

Soil contamination by crude oil: impact on the mycorrhizosphere and on the revegetation potential of forest trees

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Abstract

In vitro and greenhouse biotests were carried out to study the effects of various concentrations of crude oil on the mycorrhizosphere and the ability of ectomycorrhizal fungi to colonise Norway spruce and poplar seedlings grown on contaminated soil. Ectomycorrhizal fungi grown in pure cultures showed a variety of reactions to crude oil, ranging from growth stimulation to total inhibition of growth, depending on the species of fungi. Germination of poplar and spruce seeds was not significantly affected. The growth of spruce seedlings was not affected by crude oil, whereas that of poplar seedlings was significantly reduced at high concentrations. None of the concentrations had any effect on the degree of ectomycorrhizal and endomycorrhizal colonisation of poplar. With spruce, however, the ectomycorrhizal fungi showed species-specific reactions to increasing concentrations, in accordance with the results of the pure culture test. The length of time between soil contamination and seeding affects both seedling growth and the mycorrhizal infection potential of the soil. The results confirm the importance of mycorrhizal fungi in the bioremediation of soils contaminated by crude oil. © 1998 Elsevier Science Ltd. All rights reserved.

In Labor- und Gewächshausversuchen wurde die Wirkung von Erdöl auf die Mykorrhizafloora und ihr Potential, Feinwurzeln von Fichten und Pappelsämlingen zu besiedeln, durchgeführt. In Reinkultur zeigten die 10 untersuchten Ektomykorrhizapilze unterschiedliche Reaktion auf Erdölkontamination, von Stimulation bis zu totaler Hemmung des Wachstums. Ein Einfluss von Erdöl auf die Samenkeimung war bei keiner der beiden Baumarten festzustellen. Das Sämlingswachstum wurde bei Pappel durch hohe Erdölkonzentrationen signifikant gehemmt, währenddem es bei Fichte praktisch unbeeinflusst blieb. Die Besiedlung der Feinwurzeln durch Endo- und Ektomykorrhizapilze wurde bei Pappel durch Erdöl nicht beeinflusst, bei Fichte zeigten Ektomykorrhizapilze—in Übereinstimmung mit den Resultaten der Reinkulturversuche—artspezifische Reaktionen auf Erdölkontamination. Die Wartezeit zwischen der Erdölkontamination des Bodens und der Aussaat beeinflusst sowohl das Sämlingswachstum wie das Mykorrhiza-Infektionspotential des Bodens. Die Resultate bestätigen die Bedeutung der Mykorrhizapilzfloora im Zusammenhang mit der Wiederbesiedlung von erdölkontaminierten Böden. © 1998 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Crude oil is extracted world-wide at the rate of more than 65 million barrels per day and meets the bulk of the world's energy requirements. At the same time, however, it is a potential hazard and can cause severe and lasting damage if it is accidentally discharged into the environment. Such accidents happen despite the fact that the distribution and storage network for crude oil is closely monitored.

An accident of this kind occurred at Trecate, N. Italy, in 1994, when an oil well became uncontrollable and blew out some 18 000 Sm³ (standard m³, its gas volume at 15°C) of crude oil over an area of 1500 ha (AGIP,

1995). The area consists mainly of farmland and forest. Since oil spills have rarely affected areas with abundant terrestrial vegetation, new methods for assessing the harm caused to the ecosystems in Trecate's affected area had to be sought.

Most oil-wells are situated in deserts and oceans, which is where the majority of the world's documented blow-outs have occurred. Tanker accidents have been the most frequent cause of oil spills, resulting in pollution in coastal regions. Knowledge about the effects of oil pollution on vegetation has, therefore, been largely based on aquatic biocoenoses, such as salt marshes (Baker et al., 1993), mangrove forests (Wardrop et al., 1987; Teas et al., 1989; Garrity et al., 1993) or other

aquatic organisms (Østgaard et al., 1987). Jenkins et al. (1978), however, report on the effects of large, hot crude-oil spills upon a black spruce (*Picea mariana*) site.

