equilibrates the pressure difference between inside and outside the nest. The moved air volumes in both *Trigona* species are small in comparison to honeybee colonies, but in the light of the minute size of the stingless bees and their nests the volumes may not be so small. Since the ventilation is due to fanning of workers, the wing surface area may be an important parameter (among others) for the determination of tidal and minute volumes. If we relate the min-volumes of the stingless bee colonies to wing surface area, we obtain results in the same range than previously found for honeybee colonies[12] (min volume/wing surface area: *Apis mellifera:* wing surface approx. 15 mm^2, 8 ml/min/mm^2; *T. denoiti:* wing surface approx. 1.52 mm^2 0.55 ml/min/mm^2; *T. gribodoi:* wing surface approx. 1.42 mm^2, 4.4 ml/min/mm^2). If we look at the nest volumes we see that it takes a long period of time to exchange the complete gas volume in *T. denoiti*. The ventilatory activity in *T. gribodoi* exchanges the total nest volume (< 500 ml) in less than 1.5 h. This rate may be sufficient to prevent the accumulation of CO_2 in the colony. Strong nests of *T. gribodoi* consist of only 700–800 bees and a few hundred brood cells[11]. Indeed the recorded O_2 concentrations were high during the day. However, nests of *T. denoiti* have a gas volume of approx. 1000 ml[10] and it would take 7–8 h to exchange this volume at the observed breathing rates. Colonies consist of more than 10,000 bees, and of 10–14 brood combs each containing about 500 brood cells[10]. Even considering that the metabolic activity of bees and brood inside the nest is very low, the minute daytime ventilation should cause an accumulation of CO_2 in the nest cavity. This is supported by the very low levels of oxygen during the day, which most likely result from a high CO_2 concentration in the nest. During our recordings at night, the oxygen level was substantially higher than during the day. Apparently the bees had flushed the nest before we started our night-time recordings. Initially we could observe large tidal volumes resembling yawns in mammals, which became rare once the oxygen concentration was higher than 20.7%. It would need only 1–2 h of ventilation with those large tidal volumes to exchange the complete nest volume. Like in honeybees the activity of the bees was low during the night. The nest temperature which was constant day and night may mainly be an indication for the insulative value of the soil rather than metabolic activity of the bees[10].

The colonial breathing pattern discovered in stingless bees is very much the same as in honeybee colonies housed in airtight containers with a single entrance hole only. The breathing frequencies of honeybee colonies were in the same range (2.9 ± 0.84 bpm) and they also reduce the ventilatory activity during the night substantially. Min-volumes were about 10% of the daytime values, a reduction more drastic than in *T. denoiti* nests (39%).

The small tidal volumes of *T. denoiti* during daytime prevents the nest from overheating. The cool air stays in the nest and only as little as possible hot fresh air is introduced. During the night, when the outside air is cool, the tidal volumes are significantly higher.

The higher minute volume in the arboreal *T. gribodoi* may also result from the less severe temperature conditions in comparison to the ground nesting species. Ambient temperatures for *T. denoiti* nest entrances were as high as 60° C at the soil surface, whereas the outside air in the shade of the tree was only 36° C. The influx of fresh air is a small thermal danger to the brood for *T. gribodoi*. The brood is particularly sensitive to high nest temperatures and dies in the case of *T. denoiti* at 35.5° C, which is just 1° C above the optimal brood nest temperature[8]. This may also explain why *T. denoiti* nests ventilated at night-time with relatively large tidal volumes. Because of the low outside temperature there was no danger of overheating the brood.

In general we found that under physiological conditions social insects are able to control the concentration of their respiratory gases in a similar fashion to that of mammalian organisms. It seems that 'colonial breathing', originally found in honeybees, is a general phenomenon for social bees with nests with single entrance tubes. Colonial breathing is yet another physiological trait of colonies of social bees similar to mammalian organisms and may give further support to the physiological superorganism model[15] of colonies of social insects.

