 4
24	Valsa sordida (Cytospora chrysosperma)	Vitis vinifera vinifera	Trentino/Alto Adige, Italy	2011	ITS 1 - ITS 4
26	*Valsa malicola*	Vitis vinifera vinifera	Trentino/Alto Adige, Italy	2011	ITS 1 - ITS 4
27	*Valsa ambiens / Cytospora carbonacea*	Vitis vinifera vinifera	Trentino/Alto Adige, Italy	2011	ITS 1 - ITS 4
30	*Dothideomycetes* sp. *(Microsphaeropsis arundinis)*	Vitis vinifera vinifera	Trentino/Alto Adige, Italy	2011	ITS 1 - ITS 4

2.2. Growth conditions of fungi:
Endophytic fungi and test bacteria were initially grown on three agar media (Nutrient medium, biomalt medium- and CP medium). After incubation for 5 days, CP agar medium (yeast extract 1g, glucose 1g, agar 1.5 g, 100 ml H_2O, pH 6.2) was chosen as the best medium for growth of both types of organisms. Stocks of endophytic fungi were kept at room temperature in CP agar supplemented with grapevine leaves and antibiotics (Penicilin 60 µg/ml, Streptomycin 80 µg/ml, Tetracyclin 50 µg/ml). Bacterial stocks were kept at -80° C in LB supplemented with 90% glycerin.

2.3. Antagonism tests in dual cultures:
Endophytic fungi were inoculated on CP agar medium by inserting plugs from ca. 10 mm diameter on one side of the petri dish. The plugs were incubated until the colony had grown at least 2 cm in diameter. Then, the bacterial reference strains were inoculated on the opposite side of the petri dish (Fig. 1). Both organisms were incubated at 30°C for 3 to 4 days (for the fast growing fungi) and up to 7 days (for the slow growing fungi). Growth assays in dual culture were done in triplicate.