Investigations into the effects of crude oil on plants have concentrated on the direct toxic effects of oil on the upper parts of plants, and a complete overview of these is given by Baker (1970). However, very little is known about the effects of oil on the soil and its micro-organisms, such as mycorrhizal fungi, and the resulting indirect effects on plants. In addition, owing to the great variety and complexity of the biological systems affected by the release of crude oil into the environment, investigations into its effects have usually been confined to laboratory tests that have focused on just a few of the many chemical compounds that make up crude oil.

The degradation rate of hydrocarbons is determined by the complexity of their molecules. Linear compounds up to C30, for example, may disappear in a month, whereas those up to C37 need at least 200 days. Indeed, the entire tar fraction can persist for 4 years or longer in the northern temperate zone (Gudin, 1978).

The C/N ratio tends to favour carbon in oil-contaminated soils. This promotes intensive metabolic activity on the part of all the micro-organisms not inhibited directly by the oil. The severity of the damage caused to the vegetation by the rupture of the long hydrocarbon chains and by the mobilisation of all the nitrogen present increases as the amount of nitrogen available decreases. Only nitrogen-fixing plants can survive under these conditions. The result is a *de facto* natural selection of crude-tolerant species (Antoniewski and Schaefer, 1972). Oxidative degradation also changes the composition of the soil bacteria population, so that aerobic cellulolytic and proteolytic species decrease and anaerobic nitrogen-fixing species increase (Anderson et al., 1993).

Similar effects, namely a drastic reduction in the ectomycorrhizal biomass and in its infection potential, have been noted in the aerobic microflora of the Arctic tundra (Miller et al., 1978), whereas some fungi appear to be almost fully resistant to gas oil. Indeed, *Trichoderma harzianum* actually benefits from its presence (Gudin and Chater, 1977).

This paper describes a study of the effects of crude oil on the mycorrhizosphere and the ability of 10 different ectomycorrhizal fungi to recolonise Norway spruce (*Picea abies*) and black poplar (*Populus nigra*) seedlings grown on contaminated soils.

2. Materials and methods

2.1. In vitro growth tests with pure cultures of ectomycorrhizal fungi

The reaction to crude oil of 10 ectomycorrhizal fungi (*Amanita pantherina*, *Amphinema bissoides*, *Armillaria*

mellea, *Cenococcum geophilum*, *Hebeloma crustuliniforme*, *Laccaria amethystea*, *L. bicolor*, *L. laccata*, *Pisolithus tinctorius*, *Tricholoma vaccinum*) was tested by inserting agar disks with their pure cultures in Petri dishes containing either no crude oil or six different concentrations of oil (0.1, 1, 5, 10, 20 or 50 g kg⁻¹). The oil was added just before the dishes were filled with nearly solid MMN-agar (prepared according to Marx and Bryan, 1971) to prevent the oil from rising to the surface and to ensure that it was well-distributed within the medium. The Petri dishes were kept in the dark at 18°C. Fungus growth was measured weekly for a maximum of 35 days or until the edge of the dish was reached. Ten replicates were taken for each fungal species and each concentration.

2.2. Biotest with contaminated soil

Soil collected at a depth of 0–30 cm from the Trecate area (Novara, Italy) and at a depth of 0–10 cm from a spruce stand in Schwanden (Canton Glarus, Switzerland) was passed through a 2-mm sieve, homogenised with a gardening mixer and then contaminated with crude oil from the AGIP Oils Centre Trecate by hand spraying and continuous mixing in until the appropriate concentration level (0.1, 1, 5, 10, 20 or 50 g kg⁻¹) was reached. The chemical composition of the crude oil is described in Table 1. An uncontaminated control was set up for both soils. The soil was then placed into 'Spencer LeMaire' plastic rootainers[®] divided into 32 pots measuring 3.5×3.5×11 cm (135 cm³ soil per pot) and bedded down by watering.