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Specific effects of diterpene resin acids on spore germination of ectomycorrhizal basidiomycetes

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Summary. Six diterpene resin acids out of eight tested possess the capacity to induce spore germination in all ten Swedish and American species of *Suillus* tested. Species from other genera did not respond. A method for isolation of homokaryons (monosporous mycelia) of *Suillus* species is described.
Key words. Diterpene resin acids; ectomycorrhizal basidiomycetes; spore germination; *Suillus*.

Roots of many trees contain extractives and produce exudates which induce spore germination of ectomycorrhizal fungi[1]. In the case of Scots pine (*Pinus sylvestris* L.) it was demonstrated that an active component of the (chloroform-methanol) root extract is identical with abietic acid, a diterpene resin acid, which triggered germination of basidiospores from four species of the boletaceous genus *Suillus*[2]. It has been presumed, although not proven, that abietic acid is also the germination-inducing substance present in pine root exudate. Furthermore, it is known that pine roots contain several diterpene resin acids[3]. This raised the question whether abietic acid is unique in its germination-inducing activity among the diterpene resin acids. Another question to be considered is the possible specificity of the reactive spores: do only spores from *Suillus* species respond to abietic acid? The project included the development of a new and simple method for the production and isolation of monosporous mycelia of *Suillus* species on agar plates after germination activation of the spores by abietic acid.

Material and methods. The spore samples used in the germination experiments were derived from sporocarps collected in Sweden (the Uppsala region) and the USA (Virginia, N. Carolina, Tennessee, and California) during the fall of 1987 (table 3). The spores were stored in plastic petri dishes at 5 °C in darkness and remained germinable for at least six months. The tests for germinability were performed on water agar strips according to the 'diffusion gradient method' described earlier[4]. Liquid, hot water agar (Difco Bacto; 1.5%) was poured into plastic petri dishes (Ø 9 cm), 10 ml per dish. When the agar plate had solidified its surface was covered with a thin cellophane film sterilized in 70% ethanol and then washed in sterile distilled water. A very thin cover of activated charcoal powder (Aktivkohle, pro analysi, Merck) was sprinkled over the cellophane. About a week later the cellophane film (including the charcoal) was removed. The agar plate was now free from germination inhibitors. The size of the strips cut out from these agar plates was usually $6 \times 1 \times 0.16$ cm.

The diterpene resin acids to be tested were isopimaric, levopimaric, neoabietic and palustric acid from Helix Biotech Ltd., Vancouver, Canada. Palustric acid had a purity of 90–95%, the others 99%. Abietic, dehydroabietic and pimaric acid of high purity were obtained from Prof. O. Theander's laboratory at the Swedish University of Agricultural Sciences. These seven resin acids represent the major part of those present in the roots of pine[3]. Finally, also pinifolic acid was tested, a resin acid which was isolated from pine needles in Prof. O. Theander's laboratory[5].

The percentage germination of spores on the agar slip surface was scored by counting the number of germinated spores among 100–200 spores on photomicrographs of the agar strips. The systematic nomenclature follows Moser[6] for the Swedish and Miller[7] for the American collections.

Results and discussion. The effect of abietic acid was first tested on Swedish spore collections of *S. granulatus*, *S. luteus* and *S. variegatus* (table 1). *S. granulatus* responded faster, at a higher percentage germination and at lower abietic acid concentrations than the other two species. American material of *S. granulatus* and *S. luteus* reacted similarly, but their germination rate was not exactly recorded. The other diterpene resin acids were tested only on spores of *S. granulatus*. As seen in table 2, palustric acid produced about the same percentage of germination as did abietic acid. Both acids were also active at the concentration of 10^{-6} M. The other four resin acids were less active but released at least a few percent of germinations at 10^{-5} M. Pimaric and pinifolic acid did not affect the germination in *S. granulatus* and *S. luteus*. *S. variegatus* germinated sparsely and slowly as usual but only under the influence of abietic, palustric and neoabietic acid.

