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Effects of microflora composition in the phyllosphere on biological regulation of grapevine fungal diseases

by

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S u m m a r y : Aqueous fermentation extracts of composted microbiologically active substrates reduced significantly the infestation of grapevine leaves with *Plasmopara viticola* under growth chamber conditions. Enhancement of efficacy was available through the enrichment of the extracts with nutrients - like sucrose and brewer's yeast - or with a natural surfactant (methylized cellulose). Studies on the microbial composition of the phyllosphere showed that the quantity of the total number of colony forming units, of yeasts/filametous fungi, of enterobacteria, of pseudomonads and of aerobic bacilli depends on the specific amendments to the extracts and on the microclimatic conditions. They are responsible for the differences in efficiency of the various extracts against *P. viticola*. Appropriate investigations on the phyllosphere in a field experiment resulted in a different composition of the microflora, though the same combinations of extract amendments were used. This is caused by the varying environmental conditions. In spite of the changed microflora, the disease suppressing effects against *Uncinula necator* were preserved.

Wirkungen der Mikroflorazusammensetzung in der Phyllosphäre auf die biologische Regulierung von Pilzkrankheiten der Weinrebe

Z u s a m m e n f a s s u n g : Wässrige Extrakte aus kompostierten mikrobiologisch aktiven Substraten reduzieren nach prophylaktischer Applikation den Befall von Rebblättern mit *Plasmopara viticola* im Klimakammerversuch signifikant. Eine Steigerung der Wirkung kann durch Nährstoffanreicherungen mit Saccharose und Bierhefe sowie durch Zugabe einer natürlichen Formulierungshilfe (methylierte Cellulose) erreicht werden. Mikroflorauntersuchungen in der Phyllosphäre auf die Gesamtkeimzahl, Hefen/filamentöse Pilze, Enterobacteriaceen, Pseudomonaden und auf Aerobe Sporenbildner zeigen, daß es in Abhängigkeit von der Extraktanreicherung sowie von den mikroklimatischen Bedingungen zu einer spezifischen Förderung unterschiedlicher Mikroorganismengruppen kommt. Dadurch lassen sich die Unterschiede in den Wirkungsgraden der einzelnen Varianten im Biotest mit *P. viticola* erklären. Entsprechende Untersuchungen in einem Freilandversuch zeigen ein differenziertes Bild der Mikroflorazusammensetzung bei Zusatz der gleichen Nährstoffkombinationen, was auf die unkontrollierbaren Bedingungen im Freiland zurückzuführen ist. Die antiphytopathogene Wirkung der mikrobenreichen Kompostextrakte gegen *Uncinula necator* bleibt jedoch auch im Freiland erhalten.

K e y w o r d s : microflora composition, phyllosphere, compost extracts, nutrient enrichments, climatic conditions, Plasmopara viticola, Uncinula necator.

Introduction

For an ecologically oriented viticulture synthetic pesticides should not be used. Nevertheless, an economically efficient wine-growing is hardly possible without an effective regulation of pests and diseases. Therefore it is necessary to develop an alternative pest and disease management, which leads to a sufficient control of pathogens of grapevine in an ecologically harmless way by using the naturally occurring ecological structures. One way of acting could be the application of microbiological control agents. Their modes of action is based on antagonistic properties of microorganisms, like competitive behaviours, direct antibiotic inhibition and hyperparasitism (Garrett 1956) or they promote the natural ocurring defense reactions of host plant tissues (Schönbeck *et al.* 1980). Already a wide range of individual microorganisms were found which possess strong antagonistic properties against fungal pathogens of grapevine, for example against *Botrytis cinerea* (Dubos *et al.* 1982, Shimshoni *et al.* 1989), against *U. necator* (Gadoury and Pearson 1988, Sztejnberg *et al.* 1989, Puzanova 1990) and against *P. viticola* (Henner 1962). The promotion of such antagonists through additions of nutrients is repeatedly described (Elad and Zimand 1990, Tilcher and Brendel 1991). However, in most cases positive results in the laboratory were not confirmed under field conditions, because most of the microbial isolates can not be established under natural conditions to develop their antagonistic effects. The optimal conditions for such microorganisms are very scarce under uncontrollable outdoor conditions and they are hardly never congruous with the climatic demands of the appropriate pathogens (Blakeman and Fokkema 1982, Andrews 1985, Blakeman 1985). A promising alternative is offered by complex microbiological media, like the aqueous fermentation extracts of composted organic materials. These liq-