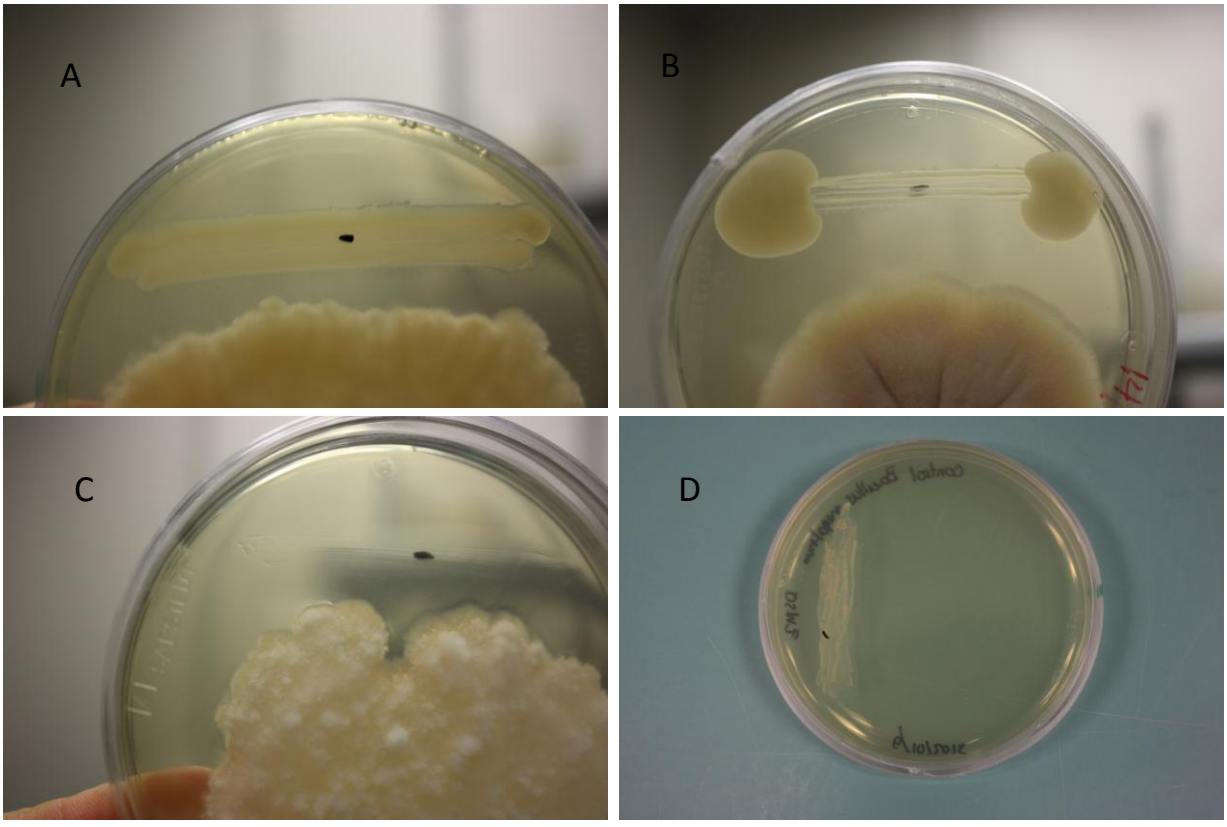

Figure 1. Examples of antagonism tests rated as A. no antagonism (-); B. mild (+) and mild to strong (++) antagonism ; C. strong antagonism (+++) as compared to D: growth control of the test bacterium.
Strains shown: A. *Fusarium* sp. (strain 10) vs. *E. coli* K12; B. *Emericella* sp. (strain 14) vs. *B. megaterium* DSMZ 319; C. *Fusarium* sp.(Strain 10) vs. *B. megaterium* DSMZ 319

2.4. Extraction of secondary metabolites in liquid phase:

After inoculation in dual culture, cultures in petri dishes were frozen at -20°C. Three replicates for each antagonism test were pooled and blended for 3 minutes after which 25 ml of distilled water were added and blended for another 2 minutes. The blended cultures were transferred to extraction funnels and another 25 ml of distilled water were added. Then, 80 ml of ethyl acetate were added and the mix was gently shaken for 10 minutes avoiding emulsification. The diphasic extracts were incubated for 24h at room temperature and the organic phases were recovered in 500 ml Erlenmeyer bulbs. A second extraction of the aqueous phase was performed with each sample by adding 50 ml of ethyl acetate and gently shaking for 5 minutes. The samples were incubated for 2 h and the organic phase recovered. The organic phases from the first and the second extraction were pooled and freed of remnant water by adding 20 g of anhydrous sodium sulfate. After incubation for 1 minute, the extracts were filtered through filter paper (150 mm diameter). The organic phases were subsequently flash evaporated in a rotary evaporator coupled to a vaccuum pump and a pressure controller. Extracts were evaporated in a water bath at 40°C, 90 rpm and 400 mbar.

2.5. Bioactivity tests in 96-well plates:

The bioguided assays were performed in 96-well plates with concentrations of the extracts as shown in Fig. 2.

	Extracts										(+)	(-)	
	1	2	3	4	5	6	7	8	9	10	11	12	CONCENTRATION mg/ml
A	O	O	O	O	O	O	O	O	O	O	O	O	2,5
B	O	O	O	O	O	O	O	O	O	O	O	O	1,25
C	O	O	O	O	O	O	O	O	O	O	O	O	0,625
D	O	O	O	O	O	O	O	O	O	O	O	O	0,3125
E	O	O	O	O	O	O	O	O	O	O	O	O	0,15625
F	O	O	O	O	O	O	O	O	O	O	O	O	0,078125
G	O	O	O	O	O	O	O	O	O	O	O	O	0,0390625
H	O	O	O	O	O	O	O	O	O	O	O	O	0,01953125

Figure 2. Representation of the bioactivity assays using bacteria or yeast as test organisms. Concentration of the extracts is shown in red. (+): Positive control with antibiotic against the

test organism (namely gentamycin for bacteria or nystatin for yeast). (-): Negative control with organic solvent.

The evaporated extracts were recovered in 1 ml of a methanol-acetone solution (1:1) and transfered to weighted brown screw-cap vials. The extracts were left to dry overnight and then 500 µl of petroleum ether were added and the lipophilic contents of the extracts were kept in separate vials for further analysis. After drying, the lipid-depleted extracts were resuspended to a concentration of 20 mg/ml or 10 mg/ml if the quantity of the extract was not adequate. After resuspending the extracts in a methanol-acetone solution (1:1), the bioactivity tests were performed. Reference strains *E. coli* K12, *B. megaterium* DSMZ 319 or the dimorphic yeast *Candida maltosa* 6/15 were grown overnight in petri dishes with appropriate medium (LB agar for the bacteria and CP agar for the yeast) and these were used as inoculum for the bioactivity assays.

