Micro-organisms of the Leaf Surface: Estimation of the Mycoflora of the Barley Phyllosphere

By H. G. DIEM

Laboratoire de Botanique et de Microbiologie, Université de Nancy, France

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SUMMARY

During a 17-day period the mycoflora of the barley phyllosphere was assessed by microscopic observation of leaf impressions. The frequency of fungal colonies detected was 0.1 to 17.2 per cm² of leaf. In favourable conditions the leaf area occupied by each colony was about 0.05 mm². The total surface of colonization could thus reach 2 mm²/cm², but generally varied between 0.002 and 0.774 mm². Only a limited range of species was observed, predominantly *Cladosporium* spp. The highest active populations coincided with a period of massive emission of pollen.

The quantitative results obtained bore little relation to those from leaf-washing counts: the reasons for this are discussed.

INTRODUCTION

In the study of the phyllospheric microflora, many techniques have been used. Some of these are based on the isolation of micro-organisms after leaf washing, spore fall, leaf impression on nutrient medium, and the 'balloon' technique of Rusch & Leben (1968). Others involving observation of the leaf surface *in situ* include leaf bleaching and cuticle prints with collodion or nail varnish (Last, 1955; Ruinen, 1961; Daft & Leben, 1966; Dickinson, 1967; Rusch & Leben, 1968).

Some authors (Barnes & Neve, 1968; Leben, 1969; Davenport, 1970; Kilbertus, 1970) have examined the phyllosphere by scanning electron microscopy. So far, however, such investigations have not given more information than the interesting results obtained with the light microscope, which include the precise distribution of micro-organisms, the role of pollen, and even the cuticular decomposition by phyllosphere inhabitants (Leben, 1965; Ruinen, 1966; Fokkema, 1968; Diem, 1970; Pugh & Buckley, 1971).

Some of these methods, useful in the study of unicellular organisms, may not be as valuable for filamentous species, for which there are advantages in direct observation. The present investigation was done to determine precisely the behaviour of this population and, at the same time, its activity in relation to environmental factors.

METHODS

Analysis of natural mycoflora. In 1971, barley leaves from 2 to 3 weeks old were periodically sampled at two stations about 3 km apart near Tomblaine (Meurthe et Moselle, France). One, A, was in the centre of a barley field; another, B, was surrounded by various Gramineae (*Dactylis glomerata* and *Lolium* spp.). In order to compare the results obtained by the usual technique of leaf washing with those given by direct observation, a leaf sample (about 500 cm² of leaf) was shaken for 1 h with 500 ml of sterile distilled water containing one drop

of Tween 80. The suspension, after appropriate dilution, was then plated on the following medium (g/l): glucose, 20.0; asparagine, 1.5; KH_2PO_4 , 1.0; KCl, 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; yeast extract, 1.0; chloramphenicol, 0.25; agar, 15.0.

At the same time, the upper surfaces of some leaves were covered with a solution of water containing agar (1 %) previously sterilized and maintained at about 40 °C. After desiccation, a thin pellicle of agar formed on the leaf surface. A strip of transparent adhesive tape was pressed on this pellicle to remove it intact. With this method, epiphytic microflora were embedded in the agar strip which could then be observed in its entirety. After counting the number of pollen grains and fungal colonies present, the outlines of these colonies were drawn with the aid of a drawing chamber in order to estimate their surface area, represented by the polygon obtained after connecting all hyphal tips of the colony. The total surface of these polygons was measured with a planimeter. The frequency of colonies/cm² leaf and the colonization surface, either per cm² or per colony (respectively c.s./cm² and c.s./col), were then calculated.

The influence of humidity. In this study, leaves of barley seedlings were sprayed with a spore suspension of *Cladosporium cladosporioides* in sterile distilled water (5000 spores/ml). This species was selected because it is amongst the commonest inhabitants of the barley phyllosphere (Diem, 1967), and its germination rate is higher than that of *Cladosporium herbarum*. Seedlings were then divided into five lots, each lot comprising about 200 cm² of inoculated leaves. Four lots were immediately incubated in a transparent plastic box with relative humidity near saturation. Leaves of the remaining lot were washed with 200 ml of sterile distilled water to determine the number of propagules present at the beginning of the experiment. After incubation at room temperature for 20 h to allow spore germination, two lots of plants were removed. As soon as they had dried by evaporation, they were again placed in the box. After incubation for 2 and 4 days, the population of *C. cladosporioides* was assessed either by the washing method or by direct examination as previously described.

