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Endophyte-Host Interactions. II. Defining Symbiosis of the Endophyte-Host Interaction

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Abstract

We provide evidence that the symbiosis of fungal endophyte and plant host should only be defined in the broad sense as originally used by De Bary to mean the living together of organisms of different species. Using endophytic fungi that were isolated from healthy plant tissue, we tested for the potential pathogenicity of the fungal isolates and did physiological experiments to understand the endophyte-host association. Due to the variability of the interaction with respect to the role of the endophyte and with respect to the physiological status of both partners, only a definition of symbiosis that does not specify the advantages and disadvantages for the individual partners can accurately describe this interaction.

Keywords: Endophyte, dual cultures, plant defense reactions, symbiosis, herbicidal metabolites

1. Introduction

According to the most prevalent current definition, "fungal endophytes include all organisms inhabiting plant hosts at some time in their life and can colonize internal plant tissues without causing apparent harm to their host".

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This definition includes organisms that have a more or less lengthy epiphytic phase and also latent pathogens (Petrini, 1991). Nevertheless, some mycologists have come to speak of endophytes as being non-aggressive and not causing disease (Freeman and Rodriguez, 1993; Tyler, 1993; Sinclair and Cerkauskas, 1996) also often presuming a mutualistic role within their hosts (Carroll, 1988; Freeman and Rodriguez, 1993; Stone et al., 1994; Sinclair and Cerkauskas, 1996). However, when endophytes are isolated from host tissue following surface sterilization, a conglomeration of fungi is obtained that may play very different roles within their hosts. These isolates include those causing infections that are localized to single cells until senescence of the host tissue occurs, e.g. *Rhabdocline parkerii* (Stone, 1987), weak parasites such as *Pezicula* (Kehr, 1992) and *Colpoma quercinum* (Kehr and Wulf, 1993), quiescent infections of pathogens (Williamson, 1994) and pathogens that are developing within their hosts during a predetermined latent period, for example *Mycosphaerella* (Götz et al., 1993) or *Botrytis* (Jersch et al., 1989) with long latent periods, more aggressive pathogens with short periods of latency such as *Bipolaris* (Agrios, 1997), hemibiotrophs such as *Colletotrichum* (Williamson, 1994), but also, we assume, incompatible pathogens.

In discussing our results on the symbiosis of fungal endophytes and plant hosts, we are defining symbiosis in the original broad sense as used by De Bary (1879) as "the living together of dissimilarly named organisms" (...des Zusammenlebens ungleichnamiger Organismen...), i.e. an association between two or more organisms of different species. Since the advantages and disadvantages of the association for the participants are not specified, this definition includes mutualists, commensalists and parasites (e.g. Ahmadjian and Paracer, 1986). Symbiosis has been defined and redefined in the course of the past 100 years (including Starr, 1975; Lewis, 1985; Ahmadjian and Paracer, 1986; Margulis, 1991; Smith, 1992; Saffo, 1992). To cite Lewis (1985) concerning the never-ending discussion on the definition of symbiosis, "...I would like to hope that what follows will be the last word but am under no illusions that this will be the case!" His prediction was verified at the Second International Meeting on Symbiosis where different definitions of symbiosis were still in use. As Saffo (1992) and others have noted, this is due to the discrepancy between the popular usage of the term symbiosis meaning "mutualism" and De Bary's original definition of symbiosis which does not specify the benefits of the interaction for the partners involved.

In the following, we report on our investigations concerning interactions of fungal endophytes with their hosts, both with respect to the flux in the role of the fungal partner within its host and with respect to the variability of the physiological interactions between host and fungus. In the context of these results we discuss the definition of symbiosis for the endophyte-host interaction.

2. Materials and Methods

Isolation of fungi

Endophytic strains were isolated from Canadian thistle (*Cirsium arvense* L.) and barley (*Hordeum vulgare* L.) plants growing in Lower Saxony (Germany) either under field conditions or in the case of barley also from plants growing under greenhouse conditions. Surface sterilization of leaf, stem and root segments was with ethanol and sodium hypochlorite, concentrations and times of sterilization being varied depending on the tissue to be sterilized (Schulz et al., 1993). The effectiveness of the surface sterilization was checked by making an imprint of the sterilized tissue on antibiotic malt-peptone-yeast extract (MPY) agar medium (20.0 g malt extract, 2.5 g yeast extract, 2.5 g peptone from meat extract, 12.0 g agar, 1000 ml H_2O). The sterilized segments were cultivated on antibiotic agar media (Schulz et al., 1993) at 20–22°C for approximately 6 weeks. Mycelia were isolated and separately cultivated on MPY agar as they appeared. Plant pathogenic strains were isolated following surface sterilization as above for the endophytic strains, but from diseased plant tissue.