100 µl of liquid LB medium were added to the first row of a flat bottom 96-well microtiter plate. Then 25 µl of each extract were added to the wells in the first 10 columns of the plate and mixed thoroughly with the LB medium. The mixture was dried for 20 minutes under the flow hood. The last two columns of the plate were used for the positive and negative inhibition controls: 25 µl of gentamicin (1mg/ml) or 25 µl of nystatin (50mg/ml) and 25 µl of methanol-acetone (1:1) respectively.

The bacterial and yeast cultures were suspended into 1 ml of sterile water and then two cell suspensions (one for the first row and one for the remaining rows on the plate) were prepared by adjusting the optical density at 600nm (OD_{600}) to 0.01 and 0.05 for the bacteria and the yeast, respectively. Then the first cell suspension was distributed in 100 µl aliquots per well in the first row of the plate and mixed together with the previously poured LB medium and extracts. The second cell suspension was distributed in the remaining wells. Finally 100 µl from the mixture in the wells of the first row were serially diluted into the wells of the following rows and the surplus volume from the last row of the plate was discarded. Plates were incubated at 30°C for 24h for the bacterial strains and at 37°C for the yeast strain. Incubation was carried out at 600 rpm.

2.6. Selecting the mininal inhibitory concentration (MIC):

The MIC was selected as the lowest concentration of the extract at which growth of the test organism was inhibited more than 90%. Inhibition was confirmed visually uner the light miscroscope at 10x and 40x without any specific staining.

3. Results

Extraction of the dual cultures resulted in the selection of candidate extracts with inhibitory activity. Most of the extracts from the dual cultures inhibited growth of the gram positive bacterium *B. megaterium* DSMZ 319, but only 5 inhibited growth of *E. coli* K12 and 3 inhibited growth of *C. albicans* 6/15.

Our results suggest that confronting a gram negative bacterium against a fungal endophyte induces the production of metabolites with biological activity that works also against the gram positive counterparts. It is exemplified on the extracts of *Giberella pulicaris* vs. *E. coli* K12 that inhibited the growth of *B. megaterium* DSMZ 319 at 312 µg/ml. In this example, the fungus alone did not produce the inhibitory substance suggesting that only the presence of the gram negative bacterium is able to trigger the synthesis of the antibiotic substance in *G. pulicaris*.

The best inhibitor (with the lowest MIC) was the extract of the dual culture of *Paecilomyces variotii* against *E. coli* K12 that efficiently arrested the growth of the *B. megatrium* strain at a concentration of 78.1 µg/ml.

Unfortunately, due to an unexpected medical operation, not all the extracts obtained during the STSM could be tested. For example, the extracts from the mono cultures of the bacteria and the yeast remain to be tested. This work is currently ongoing.

Table 2. Growth inhibition of reference strains in 96-well microtiter plates. NT :Not tested. NA: No activity.
[a] Code used for the extract (does not correspond to the number of the fungal strain).
[b]Bacteria and fungi tested in dual culture, from which the extracts were obtained.
[c]Replicates are represented by R1-3.
-: no inhibition recorded; +: incomplete inhibition of the of bacterial growth. Growth is reduced as compared to the control. ++: mild to complete inhibition of bacterial growth; +++: complete inhibition of bacterial growth with no apparent colonies on the agar medium.