RESULTS

The microscopic examination of the phyllosphere gave valuable information on the development, distribution and sequence of the natural mycoflora of the barley leaf surface (see Fig. 1). This population was particularly active at the two first sampling dates, especially in station B (frequency of colonies/cm² = 11 to 17, the highest value being 34). By this time fungal growth was important because each colony covered a mean area of 0.045 to 0.051 mm² and the c.s. was 0.774 and 0.594 mm²/cm² on 16 and 20 June respectively. The ability of epiphytic fungi to spread on the leaf surface was very variable: some colonies were very small and measured about 0.015 mm², others reached 0.41 mm². At station A, only on 20 June was a noticeable development of fungi observed and this was still lower than that found in the other station. After 26 June, very few fungal colonies were observed on leaves and the growth of those present was very limited (Table 1): their occurrence on leaves coincided with a large number of pollen grains, e.g. leaves from station B showed maximal development of fungi on 16 June and bore 300 to 600 pollen grains/cm² which had fallen from the various grasses, thus indicating a stimulatory role of pollen in the development of the leaf surface mycoflora. When leaves bore 600 pollen grains/cm² (normal number at flowering period) the number of fungal colonies/cm² was greater by a factor of 57 and the c.s./cm² by 154. Early in June 1971 the r.h. frequently rose above 80 %: this prolonged humid period coupled with the presence of pollen favoured the germination and growth of fungi (as found on 16 and 20 June). The sampling on 26 June was, in contrast, preceded by a period

Fig. 1. Microbial development on the surface of barley leaves. (a) General view of leaf surface microflora. Note the tendency for yeast growth in the depression between leaf epidermal cells. (b) A sporulating colony of *Cladosporium* sp. amongst pollen grains. P, Pollen; C, conidiophores; S, spores.

of dry weather interrupted by only two showers (18 and 19 June) which could have removed recently-deposited pollen grains. These unfavourable meteorological and nutritional conditions might explain the weak development of the fungal population found in the analysis on 26 June. Qualitatively, *Cladosporium* spp. seemed to be the only important colonizers of the phyllosphere. This was probably because of their remarkable resistance to meteorological fluctuations as well as their ability to produce readily either conservation organs or propagation structures such as spores (Diem, 1970; 1971).

Different results were obtained by the washing method (Table 2): the number of propagules increased by the time the fungal activity (as determined microscopically) was

Table 1. *Fungal colonization on the surface of barley leaves: direct-observation method*

Date (1971)	Frequency of colonies/cm ²		C.S./cm ²		C.S./colony		Pollen grains/cm ²	
	A	B	A	B	A	B	A	B
16 June	0.3	17.2	0.006	0.774	0.019	0.045	< 50	300-600
20 June	1.2	11.7	0.027	0.594	0.022	0.051	50-100	100-300
26 June	0.4	0.5	0.003	0.014	0.009	0.028	< 50	< 50
30 June	0.2	0.1	0.003	0.002	0.015	0.018	< 50	< 50
2 July	0.3	0.2	0.005	0.002	0.017	0.011	< 50	< 50

C.S., colonization surface (mm²); A, station in the centre of a barley field; B, station surrounded by Gramineae.

Table 2. *Phyllosphere mycoflora assessed by washing and direct observation methods*

Date (1971)	Station A		Station B	
	Washing*	Observation†	Washing*	Observation†
16 June	17	0.3	96‡	17.2‡
20 June	46	1.2‡	128‡	11.7‡
26 June	29	0.4	19	0.5
30 June	42	0.2	68	0.1
2 July	65	0.3	51	0.2

* No. of colonies isolated/cm².

† No. of colonies observed/cm².

‡ Presence of more than 50 pollen grains/cm².

Table 3. *Influence of humidity on the growth of Cladosporium cladosporioides on barley leaf surface*

Washing*	Incubation time (days)							
	0				2			
	Washing*		Observation†		Washing*		Observation†	
	W	D	W	D	W	D	W	D
42	57	0	26	‡	132	0	34	‡

* No. of colonies isolated/cm².