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uids are characterized by high microbial activity and a varied microbial spectrum which let expect a certain stability and a wide range of efficacy. Antiphytopathogenic effects of such compost extracts were found for a remarkable number of host-pathogen-systems if applicated on plant surfaces. This results were summarized by WELTZIEN (1989, 1990, 1992). For grapevine, beneficial effects of compost extracts under field conditions were proved against *P. viticola* and *Pseudopezicula tracheiphila* (KETTERER and WELTZIEN 1987, KETTERER 1990), against *U. necator* (SACKENHEIM *et al.* 1990, KETTERER and SACKENHEIM 1991) and against *B. cinerea* (SACKENHEIM *et al.* 1992). In this paper we report about the possibilities to strengthen the efficiency of watery compost preparations with nutrient enrichments. Furthermore, the links are studied, which exist between suppressive effects on fungal pathogens and the mircoflora composition in the phyllosphere.

Materials and methods

Extract production: A compost produced out of horse manure as raw material was mixed with water in ratio of 1 : 10 and was fermented at ambient temperatures varying from 20 to 25 °C for 7 d in open containers. 3 d before end of fermentation the nutrients sucrose (beet sugar) and brewer's yeast (Bio-Bierhefeextrakt pills, Biolabor Company, Bremen) were added to the appropriate compost broth. The extracts were obtained by filtering the broths through a sieve immediately before application. One treatment was additionally enriched with a methylized cellulose (KWS-Kleber, Kleinwanzlebener Saatzucht Company, Einbeck) as a natural surfactant. The following treatments were applied: control (tap water) - horse manure compost extract - horse manure compost extract + sucrose (3 %) - horse manure compost extract + sucrose (3 %) + brewer's yeast (1 %) - horse manure compost extract + sucrose (3 %) + brewer's yeast (1 %) + surfactant (0.1 %).

Growth chamber experiments: For every treatment 20 cuttings of grape (cv. Trollinger) were applied using ordinary spray equipment. Subsequently 10 cuttings per treatment were set up randomized in a growth chamber under humid (RH: 90 - 95 %) and dry conditions (RH: 50 - 60 %). Light and temperature were equal under both humidity stages (3 klx for 16 h at 22 °C). 3 d after application the leaves of the cuttings were detached. Through a parallel set of experiments the connection between the composition of microflora and the supression of *P. viticola* on grapevine leaves could be investigated.

Bioassay with *Plasmopara viticola*: 7 detached leaves per treatment were placed in petri dishes on moist blotter paper and were artificially inoculated on the lower leaf surface with a watery suspension of 8 x 10^4 sporangia/ml. The petri dishes were incubated at 18 - 20 °C with 16 h of light of 3 klx. 10 d later the coverage of the leaf surface by sporangiophores in % of leaf area was determined. The statistics were done seperatly for both humidity conditions.

Studies on the microflora composition in the phyllosphere: 5 g of the remaining leaf material of each treatment were handled as described by HISLOP and COX (1969). They were mixed with 20 g glas beads (2-3 cm in diameter) and with 100 ml Ringer solution. These samples were moved on a shaker (100 rpm) for 2 h and afterwards the microflora composition was studied by plating the washwater on the following culture media (Merck Company):
Total number of colony forming units (cfu): Caso-Agar (casein-peptone soymeal-peptone agar) — yeasts and filametous fungi: Wort-Agar — enterobacteria: VRBD-Agar (Violet-red-bile-dextrose agar) — pseudomonads: GSP-Agar (pseudomonas aeromonas selective agar) — aerobic bacilli: Caso-Agar (casein-peptone soymeal-peptone agar).