The spores of other species were tested in a series of experiments where the germination-inducing effect of only abietic acid was tested. Since germination, if any, was usually sparse and irregular, exact estimations of germination percentages were impossible. Since the main point in the germination experiments was to establish whether germination occurred or not, it was considered sufficient to evaluate the results simply as 'good' (++), 'poor' (+) or 'no' (−) germination (table 3). The spores of *S. granulatus* and *S. luteus* started to germinate within the first week of incubation. The other species, notably *S. grevillei*, *S. bovinus* and *S. pictus*, did not germinate until after three to five weeks on the agar strips. Only spores from species of *Suillus* were brought to germination by abietic acid.

Isolation of homokaryons. The results of these experiments made it possible to facilitate a subsequent production and isolation of homokaryons (monosporous mycelia) in species of *Suillus*. A convenient access to homokaryons is a necessary prerequisite for genetic studies in ectomycorrhizal basidiomycetes. Homokaryons develop from spores which have been induced to germinate, a process that has always been difficult to achieve in these fungi. The complicated methods employed earlier included the use of living activators like *Rhodotorula* yeast, 'self' mycelium or tree seedling roots[8]. When such activators can be exchanged for a chemically defined compound active on the species of *Suillus*, it

Table 1. The germination-inducing effect of abietic acid on spores from *Suillus granulatus*, *S. luteus* and *S. variegatus* after different times of incubation. The percentage germination was estimated (as described in 'Material and methods') after an incubation time of seven days. The figures for the abietic acid concentrations refer to the solution administered to the agar strips in amounts of 0.1 ml per strip. + = less than 1% of the spores germinated. − = germination not tested.

Abietic acid in $mg \cdot l^{-1}$	Percentage germination after different times of incubation					
	3 days	4 days	5 days	6 days	7 days	10 days
	Suillus granulatus					
1000	5	8	20	26	25	28
100	0	14	28	43	40	47
10	0	14	10	24	24	28
1	0	6	4	7	8	7
0.1	0	0	2	–	–	3
	Suillus luteus					
1000	0	18	28	33	29	37
100	0	0	0	4	5	10
10	0	0	0	0	0	0
	Suillus variegatus					
1000	0	0	+	6	9	40
100	0	0	0	+	+	+

Table 2. The effect of six diterpene resin acids on the spore germination in *Suillus granulatus*. The percentage germination was estimated in two independent experiments, I and II, after an incubation time of seven days. + = less than 1% of the spores germinated. – = not tested.

Concentration of added compound		Percentage germination with different diterpene resin acids											
in mg · l^{-1}	in moles	Abietic acid		Dehydroabietic acid		Levopimaric acid		Isopimaric acid		Neoabietic acid		Palustric acid	
		I	II	I	II	I	II	I	II	I	II	I	II
1000	$3 \cdot 10^{-3}$ M	26	15	17	17	12	10	16	14	17	13	15	9
100	$3 \cdot 10^{-4}$ M	21	13	16	23	15	15	13	19	7	–	19	17
10	$3 \cdot 10^{-5}$ M	13	–	5	–	1	0	6	4	3	–	14	12
1	$3 \cdot 10^{-6}$ M	4	–	0	–	0	–	+	–	0	–	5	–
0.1	$3 \cdot 10^{-7}$ M	1	–	0	–	0	–	0	–	0	–	+	–

Table 3. The effect of abietic acid (100 mg · l^{-1}) on the spore germination of 16 ectomycorrhizal fungi. + + = good, + = poor germination. 0 = no germination within five weeks.

Tested species with stock number	Region where the sporocarp was collected	Type of response
S. granulatus 303	Sweden, Uppsala	+ +
S. granulatus 378	USA, N. Carolina	+ +
S. granulatus 383	USA, Virginia	+ +
S. luteus 302	Sweden, Uppsala	+ +
S. luteus 392	USA, Virginia	+ +
S. variegatus 310	Sweden, Uppsala	+
S. grevillei 373	Sweden, Uppsala	+
S. bovinus 311	Sweden, Uppsala	+
S. hirtellus 338	USA, Virginia	+ +
S. americanus 384	USA, Virginia	+
S. pictus 385	USA, Virginia	+
S. lakei 394	USA, California	+ +
S. coerulescens 395	USA, California	+
X. badius 312	Sweden, Uppsala	0
X. subtomentosus 343	Sweden, Uppsala	0
B. piperatus 367	Sweden, Uppsala	0
Paxillus involutus 350	Sweden, Uppsala	0
Hebeloma mesophaeum 328	Sweden, Uppsala	0
Thelephora terrestris 141	Sweden, Uppsala	0