Seeds of black poplar (*Populus nigra*) from the seed bank of the Casale Monferrato Experimental Poplar Research Station and Norway spruce (*Picea abies*) seeds from WSL seed bank were surface-sterilised for 2 min (poplar) or 30 min (spruce) in 30% H₂O₂. Then 10 poplar seeds were sown in each pot on the Trecate soil and 5 spruce seeds on the Schwanden soil. The number

Table 1
Composition of the crude oil of Trecate (AGIP, 1995)

Water	0.10%
H ₂ S	0.01%
Hydrocarbons	88%
Gaseous fraction	11% (4% methane, 7% C ₂ –C ₄ compounds)
Liquid fraction	77%
Polycyclic Aromatic Hydrocarbons	8%
Sulfur organic compounds	0.16%
Light fuel	6.5%
Total fuel	32.5%
Kerosene	20.0%
Diesel fuel	11.0%
Lubricating oils	16.5%
Residue	18%
Loss	2%

of the seedlings was reduced later to 3 per pot. Seeding was performed at four different times: Time 0 (moment of oil contamination), Time I (7 days after contamination), Time II (21 days) and Time III (42 days). Eight replicates (pots) were performed for each treatment and placed in a greenhouse with the temperature maintained at 20°C with a relative humidity of 80% in daylight.

Germination percentages were determined 7 days and 14 days after sowing. After 4 months, the height and dry weight of the seedling shoots were measured. The rootlets were washed carefully and fixed in 6% formalin for microscopic examination. On spruce roots, ectomycorrhizal morphotypes were determined and quantified. Poplar roots were stained according to Rajapakse and Miller (1991) to assess the percentage of ectomycorrhizal tips and the percentage of roots with arbuscular mycorrhizas. Student's two-sample *t*-test was used to evaluate the effects of each treatment.

3. Results

3.1. In vitro growth tests of pure cultures of ectomycorrhizal fungi

Three types of reaction were noted: (1) the growth of *A. pantherina*, *T. vaccinum* and *P. tinctorius* was reduced by increasing crude-oil concentrations, and 50 g kg⁻¹ was lethal for *A. pantherina* and *T. vaccinum* (Fig. 1); (2) the growth of *L. bicolor*, *L. amethystea* and *L. laccata* increased with oil concentrations between 1 and 20 g kg⁻¹, and decreased slightly at 50 g kg⁻¹ (but it was still more than the control up to 5 g kg⁻¹); (3) the growth of the other four species was unaffected and they

displayed no conspicuous response to the presence of crude oil.

3.2. Biotest with contaminated soil

3.2.1. Poplar

The germination percentages of the poplar seeds were rather low, even in the control, and their great variability made it impossible to establish any statistically significant differences. Seedling shoot lengths were severely affected by concentrations higher than 5 g kg⁻¹ and growth was greatly reduced at all seeding times by those concentrations higher than 10 g kg⁻¹. Significant growth and shoot biomass (dry weight) differences between Time 0 and Time III were found only for concentrations up to 1 g kg⁻¹ (Table 2). At lower concentrations, growth was actually stimulated, especially between Time 0 and Time I.

None of the oil concentrations had any effect on the degree of ectomycorrhizal and endomycorrhizal infection, but the length of time between contamination and seeding did make a difference. The percentages of ectomycorrhizal colonisation decreased between Time 0 and Time III, and those of endomycorrhizal colonisation increased (Fig. 2).

3.2.2. Norway spruce

The germination rate of Norway spruce seeds was reduced significantly only at the highest oil concentration (50 g kg⁻¹). At lower concentrations there was no effect. Seedling growth was not influenced by the oil concentration (Table 2), but it was by the seeding time. Shoot length and biomass were significantly reduced when seeded 42 days after contamination (Time III)

