Staling growth products of phyllosphere fungi

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Summary. Pestalotiopsis funerea Desm. causing leaf spot of *Eucalyptus globulus* Labill. failed to colonize strongly staled agar discs of phyllosphere inocula. Antibiotics, besides alteration in pH, and nutrient impoverishment of the staling growth products, may play a major role in the mycostasis phenomenon leading to colonization of staled agar by phyllosphere microfungi.

Phyllosphere micro-organisms may influence the development of invading pathogens by competing with them for nutrients or by producing volatile and non-volatile inhibitory substances[2-6]. In this connection, however, very little work has been done on the effects of staling on antagonistic interactions in the phylloplane. In the present study several phyllosphere fungi including a pathogen, *Pestalotiopsis funerea* Desm., the causal agent of leaf spot disease of *Eucalyptus globulus* Labill., were studied for their ability to colonize staled nutrient agar after different periods of staling by phyllosphere organisms. The effect of staled culture filtrates of various phyllosphere fungi on the growth of *P. funerea* has also been studied.

Materials and methods. Leaves of *E. globulus* at a similar state of maturity (4 months old) were collected and discs of 11 mm diameter were cut with a sterilized cork borer. Conical flasks, each containing 100 ml sterilized water and 100 discs, were shaken for 10 min on a mechanical shaker (100 rpm). 1 ml of the washings containing the phyllosphere micro-organisms was uniformly spread over solidified agar plates (Czapek-Dox + 0.05% yeast extract + 30 $\mu g \cdot ml^{-1}$ streptomycin). 1 series of 10 replicate plates was employed to record the species spectrum of fungi commonly colonizing the agar from the washings, and the percent occurrence and the number of fungi per cm^2 area of leaf surface were recorded. 2 series of 10 plates were incubated at 24 ± 1 °C, 1 for 96 h and the other for 120 h. After incubation, the agar in the plates was inverted and the lower surface thus exposed was inoculated with 1 ml of phyllosphere washings prepared as previously described, and the plates incubated until all fungal species could be identified. This experiment was repeated twice.

The metabolites of all the phyllosphere fungi were prepared in 250-ml Erlenmeyer flasks containing 100 ml liquid nutrient Czapek-Dox + 0.05% yeast extract. Each flask was inoculated with 2 6-mm agar discs cut from the margin of a vigorously growing fungal culture. The flasks were incubated for 120 h at 24 ± 1 °C and culture filtrates prepared by Seitz filtration. The effect of these metabolites on the growth of *P. funerea* was then tested by hyphal dry weight and spore germination methods, and percent inhibition was calculated.

Results and discussion. Fungi from phyllosphere washings have been arranged in groups I–III according to their ability to colonize the staled agar plates (table 1). The percentage occurrence of fungal species of groups I and II colonizing the reverse side of the staled agar decreased or became zero as the staling period increased. Only a few fungal species, viz., *Aspergillus niger, A. flavus, Alternaria alternata, Aureobasidium pullulans, Curvularia lunata, Fusarium chlamydosporum* and *Penicillium oxalicum* could colonize the strongly staled agar discs with an increased percentage of occurrence. This increase was not due to increase in the population level of these fungi (group III) but it was due to disappearance of the fungal species of groups I and II. Thus, these 2 groups (I and II) exhibited a lower tolerance capacity against the staling growth substances of composite phyllosphere microfungi as compared to group III. Dwivedi and Garrett[7] reported that the species spectrum of fungi colonizing nutrient agar changed pro-

Table 1. Effect of staling on species spectrum of fungi colonizing nutrient agar from phyllosphere inocula

Fungi	Species spectrum (% occurrence) colonizing staled agar after different period of staling			Fungi per cm^2 area of leaf surface
	0 h	96 h	120 h	
Group I				
Torula herbarum	0.95	0	0	0.21
Epicoccum nigrum	0.83	0	0	0.25
Bipolaris spicifera	0.55	0	0	0.20
Cephalosporium roseum	1.65	0	0	0.38
Green sterile mycelium	1.30	0	0	0.41
Brown sterile mycelium	0.36	0	0	0.20
Group II				
Fusarium oxysporum	2.93	5.62	0	1.06
Phoma hibernica	0.57	1.50	0	0.21
Penicillium chrysogenum	2.13	6.01	0	1.06
Cladosporium herbarum	14.30	4.01	0	5.41
C. cladosporioides	13.10	3.02	0	4.32
Pestalotiopsis funerea	19.10	4.01	0	6.46
Group III				
Curvularia lunata	5.23	8.50	6.0	1.41
Aureobasidium pullulans	20.00	15.00	9.3	7.42
Aspergillus niger	1.20	10.50	25.5	0.42
A. flavus	4.90	12.10	20.4	1.32
Alternaria alternata	2.80	8.60	10.7	1.01
Fusarium chlamydosporum	3.50	10.60	13.4	1.21
Penicillium oxalicum	4.10	11.60	14.18	1.10