should be possible to radically simplify the germination procedure with these fungi. In order to produce homokaryons for isolation various methods were tried. The following version proved reliable and relatively simple. It is a two-step procedure, since germination and mycelium development require different substrates. The germination of *Suillus* spores in the presence of abietic acid is very poor or completely inhibited in water and in liquid media. Therefore, this phase of the procedure has to take place on strips (or any sort of pieces) of inhibitor-free water-agar as described above in 'Material and methods'. A suspension in distilled water of the spores to be germinated is pipetted on the strip(s), about 0.05 ml · cm^{-2}. The following day the water layer of the spore suspension had evaporated, while the spores were fixed onto the agar surface. A sterile solution of abietic acid, e.g. 0.1 mg · ml^{-1}, is then pipetted onto the strip, about 0.1 ml · cm^{-2}, and preferably placed on the one end of the strip to produce a diffusion gradient. The petri dishes with these pieces or strips of agar are incubated at 25 °C in darkness in a place with 100% relative humidity. When germinations seem to be numerous enough the spores are washed off the surface of the strips with water (ca 0.2–0.4 ml). The suspension, diluted or not, is transferred to a nutrient agar plate containing 1 mg of abietic acid in another petri dish. The suspension, which contains a number of germinating spores, is spread out over the new agar plate by means of a glass rod curved like a hockey stick. One or two weeks later developing homokaryons should be visible to the naked eye. The isolation of homokaryons can be performed in about 2–3 weeks after the start of the experiment.

Discussion. From the results of this study it is evident that several diterpene resin acids possess the capacity to induce spore germination in ectomycorrhizal species of the genus *Suillus*. Of the resin acids tested, abietic and palustric acids are the most active ones in this respect, dehydroabietic, levopimaric, isopimaric and neoabietic acids being less active, whereas pimaric and pinifolic acid proved inactive. Spores from all ten *Suillus* species tested, Swedish as well as American, were brought to germination under the influence of abietic acid, although in some cases slowly and at a low percentage. Spores from six other species representing five genera, both boletes, agarics and one Aphyllophorales, did not respond to abietic or palustric acid. Four of these species, viz. *Xerocomus badius, Hebeloma mesophaeum, Paxillus involutus* and *Thelephora terrestris,* have been found to germinate when exposed to a pine root exudate [9–11], while the two remaining species, *Boletus piperatus* and *Xerocomus subtomentosus* have never been tested in that respect. In all probability pine root exudates and extracts contain not only diterpene resin acids functioning as germination activators in *Suillus* but also other compounds active on germination in other genera of ectomycorrhizal fungi. Hence, it is not surprising that recent experiments have demonstrated the presence in pine root exudate of a compound, as yet unidentified, which triggers the spore germination of *Hebeloma mesophaeum* and is clearly different chemically from a diterpene resin acid (Fries, in preparation).

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Flux of singlet oxygen from leaves of phototoxic plants

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Summary. Detached leaves of *Zanthoxylum americanum* and *Pastinaca sativa*, plants known to produce phototoxins, generate singlet oxygen when illuminated by a xenon arc lamp that simulates sunlight. Other species tested did not produce detectable amounts of singlet oxygen. Calculations of the rate of production of singlet oxygen indicate a flux of up to 4×10^{12} molecules $cm^{-1}\,s^{-1}$. This level is sufficiently high to induce damage in the cells of organisms near the leaf surface. Photodynamic action may thus provide for plants an evolutionary advantage in the form of preemptive protection against predators without tissue loss.
Key words. Phototoxicity; singlet oxygen; *Pastinaca sativa; Zanthoxylum americanum;* plant defense.