For the selection of the aerobic bacilli the washwater samples were incubated for 20 min at 80 °C. The samples were laid on the media with a Spiral plater (Modell CU, Spiral Systems Inc., Cincinnati/USA) in three replications. After the respective incubation times the number of colony forming units (cfu/g leaf material) was determined.

Phyllosphere studies in a field experiment: The field trial was performed in a cv. Kerner vineyard named „Glückenhälde“ located in Weinsberg/Württemberg. The experiment was set up in a randomized block design and all treatments were replicated 6 x. The extracts were applied with regular pesticide spray equipment 6 x in intervals of 10–11 d between June 3 and July 22, 1991. The treatments were the same as in the growth chamber experiment. 3 d after the last application 20 leaves of each replication of every treatment were taken and 15 g leaf material was reduced to small pieces, mixed with 50 g glas beads and with 300 ml Ringer solution and handled as mentioned above (microflora investigations of the growth chamber experiment).

Results

Growth chamber bioassay with *P. viticola*: Under moist conditions 26 % disease reduction was obtained with the pure compost extract while all amendments to the extracts lead to an increased efficacy against *P. viticola* (Fig. 1). Thus, the extract with the admixture of sucrose plus brewer's yeast achieved an efficiency of 58 %. Under dry conditions the pure extract and the one enriched with sucrose have no significant effect on *P. viticola*. Only the extracts with the addition of sucrose plus brewer's yeast reduced the incidence of the pathogen significantly by up to 41 %.

Growth chamber studies on the microflora composition in the phyllosphere: Comparing the microflora, it is evident that under dry conditions all groups of microorganisms were found in lower densities than under humid conditions, with the exception of the aerobic bacilli. They also occurred with high populations under dry conditions (Figs. 2 + 3).

The application of the pure compost extract under humid conditions raised the total number of colony forming units (cfu) and the number of enterobacteria, pseudomonads and aerobic bacilli compared to the control. Under dry conditions only the total number of cfu increased.

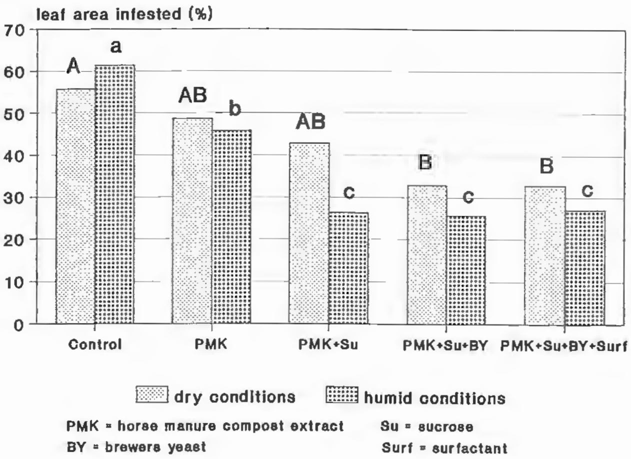
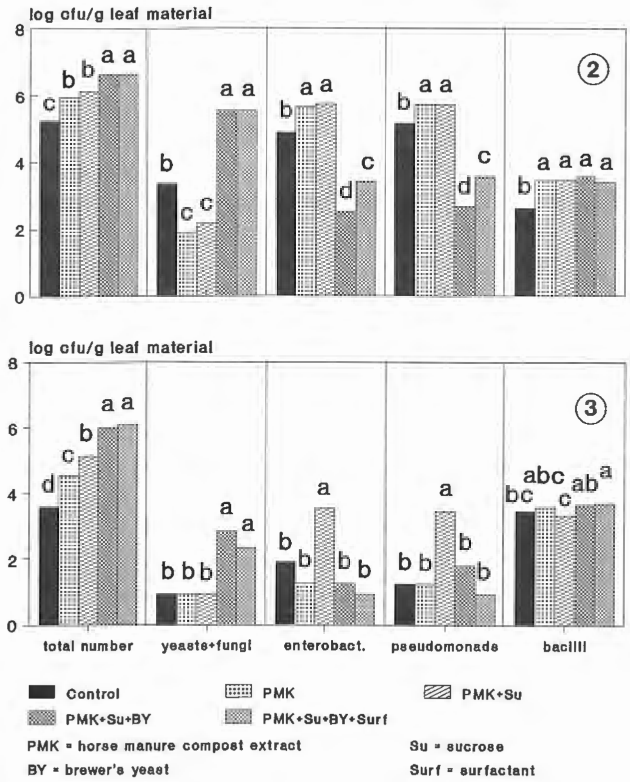