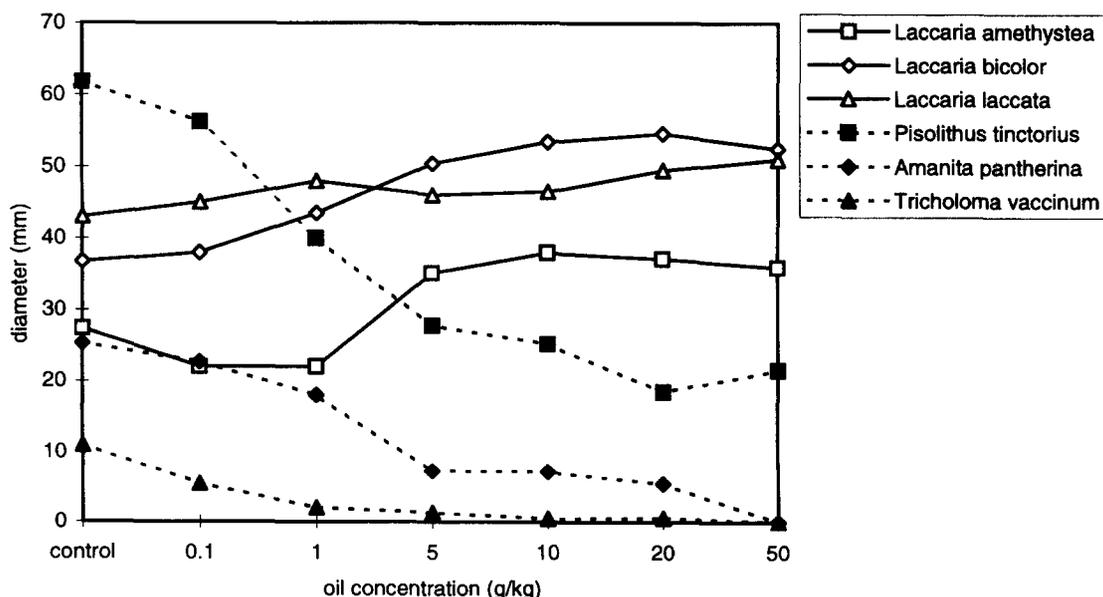


Fig. 1. In vitro growth of some ectomycorrhizal fungi at different concentrations of crude oil (35 days after inoculation).

Table 2

Growth response of poplar and spruce seedlings to crude-oil concentration and time of sowing after contamination

	Time of sowing	Control	Crude oil concentration (g kg ⁻¹ soil)					
			0.1	1	5	10	20	50
Poplar								
Shoot length (cm)	Time 0	6.1	6.0	7.2	5.7	2.4**	1.7**	2.0**
	Time I	5.2	6.9**	7.1*	4.8	2.3**	1.7*	1.7**
	Time II	5.3	4.6	5.0	4.7	2.5**	2.0**	2.2**
	Time III	3.7	4.1	4.7*	4.6	3.1	2.1**	2.4
	Time III-Time 0	-2.4**	-1.9**	-2.5**	-1.1	0.6	0.4*	0.4
Shoot biomass (mg plant ⁻¹)	Time 0	41.4	40.1	61.9*	40.2	8.3**	4.6**	4.6**
	Time I	29.5	48.3**	46.6*	24.6**	7.1**	4.7**	3.9**
	Time II	31.9	26.6	36.2	35.8	8.6**	4.1**	4.4**
	Time III	15.9	23.8**	24.8**	30.3	14.8	6.2**	3.3**
	Time III-Time 0	-25.5**	-16.3**	-37.1**	-9.9	6.5*	1.6*	-1.3*
Spruce								
Shoot length (cm)	Time 0	3.1	3.3	3.2	3.2	3.3	3.3	2.9
	Time I	3.8	2.8**	3.2	3.2	3.3	3.3	3.1*
	Time II	2.7	2.4	2.4	2.5	2.4*	2.4	2.3
	Time III	2.4	2.1	2.1	2.6	2.4	2.2	2.5
	Time III-Time 0	-0.7**	-1.2**	-1.1**	-0.6**	-0.9**	-1.1**	-0.4**
Shoot biomass (mg plant ⁻¹)	Time 0	15.5	16.3	18.1	12.4	19.5*	18.5	9.1**
	Time I	13.9	15.9	15.7	14.2	17.3	17.2	9.2
	Time II	13.7	13.3	14.7	14.0	15.1	15.0	8.7**
	Time III	10.1	8.7	8.3	12.1	11.0	9.4	8.0
	Time III-Time 0	-5.4*	-4.6**	-9.8**	-0.3	-8.5**	-9.1**	-1.1

Means of 8 (biomass) or 24 (length) replicates. Significant differences (*t*-test) between concentrations and the control and between Time 0 and Time III are indicated by **p* < 0.05 and ***p* < 0.01.

compared with when they were seeded at the time of contamination (Time 0).