At least eight distinct classes of plant chemicals are photoactive, that is, capable of absorbing sunlight energy to increase their toxicity to living organisms[1]. The efficacy of these photoxins against a diverse array of plant pathogens and herbivores has given rise to the suggestion that these chemicals function in defending plants against potential enemies[2–4]. Many photosensitizing plant chemicals are present primarily in epidermal tissues[5] and thus are in close contact with the atmosphere, making feasible energy transfer from their photochemically excited states to atmospheric oxygen to form singlet oxygen. Singlet oxygen is reactive toward some constituents of DNA, cell membranes, enzymes, and other essential biomolecules[6,7]; because of its long lifetime in the gas phase (roughly 1000 times greater than in the liquid phase), it can diffuse through air for distances of several millimeters to react with substrates in solution[8]. Singlet oxygen generated at a leaf surface could potentially persist long enough to interact with invaders of the phylloplane such as fungal spores, bacteria, yeasts, and plant-feeding arthropods and their eggs. In this study, we document for the first time the presence of singlet oxygen at distances 1–2 mm from the surfaces of leaves of some phototoxic plants and its apparent absence on or near the surface of nonphototoxic plant leaves.

A modification of a previously reported method[8] was used to generate singlet oxygen in the gas phase; an intact plant leaf was secured over the well of a microscope slide containing 100–200 µl of a 2×10^{-4} M aqueous solution of furfuryl alcohol (FFA), a reactive acceptor of singlet oxygen, such that its surface was 1–2 mm above the droplet. The leaf was illuminated from beneath using a mirror to reflect the beam of a 100 W xenon arc lamp (filtered through water, pyrex, and cellulose acetate filters to remove infrared and nonsolar UV wavelengths) up through the underside of the glass slide (fig.). The UV (320–400 nm) intensity on the slide was approximately 300–400 $\mu W/cm^2/min$; while this intensity is relatively low for solar exposure (although typical of overcast or winter conditions), evaporation of the droplet due to increasing temperatures became a problem at higher lamp outputs. At timed intervals, 20 µl samples of FFA were taken and quantified by high-pressure liquid chromatography[9]. Benzyl alcohol (1.8 mM) was used as an internal standard. Initial observations of plants were made after 15 min of illumination. Five species were tested (table 1); of these species, only *Pastinaca sativa* (wild parsnip) and *Zanthoxylum americanum* (prickly ash) showed a decline in FFA concentration of 10% or greater. These results are consistent with the chemistry of these plants; wild parsnip foliage contains high levels of phototoxic furanocoumarins[10] and prickly ash leaves contain furanocoumarins as well as the phototoxic furanoquinoline and β-carboline alkaloids[11,12]. In solution, many of these compounds are known to produce singlet oxygen[13,14]. Although furanocoumarins are reported to occur in *Citrus sinensis*[12], no loss of FFA was observed in experiments with its leaves.

Three species were examined further for time intervals of 45–80 min. From 4–6 determinations were made for each sample. Some loss of FFA relative to the benzyl alcohol standard occurs over the assay interval (table 2); therefore, only regressions with slopes significantly greater than that of the control (0.0009) were considered meaningful. Slopes of FFA loss against time in minutes were significant and negative for both wild parsnip (slope = −0.007, r = 0.92) and prickly ash (slope = −0.002, r = 0.99). A second experiment with a different prickly ash leaf gave a steeper slope (−0.008, r = 0.90) which due to high variance was marginally nonsignificant (p = 0.096). To test whether factors other than singlet oxygen (e.g., plant volatiles) were causing FFA depletion, well slides were set up with wild parsnip leaves as before except that the xenon lamp was left off for the 80 min

Table 1. Changes in peak height of furfuryl alcohol after 15 min of exposure to xenon arc lamp (330–400 $\mu W/cm^2/min$)

Plant species	Peak height relative to time zero (avg. of 2 replicates with SD)
Pastinaca sativa	0.915 (0.007)
Zanthoxylum americanum	0.795 (0.077)
Liquidambar styracifolium	1.040 (0.042)
Ailanthus altissima	1.060
Citrus sinensis	1.090
Glass slide only	1.005 (0.077)