Culture conditions, inoculation of plants or leaf segments and pathogenicity test

Thistle plants derived from root segments (6 cm) were cultured in standardized soil (Composana) : sand mixture (3:1) at 18°C, 70% relative humidity under long day condition (16 h light) at a PAR of 210 $\mu E/m^2$ sec in a greenhouse.

For leaf segment tests, thistle plants were grown in the greenhouse under conditions that minimized the chance of infection. Segments (2.5 cm^2) were cut out of young fully developed leaves and placed on water agar plates, alternately with upper and lower leaf surfaces on the agar surface (n = 5). Depending on whether or not the fungus sporulated in culture, inoculation was done by spraying a spore suspension (2×10^7/ml) or a slightly homogenized mycelial suspension onto the leaf segments. The evaluation of disease symptoms was done 7 and 9 d after inoculation following cultivation under standard conditions in comparison to the controls.

For axenic culture of barley plants, seeds were surface sterilized using 0.1% (v/v) formaldehyde with 0.1% (v/v) Tween 20 for 12 h, washed for 3 × 5 min in sterile tap water and plated for germination on 5% (w/v) semi-solid biomalt medium at 20°C in the dark. After 5–7 d the seeds had germinated with coleoptile and roots and were transplanted into Phytacon growth pots containing 60 g Lecaton expanded clay substrate and 50 ml modified MS

Table 1. Genera of the endophytic fungi of barley and thistle isolated from leaves, stems and roots of healthy plant tissue following surface sterilization with ethanol and sodium hypochlorite, the concentrations and times of sterilization varied according to the consistency of the tissue.

Genus	Number of isolates barley	Number of isolates thistle
Acremonium	56	11
Alternaria	74	43
Ascochyta	80	0
Aureobasidium	0	48
Cladosporium	5	13
Drechslera	394	0
Epicoccum	6	0
Fusarium	124	6
Microdochium	67	0
Penicillium	46	3
Phialophora	11	0
Rhizopus	24	0
Phoma	2	35
Stachybotrys	9	0
Ulocladium	6	0
Verticillium	12	0
Yeasts	9	70
Mycelia sterila	77	35
Miscellaneous isolates	31	23
Total isolates	1033	287

Genera from which there were less than 5 isolates from barley: *Aspergillus, Chaetomium, Coniochaeta, Cylindrocarpon, Geotrichum, Monodictys, Myrothecium, Phoma, Ramichloridium, Rhizoctonia, Septocylindrium, Stemphylium* and *Trichurus*. Genera from which there were less than 5 isolates from thistle: *Arthrinium, Candida, Chaetomium, Cryptococcus, Colletogloeum, Exophiala, Dendryphion, Fusidium, Geomyces, Libertella, Paecilomyces, Penicillium, Polyscytalum, Pyrenophora, Stagonospora, Tricellula*.

(Murashige and Skoog, 1962) liquid growth medium. Two pots were taped together, one inverted, to allow ample room for growth. To enable gas exchange, a hole was cut in the top of the upper pot and fitted with a cotton stopper.

To test for potential pathogenicity, barley roots of 7 d old plants were inoculated with typical isolates of the most common genera parallel to transplantation, by placing a 5 × 5 mm piece of mycelial culture (after 14 d of growth on 2% w/v biomalt agar medium) in the hole for the plant and directly

under the root of the young barley plant. The potential pathogenicity of the isolates was evaluated four weeks after infection by subjectively judging chloroses of stems, shoots and leaves and necroses of the roots in comparison to the axenically cultured control plants.