Plants sown at Time 0 were extensively colonised by ectomycorrhizal fungi (Fig. 3). More than 90% of the root tips were mycorrhizal. With increasing lapses of time between contamination and seeding, this percen-

tage went down to 60% (Time III). Six morphotypes (C, D, E, F, G, H) were distinguished and described according to their macroscopic and microscopic features (Table 3). Types C and E were identified as *C. geophilum* and *Laccaria* sp., respectively. Types C and D became less frequent with increasing oil concentration. Type D

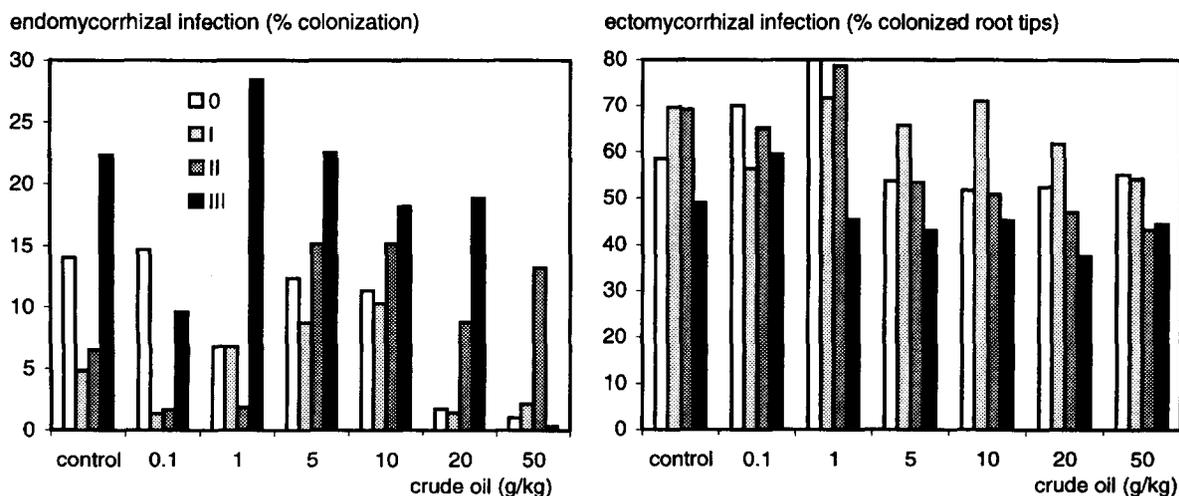


Fig. 2. Endo- and ectomycorrhizal colonisation of poplar roots.