Fig. 1: Suppression of *P. viticola* by a horse manure compost extract (PMK) with and without different amendments after incubation under humid (R.H. 90 - 95 %) and dry (R.H. 50-60 %) growth chamber conditions. Different letters mark significant differences according to Duncan's multiple range test (P = 0.05).



Figs. 2 and 3: Influence of different enriched horse manure compost extracts (PMK) on the populations of 5 groups of microorganisms (total number of cfu, yeasts/filamentous fungi, entero bacteria, pseudomonads and aerobic bacilli/g leaf material) in the phyllosphere of grape cuttings.
Under humid (R.H. 90 - 95 %: Fig. 2, above) and under dry (R.H. 50-60 %: Fig. 3, below) growth chamber conditions.
Before statistical analysis data were log-transformed (Fig. 2: yeasts+fungi, pseudomonads; Fig. 3: total number, yeasts+fungi, enterobact, pseudomonads), reziprocal-transformed (Fig. 2: enterobact.). Different letters mark significant differences according to Duncan's multiple range test (P = 0.05).

If the extract was enriched with sucrose, an additional promotion of microflora populations was not observed under humid conditions in comparison with the pure compost extract. But under dry conditions the addition of sucrose promoted the total number of cfu, enterobacteria and pseudomonads but on a lower level of population. This demonstrates the stabilization of this groups of microorganisms under unfavourable conditions through this enrichment. Inhibitional effects of the pure and the sucrose-enriched extract on the yeasts/filamentous fungi occur only under moist but not under dry conditions.

The addition of the nutrient combination sucrose plus brewer's yeast raised the total number of cfu under both humidity stages. Particularly under humid conditions the quantity of the yeasts/ filamentous fungi was increased. In contrast to this, the development of the enterobacteria and pseudomonads was strongly inhibited, but only in the moist atmosphere.

The addition of the surfactant did not influence the quality or quantity of the microflora under both simulated moisture conditions except the enterobacteria and pseudomonads under humid conditions. Their number was reduced compared to the control but without reaching the low level caused by the appropriate extract with the only enrichment of sucrose and brewer's yeast but without surfactant.

The dependence of the different groups of microorganisms with antagonistic relevance on the environmental conditions was demonstrated through the highly significant correlations between the intensity of attack of *P. viticola* and the number of yeasts/filamentous fungi and aerobic bacilli. The importance of yeasts/filamentous fungi increased under dry conditions (r = -0.737), the aerobic bacilli under humid conditions (r = -0.851). Clearly negative correlations were found for the total number of cfu, particularly in the dry atmosphere (r = -0.912), showing the highest negative correlation between increase in microbes in the phyllosphere and decrease in disease incidence. It is evident, that especially under extreme environmental conditions additional microorganisms may be involved, which were not studied in this investigation.