3. Results and Discussion

Flux in the role of the fungal partner

Fungal endophytes were isolated from roots, leaves and stems of the Canadian thistle and barley. All of the host plants were free of disease symptoms. Most of the fungal genera isolated from the two hosts were ubiquitous ones (Table 1), including genera and species with known pathogenic representatives. Of the 287 isolates from the thistle, the most common genera were *Aureobasidium, Alternaria, Phoma, Cladosporium, Acremonium* and *Fusarium,* whereby 70 of the isolates were yeasts, the latter primarily growing out of the stem segments. Of the 1033 strains isolated from barley, the most commonly isolated genera were *Drechslera, Fusarium, Ascochyta, Alternaria, Acremonium* and *Penicillium.* Riesen and Close (1987) had also isolated primarily ubiquitous genera as endophytes from the leaf blades of barley grown in the field in New Zealand, although only *Alternaria* was among the six most commonly isolated genera in both our studies and theirs. Their second most commonly isolated species, *Didymella phleina,* is, as *Drechslera* (Agrios, 1997) known to be pathogenic of barley.

To assess the role that these strains, isolated as endophytes from healthy plant tissue, play within the host we tested their potential pathogenicity on host tissue by reinfecting the roots of axenically grown plants directly with the fungi (barley) or in leaf segment tests (thistle). In the case of barley, one morphologically typical isolate was chosen from each of four of the most common genera. Only the *Fusarium* isolate caused no disease symptoms of the roots or shoots (Fig. 1A, B). Reinfection with the *Acremonium, Alternaria* and *Drechslera* isolates led to necroses, chloroses and growth inhibition. The most pronounced disease symptoms resulted from infection with *Drechslera* sp., all of the infected roots becoming necrotic and stunted in growth (Fig. 1B). In this context, it is relevant to note that isolates of *Drechslera* are not only known to be pathogenic of grains, but a strain of *Drechslera* was also isolated as an endophyte by Cabral et al. (1993) from leaves of *Juncus bolanderi,* where microscopic examination of the infected tissue showed that the infections were limited to single epidermal cells. This quiescence might be regulated by active host defense responses, since in several cases the authors observed callose formation.

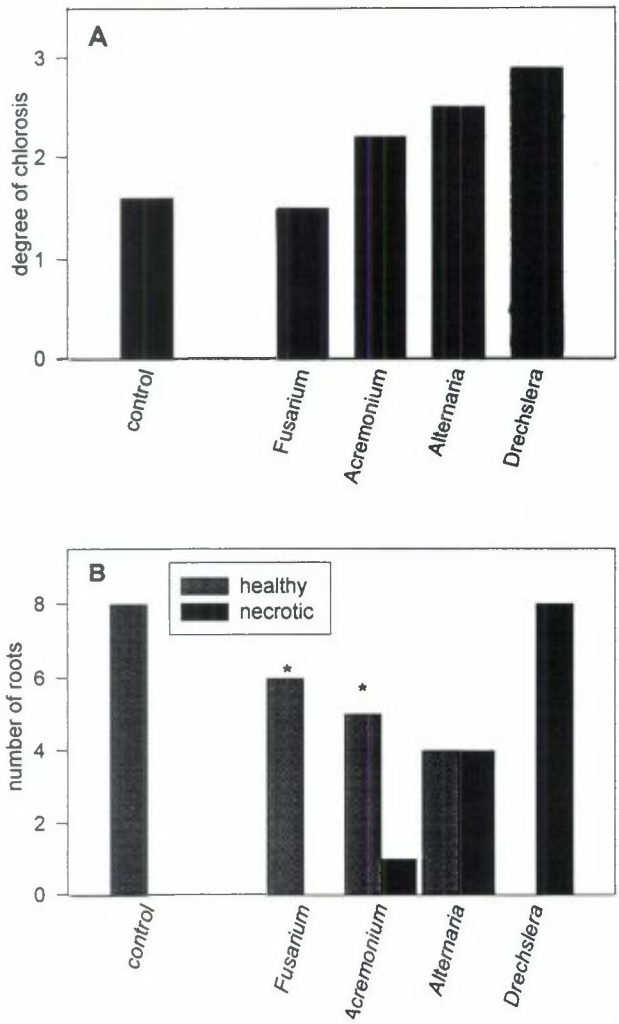

Figure 1. Disease symptoms of barley seedlings, four weeks after inoculation following reinfection with endophytic isolates at the age of one week. A) Evaluation of shoots according to a subjective scale of 1–3 to denote the degree of chlorosis (0–25% chlorosis = 1; 26–50% = 2; >50% = 3; n = 8); B) evaluation of the roots according to presence or absence of visible necrosis (n = 8; *not all roots were infected, n = 6).