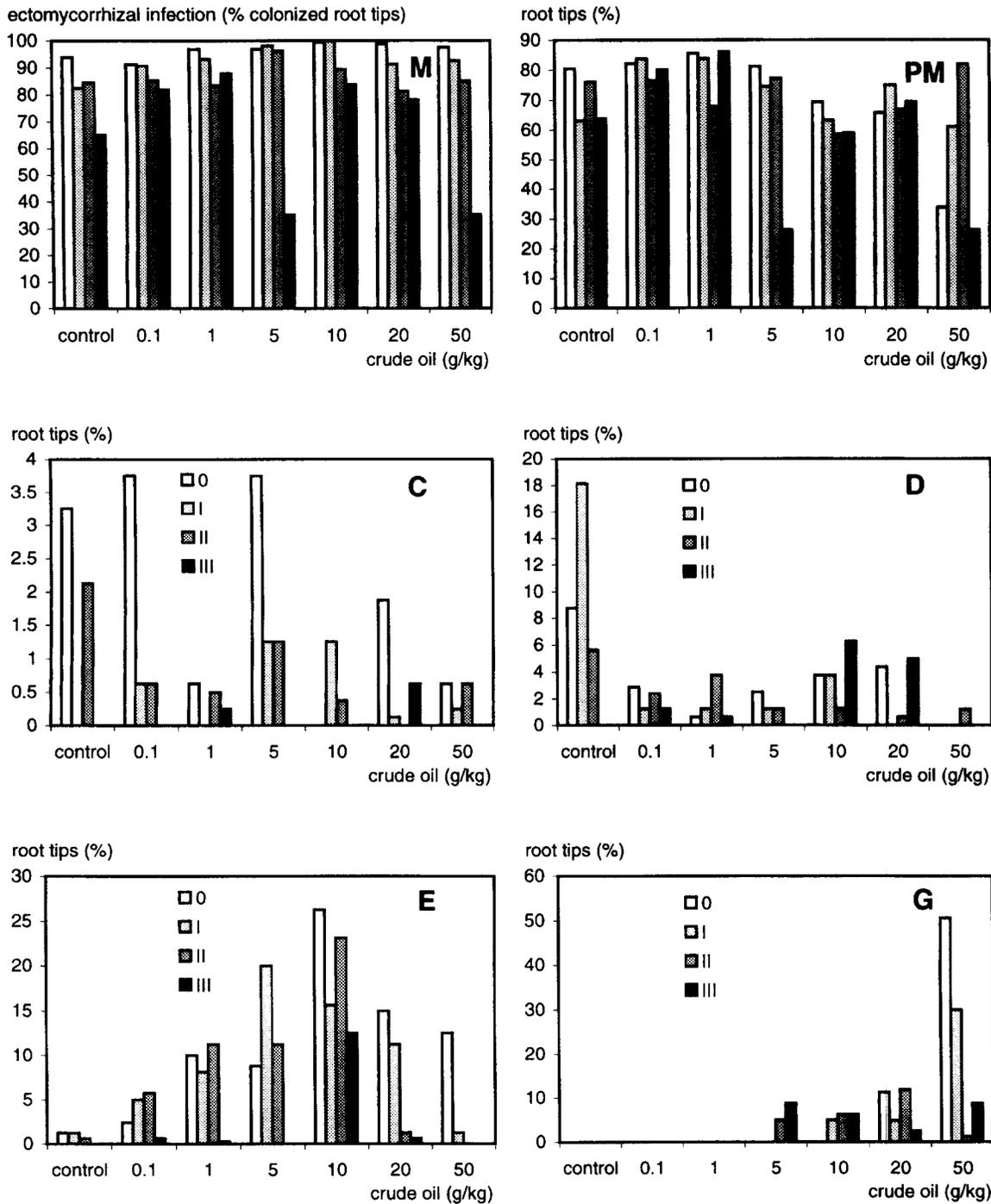


Fig. 3. Occurrence of ectomycorrhizal morphotypes on spruce (M: ectomycorrhizas; PM: pseudomycorrhizas; C, D, E, G: morphotypes).

was the most sensitive: its frequency was reduced at all concentrations compared with the control. types E and G were apparently favoured by the presence of crude oil: type G was only present at 5 g kg^{-1} and above, whereas type E displayed an optimum at intermediate concentrations.

The percentage of pseudomycorrhizas did not seem to be influenced by the presence of oil. Types F and H were

not present in sufficient quantities to enable any conclusions to be drawn.

4. Discussion

Crude oil in the concentrations applied in this study neither affects seed germination nor kills seedlings. It

Table 3

Macro- and microscopic descriptions of the ectomycorrhizal morphotypes on spruce seedlings sown on the Schwanden soil (Rhiz = Rhizomorphs)

Type	Macroscopic features			Microscopic features		Remarks
	Colour	Mantle surface	Rhiz	Ramification	Mantle structure	
C	Black	Smooth to grainy	-	Simple, often single	Plectenchymatous, star-like arrangement	<i>Cenococcum geophilum</i>
D	Yellow-brown	Gelatinous	-	Simple	Plectenchymatous	
E	Opaque white to grey to beige	Smooth, silvery, mat	+	Monopodial-pinnate	Plectenchymatous	<i>Laccaria</i> -like
F	Dark-brown to black	No distinctive mantle	-	Simple, often single	Mantle lacking	
G	Red-brown	No distinctive mantle, loose hyphae	-	Simple	Mantle lacking	Pseudomycorrhiza-like, but thickened
H	White	Cottony, with emanating hyphae	-	Monopodial-pinnate	Plectenchymatous	<i>Hebeloma</i> -like