Phyllosphere studies in a field experiment: Studies of microbial populations in a field experiment in 1991 with the same treatments like in the growth chamber trial showed different results (Fig. 4). The pure compost extract did only raise the number of aerobic bacilli significantly under field conditions. The amendment with sucrose results in a significant increase of the total number of cfu and yeasts/filamentous fungi, as compared with the pure extract, but it shows no effects on the enterobacteria and pseudomonads. The addition of brewer's yeast increases the effects on the total number of cfu, the yeasts/filamentous fungi and on the aerobic bacilli. A significant rise of the enterobacteria and pseudomonads through the amendment of the surfactant in comparison with the control was found in the field experiment in contrast to the growth chamber trial.

In the same field trial the naturally occurring incidence of *U. necator* on leaves was examined on July 22. All treatments reduced the frequency of disease attack significantly compared with the tap water treated control, which showed an infestation of about 57.6 %. However, only the extract enriched with sucrose, brewer's yeast plus surfactant differs significantly from the pure compost extract. It obtained an efficiency of 71 % compared with the control and differs also significantly from the same treatment without surfactant. The occurrence of other fungal pathogens was not important during the time of experiment.

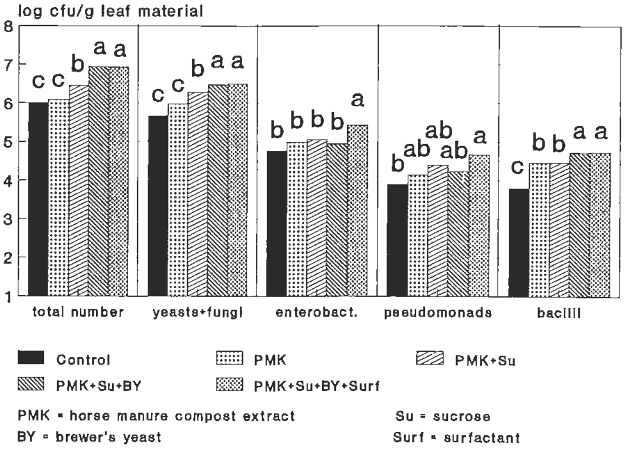


Fig. 4: Influence of different amended horse manure compost extracts (PMK) on the populations of 5 groups of microorganisms (total number of cfu, yeasts/filamentous fungi, entero bacteria, pseudomonads and aerobic bacilli/g leaf material) in the phyllosphere of grapevine at „Glückenhälde" vineyard in Weinsberg/Württemberg on 25.07.91. Before statistical analysis data were arcsin-transformed (enterobact.), reziprocal-transformed (pseudomonads). Different letters mark significant differences according to Duncan's multiple range test (P = 0.05).

Discussion

The application of compost extracts has a strong influence on the microflora in the phyllosphere of grape leaves. The amendment with nutrients - such as sucrose or brewer's yeast - changes the quantity of the different groups of microorganisms considerably and in this way the antagonistic quality of the microflora in the phyllosphere. It leads to a stabilization of the phyllosphere colonization through microorganisms under extreme climatic conditions. However, there is no general promotion and stabilization of the microflora but a selective effect according to the kind of nutrients added and to the climatic conditions. SUSLOW (1982) has reported the enhancement of drought resistance of bacteria through small amounts of sugar compounds and aminoacids, resp. The laboratory tests with *P. viticola* verify, that with increasing numbers of microbes under humid conditions or through nutrient amendments the protective efficacy is improved. The specific preference of the several groups of microbes under the different conditions is shown through the correlation tests.

GROSS-SPANGENBERG (1992) also proved correlations between the total number of cfu, bacteria, enterobacteria and fluorescent pseudomonads in the phyllosphere of apple leaves and the incidence of *Venturia inaequalis* after treatments with sucrose and brewer's yeast enriched extracts. STINDT (1990) found strong relations between the total number of cfu, enterobacteria and pseudomonads and the infection of phaseolus bean leaves with *B. cinerea*, if treated with compost extracts. KETTERER and SCHWAGER (1992) emphasized the importance of the aerobic bacilli and enterobacteria in the host-pathogen-system Phaseolus bean - *Erysiphe polygoni*, while for *Phytophthora infestans* on tomato they indicate an outstanding role for disease suppression for the enterobacteria and particularly the pseudomonads. Moreover, the authors reported about a significant promotion of the microbial populations through addition of casein to a compost extract. TRÄNKNER and LANGE (1993) propagated "caso broth" plus rape oil as effective enrichments for compost extracts. The enhancement of efficacy against *P. tracheiphila* and *U. necator* was correlated with the increase of the total number of cfu and the quantity of aerobic bacilli.