Potential pathogenicity of the endophytes isolated from thistle was tested in leaf segment tests using spores and/or mycelial suspensions. Of the 99 endophytic isolates from thistle that were tested in leaf segment tests, 59

Table 2. Leaf segment tests with endophytic isolates of thistle, cultivation for 9 d on water agar at 20°C, 16 h : 8 h (l/d) before evaluation. + = 50% or more increase in disease symptoms (necrosis, chlorosis, maceration) in comparison to the water control, – = less than 50% increase in disease symptoms in comparison to the control.

	Number of isolates	+	–
Genus			
Acremonium	4	3	1
Alternaria	5	4	1
Aureobasidium	1	1	0
Candida	1	1	0
Chaetomium	1	1	0
Cladosporium	9	5	4
Cryptococcus	1	0	1
Exophiala	2	1	1
Fusarium	5	3	2
Fusidium	1	0	1
Geomyces	1	0	1
Paecilomyces	1	1	0
Penicillium	3	1	2
Phoma	5	4	1
Polyscytalum	2	2	0
Stagonospora	2	1	1
Tricellula	2	1	1
Yeasts	10	2	8
Mycelia sterila	18	11	7
Species			
Acremonium kiliense	3	2	1
Alternaria alternata	2	1	1
Aureobasidium microsticum	1	1	0
Cladosporium cladosporioides	3	2	1
Cladosporium herbarum	2	1	1
Cryptococcus albus	1	0	1
Fusarium tabacinum	4	2	2
Paecilomyces carneus	1	1	0
Phoma destructiva	1	1	0
Phoma lingam	1	1	0
Phoma nebulosa	2	2	0
Polyscytalum pustulans	2	2	0
Tricellula inaequalis	2	1	1

caused an increase of at least 50% in the extent of necroses, chloroses and/or maceration in comparison to the controls which exhibited normal senescence (Table 2). There was no obvious correlation between pathogenicity, i.e. the

ability to cause disease symptoms in the leaf segment tests, and genus of the 18 genera tested. Some, but not all, of the *Acremonium, Alternaria, Cladosporium, Fusarium, Penicillium, Phoma, Mycelia sterila,* but also some yeast isolates caused disease symptoms. Additionally, of the species from which 3 or more isolates were tested there was no single species from which all isolates caused disease symptoms. Conversely, isolates from diseased tissue may grow as endophytes when tested *in vitro* for aggressiveness (Peters et al., this volume).

The pathogenicity that approximately 2/3 of the fungal strains isolated as endophytes from barley and thistle showed in our tests demonstrate that being isolated as an endophyte does not exclude the possibility that the isolate will be aggressive or be pathogenic at a later time or under other environmental conditions, aggressiveness being defined as the ability to infect and colonize host tissue. Some of the fungal strains that were pathogenic in our tests had presumably been latent or quiescent pathogens, as was suggested by Cabral et al. (1993) for *Drechslera* sp. in *Juncus bolanderi.* Others were perhaps weak pathogens. It is interesting to note that leaf segment tests with isolates from surface sterilized diseased tissue of thistle gave similar results: approximately 2/3 of the isolates of the same genera as those from which the endophytes had been isolated caused disease symptoms in the leaf segment tests (unpublished results).

The ability of a fungal isolate to cause disease not only depends on the pathogenicity of the fungus, but also on the disposition of the plant host. Neither the axenically cultured barley plants nor the leaf segments of thistle had the predisposition to resist disease that plants growing *in situ* have. Thus, in plants with lowered defense potential not only the fungal pathogens with a predetermined period of latency, but also the weak pathogens may have caused disease symptoms. Similar results were obtained by Wilson (1995), who observed that the endophyte *Discula quercina* only caused 12.5% infection in the field, in contrast to 100% in leaf infection tests. He also accredited the difference in infectivity to the predisposition of the host. Additionally, the inoculum density used in experiments to test for potential pathogenicity was higher than that found under field or greenhouse conditions, favoring the development of the fungal isolate. Nevertheless, the fact that only some of these isolates caused disease shows that there is great variability in their innate pathogenicity. *In situ* disease may be triggered by unfavorable environmental conditions, e.g. air pollution, changes in water pressure deficit, other pathogens or insects.