Number of the extract[a]	**Dual cultures**				**MIC (mg/ml) in 96 well plates with *B. megaterium* DSMZ 319**	**MIC (mg/ml) in 96 well plates with *E. coli* K12**	**MIC (mg/ml) in 96 well plates with *C.maltosa* 6/15**
	Organisms tested[b]	**Inhibition in petri dish[c]**					
		R1	**R2**	**R3**			
1	*Epicoccum nigrum* vs. *E. coli* K12	-	-	-	0.625	2.5	2.5
2	*Epicoccum nigrum* vs. *B. megaterium* DSMZ 319	++	++	++	0.625	2.5	2.5
29	*Epicoccum nigrum* alone	NT	NT	NT	NT	NT	NT
3	*Phoma herbarum* vs. *E. coli* K12	+	+	+	2.5	NA	NA
4	*Phoma herbarum* vs. *B. megaterium* DSMZ 319	-	-	-	NA	NA	NA
30	*Phoma herbarum* alone	NT	NT	NT	1.25	NA	NA
5	*Fusarium* sp. vs. *E. coli* K12	-	-	-	NA	NA	NA
6	*Fusarium* sp. vs. *B. megaterium* DSMZ 319	+	+++	+++	0.625	NA	NA
31	*Fusarium* sp. alone	NT	NT	NT	1.25	NA	NA
7	*Giberella pulicaris* vs. *E. coli* K12	++	+	++	0.312	NA	NA
8	*Giberella pulicaris* vs. *B. megaterium* DSMZ 319	-	-	-	NA	NA	NA
32	*Giberella pulicaris* alone	NT	NT	NT	NA	NA	NA
9	*Emericella* sp. vs. *E. coli* K12	-	-	-	NA	NA	NA
10	*Emericella* sp. vs. *B. megaterium* DSMZ 319	++	-	++	0.312	NA	NA
33	*Emericella* sp. alone	NT	NT	NT	NA	NA	NA
11	*Nectria mauritiicola* vs. *E. coli* K12	+	-	-	2.5	2.5	NA
12	*Nectria mauritiicola* vs. *B. megaterium* DSMZ 319	-	-	++	NA	NA	NA
34	*Nectria mauritiicola* alone	NT	NT	NT	NA	NA	NA
13	*Beauveria bassiana* vs. *E. coli* K12	+	+	+	NA	NA	NA
14	*Beauveria bassiana* vs. *B. megaterium*	++	++	++	1.25	NA	NA

	DSMZ 319						
35	*Beauveria bassiana* alone	NT	NT	NT	NA	NA	NA
15	*Paecilomyces variotii* vs. *E. coli* K12	-	-	-	0.0781	NA	NA
16	*Paecilomyces variotii* vs. *B. megaterium* DSMZ 319	-	+	-	0.156	NA	NA
36	*Paecilomyces variotii* alone	NT	NT	NT	0.312	2.5	NA
17	*Truncatella angustata* vs. *E. coli* K12	-	-	-	NT	NT	NT
18	*Truncatella angustata* vs. *B. megaterium* DSMZ 319	++	++	++	NT	NT	NT
37	*Truncatella angustata* alone	NT	NT	NT	NT	NT	NT
19	*Alternaria* sp./*Coniothyrium* sp. vs. *E. coli* K12	-	+	+	0.156	2.5	NA
20	*Alternaria* sp./*Coniothyrium* sp. vs. *B. megaterium* DSMZ 319	+	++	++	NA	0.625	NA
38	*Alternaria* sp./*Coniothyrium* sp. alone	NT	NT	NT	NA	NA	NA
21	*Ampelomyces humali* vs. *E. coli* K12	-	-	+	0.312	NA	NA
22	*Ampelomyces humali* vs. *B. megaterium* DSMZ 319	++	++	++	0.625	NA	NA
39	*Ampelomyces humali* alone	NT	NT	NT	NA	NA	NA
23	*Valsa sordida* (*Cytospora chrysosperma*) vs. *E. coli* K12	++	++	++	2.5	NA	NA
24	*Valsa sordida* (*Cytospora chrysosperma*) vs. *B. megaterium* DSMZ 319	+++	+++	+++	NA	NA	NA
40	*Valsa sordida* (*Cytospora chrysosperma*) alone	NT	NT	NT	0.312	NA	2.5
25	*Valsa malicola* vs. *E. coli* K12	++	+	+	NA	NA	NA
26	*Valsa malicola* vs. *B. megaterium* DSMZ 319	++	++	++	2.5	NA	NA
41	*Valsa malicola* alone	NT	NT	NT	1.25	NA	NA
27	*Dothideomycetes* sp. (*Microsphaeropsis arundinis*) vs. *E. coli* K12	-	-	-	1.25	NA	NA
28	*Dothideomycetes* sp. (*Microsphaeropsis arundinis*) vs. *B. megaterium* DSMZ 319	++	++	++	1.25	NA	NA
42	*Dothideomycetes* sp. (*Microsphaeropsis arundinis*) alone	NT	NT	NT	NT	NT	NT

4. Future work

We will continue testing the remaining extracts that were not analyzed in this set of experiments.. Subsequently, we will elucidate and characterize the structures of these molecules and search for other types of biological activity with agricultutral and medical aplications.

5. Confirmation by the host laboratory

The student achieved the goals proposed for this STSM and hereby I confirm and support the veracity of the findings. The student had surgery during the course of his STSM and thus part of the work will be completed shortly after the end of the STSM. Should you have further questions do not hesitate to contact me at any time.

Signed by head of host lab

Barbara Joan Schulz

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