Table 2. Percent inhibition of *Pestalotiopsis funerea* by non-volatile metabolites (staling growth products) of some of the phyllosphere fungi

Metabolites supplied by	pH of the culture filtrate (containing metabolites)	Inhibition of hyphal yield in the culture filtrate (containing metabolites) (%)	Inhibition due to initial pH of the culture filtrate (containing metabolites) (%)	Inhibition remained (%)	Inhibition of spore germination (%)
Phyllosphere fungi					
Alternaria alternata	4.2	70	15	55	52
Aspergillus niger	4.0	79	15	64	100
A. flavus	7.0	32	5	27	51
Bipolaris spicifera	5.0	62	7	55	52
Cephalosporium roseum	4.2	77	15	62	52
Cladosporium herbarum	4.0	56	15	41	0
C. cladosporioides	4.0	64	15	49	18
Curvularia lunata	4.0	51	15	36	10
Epicoccum nigrum	4.2	48	15	33	7
Fusarium oxysporum	4.0	62	15	47	51
F. chlamydosporum	7.5	80	6	74	6
Penicillium oxalicum	4.0	76	15	61	65
P. chrysogenum	7.0	70	5	65	76
Phoma hibernica	8.5	58	8	50	41
Torula herbarum	7.0	20	5	15	12
	pH	Hyphal dry wt (mg)	Hyphal dry wt (mg)		Spore* germination (%)
Control	5.8	655	655	0	100

* Percent spore germination in medium composed of liquid Czapek-Dox + 0.05% yeast extract.

gressively with the degree of staling caused by earlier established colonies. Park[8] states that the competitive success of any organism depends in the first place on its capacity to withstand the antagonistic substances produced by other micro-organisms. Further, success in competitive colonization of staled agar also depends on the population level of the particular fungal species. A high population level will confer a high inoculum potential and this can compensate for a low degree of competitive saprophytic ability[7]. This may have played a part in the colonization by *Aureobasidium pullulans, Cladosporium herbarum, C. cladosporioides* and *Pestalotiopsis funerea* of the staled agar.

The higher population of *P. funerea* decreased most rapidly on staled agar as compared to other phyllosphere fungi, which leads to the conclusion that factors other than population level (staling growth products) govern the colonization. To trace out the other possible factors, phyllosphere fungi were individually assessed for their mycostatic activity against *P. funerea.*

The non-volatile metabolites (staling growth products) of all the phyllosphere fungi tested differed in their ability to inhibit the growth of the pathogen *P. funerea* (table 2). More than 70% inhibition of hyphal yield was recorded with *Alternaria alternata, Aspergillus niger, Cephalosporium roseum, Fusarium chlamydosporum, Penicillium oxalicum* and *P. chrysogenum.* Similarly more than 50% inhibition of spore germination of *P. funerea* was observed with the culture filtrates of *A. alternata, A. niger, A. flavus, C. roseum, F. oxysporum, P. oxalicum* and *P. chrysogenum.*

These instances of inhibition of *P. funerea* might be attributed to a number of different causes, including antibiotic production, pH alteration and nutrient impoverishment in the medium due to growth of the fungi inoculated earlier[3-5,9]. These 3 factors were evaluated in a separate experiment, where *P. funerea* was grown at a different pH in liquid Czapek-Dox medium. Perusal of the data (table 2) shows that 15, 7, 5 and 8% inhibition of the growth of the pathogen as compared to the control (pH 5.8) at pH 4, 5, 7 and 8.5 respectively occurred. Furthermore, a significant percent inhibition remained even after the deduction of inhibition factor due to pH from the respective metabolites (a–b), which confirms that some factors other than pH were more responsible for the inhibition. Moreover, the pathogen was also found to grow normally over a wide pH-range (4–11) and thus the possibility of this factor can be eliminated. In sterile double distilled water without an external supply of nutrients, 85% spore germination of *P. funerea* occurred, as compared to 100% in liquid nutrient Czapek-Dox + 0.05% yeast extract which showed that inhibition exceeding more than 15% could not be due to nutrient depletion, and thus this factor could be excluded from consideration. It may therfore be concluded that the inhibition of *P. funerea,* and its failure to colonize strongly staled agar discs, were mainly due to antibiotics produced by previously colonizing phyllosphere fungi. This is an alternative to the view that shortage of nutrients (through leaching and uptake by bacteria) on the leaf surface leads to the mycostasis phenomenon. However, these findings are true of in vitro conditions and may not necessarily be true in vivo, i.e. on leaves. This failure could be attributed to the fact that usually laboratory experiments are carried out in optimal conditions, which is not the case in the field because of the involvement of certain ecological factors.

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