can, however, reduce seedling growth, although this varies according to plant species. In the light of the results of an earlier study on the same subject (Barolo, 1996), it seems likely that crude oil has a caustic or lethal effect only when it comes into direct contact with the tissues of a plant, as happens when an oil slick from a tanker accident reaches the coast. Crude oil's indirect effect on the soil is confined to a more or less marked reduction in plant growth and biomass, possibly attributable to the changes brought about in the soil microbe populations. On the other hand, the growth of certain plants, such as *Festuca rubra* and *Puccinellia maritima* (Baker, 1971) or *Trifolium rubra* (Klokk, 1984), can also be stimulated by crude oil. The mechanisms for this phenomenon are unclear, but growth stimulation could be the result of the direct release of nutrients or growth-regulating compounds from the oil or to the release of nutrients from animals or bacteria killed by the oil (Baker, 1971).

The results of this study reveal a very high variability in the data, especially in the mycorrhizal data. Those working in mycorrhiza research are quite familiar with the problem of the lack of homogeneity in mycorrhizal data, both in field studies and in bioassays. This makes the interpretation of such data more difficult, but it is still possible to draw some conclusions from the present study.

One striking finding is that seedling growth is reduced the longer the time between contamination and seeding. The reverse was to be expected since soil recovers with time and the concentrations of phytotoxic volatile compounds decrease. One explanation could be that seedling growth is affected by mycorrhizal colonisation, which reacts conversely. The ectomycorrhizal infection potential on spruce and poplar is highest immediately after contamination and decreases over time. It is likely that the increased activity of ectomycorrhizal fungi just after oil contamination contributes to the seedlings' better growth.

The decrease in the ectomycorrhizal infection potential with time may be due to the direct effect of the oil

on the hyphae of some more sensitive ectomycorrhizal species. On the other hand, the endomycorrhizal infection potential on poplar is highest 42 days after contamination. This could be attributed to the fact that endomycorrhizal fungi are endowed with survival spores and are, therefore, more resistant to environmental stress factors. When the oil contamination has decreased sufficiently over time through biodegradation, it is possible for new mycelium generated by germination of surviving spores to develop in the soil and colonise plant roots.

Since rapid and complete colonisation is essential for survival in the early stages of plant development, the different reactions of ecto- and endomycorrhizal fungi to oil contamination could have considerable influence on the success of recolonisation. Recolonisation with ectotrophic plants could be difficult as it would require them to be set out immediately after soil contamination to ensure that they find their symbionts, since these cannot survive long in oil-contaminated soil. This, however, is impracticable because reclamation work which disturbs the soil is always necessary after a blow-out. Endotrophic plants, on the other hand, can be set out much later since their resistant spores can survive in the contaminated soil.

The *in vitro* growth test showed a species-specific response. Some fungal species are inhibited by oil, while others actually grow better in oil-contaminated soil, even at very high concentrations (*Laccaria* sp.). Yet others display no definite reaction. A similar behaviour pattern was evident in the biotest: type G fungi only form ectomycorrhizas at very high concentrations; type E prefer intermediate concentrations of oil; other types seem to be uninfluenced.

The best colonisation rate for type E was at intermediate oil concentrations, which corresponds exactly to the growth response of the three *Laccaria* species in pure culture, indicating that type E probably belongs to this genus. A comparison of the morphology and anatomy of type E with those of the *Laccaria* mycorrhizas as described in the literature (Agerer, 1995) revealed a very

close resemblance in that both had a plectenchymatous mantle structure and smooth, undifferentiated rhizomorphs. From this it can be concluded that the probability is very high that morphotype E represents a *Laccaria* species.

The findings of this study suggest that a crude oil blow-out in a mixed agricultural and forest area does not cause long-term environmental damage of the kind associated, for example, with coastal ecosystems. This could be due to bioremediation on the part of the microflora in the soil, as already reported for certain bacteria (Miller et al., 1978) and fungi (Barr and Aust, 1994). Our results support this hypothesis. Moreover, they show that some of the ectomycorrhizal fungi that survive in contaminated soil may actually use crude oil as a nutrient and thus make their own contribution to the process of bioremediation.

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