Our own investigations also show the dependence of the microbial composition and of the effects against various fungal grapevine diseases from the type of nutrient enrichment. In laboratory and greenhouse experiments distinct effects occur against the pathogens *P. viticola, B. cinerea* and *U. necator*, depending on the added nutrients (SACKENHEIM 1993).

Comparing the results of the growth chamber experiment with the investigations in the field in 1991, the different behaviour of the single groups of microorganisms is conspicious. Outdoors the total number of cfu and the amounts of yeasts/filamentous fungi develop similarly to the growth chamber data under dry conditions. However, the populations of enterobacteria and pseudomonads were very different in both experiments. Their densities were not promoted in the field by adding exclusively sucrose to the extract. In contrast, the addition of the combination of sucrose plus brewer's yeast favoured the aerobic bacilli stronger under field conditions than in growth chamber.

These reactions of the different microbial groups are caused by environmental factors, which are especially important under field conditions. On the occasion, it is evident, that the microclimate in the phyllosphere have a decisive influence on the composition of the microflora, while the influence of different cultivars (Trollinger in growth chamber and Kerner in the field experiment) can be neglected. Humidity and available nutrients represent the most important limitation factors (BLAKEMAN and FOKKEMA 1982). Nevertheless, VERCESI *et al.* (1990) characterize the temperature as the most important factor influencing the activity of antagonists against *B. cinerea*. Also BAKER and COOK (1973) consider the different temperature conditions in growth chamber and field trials as the reason for the failure of biological control agents under outdoor conditions.

At the time of sampling in the field, dry and hot weather prevailed in day-time, while during the night high relative humidity and dew on grape leaves set in, caused by the decline of temperature. This may explain the relatively high densities of all groups of microorganisms under field conditions. Moreover, the several microbial groups react differently on the specific weather conditions and therefore another microbial structure occurs outdoors as compared with the growth chamber trial.

An other important factor influencing the phyllosphere microflora is the natural ultraviolet radiation (Dickinson 1986, Costerton 1990) and differences in sensibility of the several groups of microbes must also be expected.

Beside the microclimate also antagonistic interrelations with the epiphytic microflora play a part in the introduction of the microorganisms in the phyllosphere of outdoor plants. Smaller original microbial populations allow better establishment of antagonistic microorganisms (Lindow 1985). Clearly, under outdoor conditions higher initial densities of microbes were found than under growth chamber conditions. Accordingly, the outdoor colonization with microbes from the extracts must be weaker and again differences in the reaction of the several microbial groups can be expected.

In spite of the different population dynamics of microorganisms in the growth chambers and under field conditions, the antiphytopathogenic potential of the compost extracts is preserved and effective under outdoor conditions as shown by the suppression of *U. necator* infections.

Conclusions

The experiments show, that results from growth chamber trials on biological disease control can not easily be transferred to field conditions. Environmental factors influence largely the population dynamics of microbial antagonists. It is evident, that pure cultures of isolated micobes are much more vulnerable to hostile environmental conditions, than microbial mixtures in complex brothes, such as compost extracts. Their complexity enables an adaption to the environmental conditions by the shifting of the microbial spectrum in the phyllosphere. In this way, the suppression of various pathogens in a relatively wide range of temperature and humidity is possible (Sackenheim 1993). Therefore is is evident, that such complex microbial media have an important advantage as biological control agents if used under practical conditions in vineyards. Furthermore, amendments of the extracts with nutrients can frequently stabilize the effects of the microbes through selective increase of their numbers.

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