† No. of colonies observed/cm².

‡ Restricted growth, absence of branching colonies.

W, wet leaves; D, dry leaves.

highest, and also at the end of the experiment when this same activity had become negligible. Most colonies isolated at the final sampling probably originated from atmospheric flora, which is always denser in July than in June.

The presence of water on leaves was found to be necessary for the development of the mycoflora (Table 3). By direct observation of wet leaves, the formation of 57 branching colonies/cm² was recorded as early as the second day, whereas on dry leaves either germ tubes remained at the same development stage or colonies were still rudimentary. None of them was sporulating. On wet leaves, about 85% of observed colonies began to sporulate after 2 days and produced 5 to 20 conidia/colony after 4 days. This early sporulation of *Cladosporium cladosporioides* was also indicated by the increasing number of propagules

obtained with the washing method. Under experimental conditions in the laboratory, newly formed spores remained in their place, whereas in nature they are presumably removed by winds and rains.

DISCUSSION

Contrary to suggestions by some earlier investigators, the active epiphytic mycoflora, in these experiments at least, was qualitatively and quantitatively sparse. It became noticeable on the leaf surface only under very favourable conditions, such as extended wet periods or the presence of exogenous nutrients, e.g. from pollen (Fig. 1). The stimulatory role of pollen (Fokkema, 1968; 1971; Diem, 1970) suggests that the most active period of the phyllosphere microflora is determined by flowering time (10 to 15 June 1971 in Lorraine). Plants with extruded stamens may well contribute to the development of their own phyllosphere and of that of surrounding plants, as *Dactylis glomerata* and *Lolium* spp. probably did with the barley examined in this work. The regions in which this microbial stimulation by pollen grains occurs have been termed the 'palynoplane' and 'palynosphere'; because of the presence at the leaf surfaces of these microhabitats, new definitions have also been proposed for the terms 'phylloplane' and 'phyllosphere' (Diem, 1973).

Cladosporium is one of the most common inhabitants of the phyllosphere. Many authors (Kerling, 1964; Hollomon, 1967; Diem, 1967; Sinha, 1971; Pugh & Buckley, 1971) have accounted for this observation by the abundance of the spores of this genus in the atmosphere. But, since our microscopic examination revealed a high percentage of sporulating colonies at the time of flowering, propagules recovered by washing probably often come from the active mycoflora on the leaf itself.

The existence of active mycelium in the phyllosphere is a fundamental and much-discussed question (Kerling, 1958; Last & Deighton, 1965; Dickinson, 1965; 1967; Fokkema, 1968; Lamb & Brown, 1970; Ruscoe, 1971; Di Menna, 1971; Pugh & Buckley, 1971; Brainbridge & Dickinson, 1972). The results of this work, as well as photographs previously published (Diem, 1970; Pugh & Buckley, 1971), confirm the presence on green leaves and in natural conditions of a living mycoflora. At least in these experiments it was only active during favourable periods and consisted almost exclusively of *Cladosporium*. Warnock (1973) suggests the importance of the humidity and the anthers in the development of *Cladosporium* mycelium on the inner surface of the lemma and palea of barley grains.

The improved method of microscopic examination used in this work demonstrated not only the ability of fungi to develop on the leaf surface but also some detail on the spatial distribution of their colonies. Because this was not random, fungal effects on the leaf surface may occur only in very restricted areas, e.g. micro-sites near colonies.

The direct observation and leaf-washing methods appear to be complementary. For example, the latter may show an apparent decline in the population when, in fact, the decrease in spore numbers may be the result of leaf washing by rains (Kerling, 1958) and it may also fail to detect colonies on dry leaves (Table 3). Microscopic examination is thus essential for assessment of the number, location and activity of fungi in the phyllosphere. Conversely, the direct observation method implied that, towards 2 July, fungal development was negligible, whereas the washing technique revealed the presence of an important population (Table 2).

In these experiments, very few species were found to have an active life on green leaves and, as already noted in the literature, most fungi detected by cultural methods originate from spores. Consequently, the specificity of phyllosphere fungi in relation to host species may be more apparent than real and may well reflect the differing abilities of leaves of different plants to retain propagules deposited from the atmosphere.

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