Regarding the role of the fungal partner within its host, we have seen that the interaction of the host with a particular fungal strain may depend on the aggressiveness of the fungal strain, the developmental stage of host and fungus, as well as on the predisposition of the host. At one stage, a particular fungal strain is an endophyte and causes no visible symptoms. At another stage of

development or under different environmental conditions which might alter the predisposition of the host, the same fungal strain may become a pathogen. Thus, as suggested by Petrini (1991), the symbiosis of host and endophyte is a variable situation, one in continual flux. Viewing the interaction from a different standpoint, at one stage the endophyte may enter into a mutualistic symbiosis with its host (Carroll, 1988; Wulf, 1990; Calhoun et al., 1992; Schulz et al., 1995), but later the endophyte becomes a pathogen and the relationship can be termed parasitic. This flux not only occurs in the endophyte-host interaction, but also in other symbioses. For example, in some cases AM or ectomycorrhizal fungi, which usually enter into mutualistic symbioses with their hosts, may no longer be beneficial to the host (Janos, 1996). In extreme cases AM fungi may even function as parasites (Modjo and Hendrix, 1986). Therefore, only De Bary's definition of symbiosis, which encompasses all interactions between organisms of different species, takes into account the flux that occurs in the relationships between organisms during different developmental stages and under different environmental conditions.

Variability in the physiological interaction between host and fungus

Until now, we have considered the variable role of the fungal partner in the endophyte-host interaction. In order to understand the relationship between two organisms it is also necessary to investigate the physiological aspects involved (see also Peters et al., this volume). We compared the endophyte-host interaction with that of a pathogen, studying these interactions on four levels.

The first level for studying the physiological interactions was the screening of fungal isolates for herbicidal activity. Although there have been numerous publications on the isolation of secondary metabolites from endophytes with antimicrobial (e.g. Fisher et al., 1984a; Fisher et al., 1984b; Dreyfuss, 1986) and insecticidal (Johnson and Whitney, 1994; Calhoun et al., 1992) activities, the herbicidal potential has seldom been studied (Schulz et al., 1995). We found that 52% of the endophytes, but only 27% of the pathogens and 18% of the soil isolates produced metabolites active against our plant and algal test organisms (Schulz et al., 1996; Krohn, 1996). This suggests that particularly the endophytic isolates, assuming that they also synthesize these metabolites *in situ* (Demain, 1980), produce metabolites that are potentially toxic for their hosts. These substances could be toxins which render plant tissue more amenable to colonization (Tyler, 1993; Otani et al., 1995). They might be membrane active and *in vivo* damage host membranes so that the nutrition of the fungus is improved or they could also down-regulate plant defense reactions.

The next level was a simplified *in vitro* system using dual cultures of endophytes with calli of their hosts. We found that both fungal endophyte and host calli secreted metabolites toxic to their respective partners. The endophytes secreted non-specific herbicidal substances and the host calli secreted non-specific fungicidal metabolites (Peters et al., 1998). Others (Sieber et al., 1990; Hendry et al., 1993) had also found that in dual culture both fungi and host calli secreted metabolites that influenced growth of the respective partner. Thus, although in nature endophyte and host in some cases appear to be mutualistically cohabiting, in this simplified system they not only have the potential but seem to be mutually antagonistic.

To characterize the plant defense reaction, we first determined a rapid and two delayed defense reactions of the host *Lamium purpureum* L. to endophytes and a pathogen in three test systems, resulting in clear defense reactions against both fungi and fungal elicitors (Peters et al., this volume). When there was direct contact between plant cells and fungus, the endophytes induced greater defense responses (PAL-activity, total secreted phenols, H_2O_2) than the pathogen did. These results suggest that the endophyte may have been recognized as an incompatible pathogen (Low and Merida, 1996). A weak defense response to the pathogen *in vivo*, could result in disease of the host tissue.

In order to more closely approach the *in situ* situation, the production of phenolic defense metabolites was studied in intact plants of barley (*Hordeum vulgare* Mill.) and larch (*Larix decidua* L.) following infection with either an endophyte or a pathogen (Schulz et al., in preparation). In roots of barley, we were able to identify coumaric and ferulic acids, n-4-coumaroylputrescin and n-4-coumaroylagmatin. In the roots of the larch, we found changes in the concentrations of catechin and proanthocyanidins following infection. In all cases, the concentrations of the phenolics were higher in the plants infected with the endophyte than in those infected with the pathogen, corresponding to the results obtained for induction of the plant defense reactions in the test system using suspension cultures (Peters et al., this volume) in which there is also direct contact between host cells and fungal elicitors or fungus.

Our results studying the physiology of the endophyte-host interaction on four levels show that both endophyte and host have active defense mechanisms against their respective partners. Thus, although by definition all endophytic infections are symptomless, we see that the macroscopic condition of the host gives no indication of the metabolic interactions involved between the two symbionts.

Additionally, if these results are compiled to a composite picture (Table 3), it seems that the endophyte-host symbiosis should primarily be seen as a balanced antagonism. The evidence supporting this hypothesis:

- In dual cultures of endophyte and host calli, both partners excrete metabolites toxic to one and other.
- In test systems in which there is direct contact between host cells and fungus, the plant defense reactions are stronger towards endophytic than towards pathogenic infection.
- Endophytes produce, in contrast to other fungal isolates, a high proportion of herbicidal metabolites. These might be directed against the host, making it more amenable to colonization.

In our opinion, both host and endophyte actively defend themselves against each other. However, by maintaining an equilibrium, this mutual antagonistic symbiosis does not lead to disease. When this equilibrium is disturbed in favor of the fungus, e.g. due to senescence of the host or changed environmental conditions, the interaction becomes imbalanced, the endophyte becomes a pathogen and disease develops. In the long run, maintaining the endophytic phase for an extended period of time is advantageous for both partners and there are two winners: the endophyte that remains protected in its ecological niche until it eventually sporulates and the host that is usually not reduced in its capacity for vegetative growth and propagation.

4. Conclusion

We have provided evidence that symbiosis should be defined as originally done by De Bary to mean the living together of organisms of different species. This definition avoids anthropogenic prejudgements about variable or unknown aspects of the interaction. In the case of the endophyte-host interaction, one variable is the continual flux in the role of the fungal partner, which may, for example, at one moment be that of an endophyte and at another phase in development be that of a pathogen. The other variable is the frequently unknown physiological interactions between the two symbionts which prevents specifying the advantages and disadvantages of the interaction for the individual partners. The use of this broad definition of symbiosis does not exclude the possibility of additionally characterizing an interaction as mutualistic, commensalistic or parasitic when enough is known about the relationship to warrant a classification.

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Table 3. Comparison of the physiological interactions of endophytes or pathogens and host cells using axenic culture of barley seedlings infected with an endophyte (*Fusarium* sp.) or a pathogen (*Drechslera* sp.) and of larch seedlings infected with an endophyte (*Cryptosporiopsis* sp.) or a pathogen (*Heterobasidion annosum*); dual cultures of host callus (*Lamium purpureum*) with an endophyte (*Coniothyrium palmarum*) or a pathogen (*Alternaria* sp.); and suspension cultures with elicitors and mycelium of endophyte and pathogen (organisms as with dual cultures). In comparison to the controls: o = no effect, – = weakly negative effect, – – = negative effect, + = positive effect, ++ = strongly positive effect.

	Endophyte	Pathogen
Disease symptoms in reinfected host seedlings	o	+
Plant defense reactions		
Suspension cultures following elicitation		
PAL-activity	++	+
Oxidative burst (H_2O_2)	++	+
Total phenolics	++	+
Intact plants		
Phenolic defense metabolites		
in *Hordeum vulgare*		
Soluble:		
N-4-coumaroylagmatine	o	– –
N-4-coumaroylputrescine	o	– –
Bound:		
Ferulic acid	+	– –
4-coumaric acid	o	– –
in *Larix decidua*		
Soluble:		
Catechin	–	– –
Proanthocyanidins	+	o
Fungal metabolites		
Excretion of metabolites toxic to partner in dual culture	+	+
Herbicidal metabolites in screening (*Chlorella*)	++	+

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