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Diterpenes from *Nicotiana glutinosa* and their Effect on Fungal Growth

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SUMMARY

The diterpenes sclareol and 13-epi-sclareol were isolated as a eutectic mixture from leaves of *Nicotiana glutinosa*. These compounds were probably present in liquid droplets which occurred on the leaf surface. These diterpenes did not prevent germination of fungal spores but markedly inhibited the radial extension of colonies growing on agar. This inhibition is shown to be due to an effect on the morphology of the fungi in which the degree of hyphal branching is increased. The possibility that sclareol/13-epi-sclareol influence the growth-regulatory processes of fungal hyphae and the potential of these compounds to affect growth of fungi on the surface of leaves of *N. glutinosa* is discussed.

INTRODUCTION

Previous research has demonstrated that legumes produce antifungal compounds when they undergo necrosis as a result of virus infection (Bailey & Ingham, 1971; Klarman & Hammerschlag, 1972; Bailey, 1973; Bailey & Burden, 1973). The present investigations were started in order to determine whether similar responses occurred in other plant families. In this instance a species of tobacco, *Nicotiana glutinosa*, was used which develops a necrotic reaction after infection with tobacco mosaic virus. Preliminary experiments, however, demonstrated that extracts of uninoculated leaves possessed antifungal properties. The present paper describes the isolation, identification and effect on fungal growth of compounds isolated from healthy leaves of *N. glutinosa*.

METHODS

Organisms. Nicotiana glutinosa was grown in sterile compost in a glasshouse with a minimum temperature of 21 °C, or in a plant growth room illuminated by fluorescent lighting at 23 ± 2° °C.

The isolates of *Alternaria brassicicola*, *Aspergillus niger*, *Botrytis fabae*, *Cladosporium cucumerinum*, *Colletotrichum lagenarium*, *Colletotrichum lindemuthianum* Race γ, *Glomerella cingulata* and *Septoria nodorum* were those used previously (Bailey & Burden, 1973). *Alternaria longipes* (IMI 105920) was obtained from the Commonwealth Mycological Institute, *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotinia fructigena* were from Dr G. A Carter of this Unit, and *Alternaria alternata*, *Alternaria brassicae*, *Alternaria solani*, *Alternaria* sp., *Phytophthora cinnamomi* and *Stemphylium botryosum* came from the Agricultural Development and Advisory Service, Wye.

Extraction of plant material. Plant tissues were soaked for 48 h in redistilled benzene. The

benzene was decanted and the solvent removed by means of a rotary film evaporator at 45 °C. Any water remaining at this stage was removed in a similar way after the addition of a small quantity of ethanol. This procedure resulted in the virtually complete extraction of benzene-soluble material.

Chromatography. This was done on 0·25 mm silica-gel plates (Merck 5715) using mixtures of hexane and acetone (3:1) or ethanol and chloroform (3:100) for development. The resulting chromatograms were examined chemically by spraying with a vanillin reagent (1 g vanillin, 3 ml phosphoric acid and 30 ml methanol) or when assessing extracts for the presence of antifungal compounds by spraying the plates with spores of *Cladosporium cucumerinum* in Czapek Dox liquid medium. Column chromatography was employed to provide substantial amounts of the inhibitors. Mature leaves (3·0 kg) were kept at −20 °C for several days and extracted with benzene (4 l) for 3 days at room temperature. The benzene was removed and the residue re-extracted with further benzene (3 l). The combined extracts were evaporated, redissolved in benzene (5 ml) and chromatographed on a column of silicic acid (200 g). The column was eluted with hexane (200 ml), 5 % acetone in hexane (600 ml), 7 % acetone in hexane (600 ml) and finally with 10 % acetone in hexane (1 l). Fractions of 25 ml were collected and each fraction was examined by thin-layer chromatography using the vanillin reagent.

Extracts, which had been evaporated and redissolved in ethyl acetate, were also examined by means of a Pye 104 gas–liquid chromatogram fitted with a column containing 3 % OV-225 and operated at 215 °C. The concentrations of compounds in extracts were assessed by comparing the peak areas obtained from the extract with those obtained from standard solutions prepared from compounds which had been purified by recrystallization from hexane.

Assays of antifungal activity. Activity was assessed by measuring the germination of fungal spores on glass slides and on agar, and the growth of hyphae both in liquid medium and on agar. The methods used have been reported previously (Bailey & Deverall, 1971; Bailey & Burden, 1973); any amendments are described in the text.

RESULTS

Demonstration and isolation of inhibitors of fungal growth from leaves of Nicotiana glutinosa

Mature leaves, removed from ten-week-old plants which had been grown in a glasshouse, were extracted with benzene. The benzene was removed and the ether-soluble residue was subjected to thin-layer chromatography in hexane and acetone (3:1). The resulting chromatogram was assayed for antifungal activity by using *Cladosporium cucumerinum.* A large area of inhibition was demonstrated at R_F 0·17 and other minor areas of inhibition were visible at R_F 0·27, 0·23, 0·13 and 0·11. When similar chromatograms were sprayed with vanillin reagent and heated, several regions of the chromatogram, at R_F 0·47, 0·17, 0·14 and 0·11, became coloured dark blue. The major inhibitory area at R_F 0·17 was investigated more closely by repeating the chromatography and eluting the silica with ethyl acetate. Removal of the solvent yielded a gum which crystallized in hexane giving a compound which had a melting point of 96 to 100 °C.

Identification of inhibitor as sclareol/13-epi-sclareol

Two grams of the compound of m.p. 94 to 99 °C were obtained by column chromatography (fractions 35 to 54). Repeated crystallization from hexane did not raise the melting point. However, by using methanol, colourless needles, m.p. 127 to 128 °C, were obtained

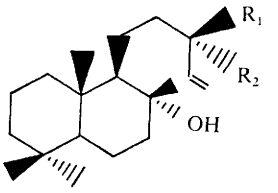

Fig. 1. Structures of sclareol and 13-epi sclareol. Sclareol: R_1 = OH, R_2 = Me. 13-Epi sclareol: R_1 = Me, R_2 = OH.

after considerable loss of material. The compound analysed well for $C_{20}H_{36}O_2$ (values found were 77·98 % C, 11·60 % H; $C_{20}H_{36}O_2$ requires 77·85 % C and 11·77 % H), but the highest peak observed in the mass spectrum was at m/e 290. This peak could result from a loss of a molecule of water from the molecular ion. In the nuclear magnetic resonance spectrum resonances were observed from 5 methyl groups (δ 0·76, 6 H, s; δ 0·86, 3 H, s; δ 1·12, 3 H, s; δ 1·23, 3 H, s), two hydroxyl groups (δ 2·70, 2 H, s, which disappeared in D_2O) and a vinyl group which appeared as a characteristic 12-line ABX spectrum (δH_A 5·07, δH_B 5·23, δH_X 5·92, J_{AB} = 2, J_{AX} = 10, J_{BX} = 8). Both hydroxyl groups appeared to be tertiary as the compound did not give an acetate on treatment with acetic anhydride and pyridine.

The nature of the substance is indicated from work by Popa & Lazurevskii (1963), who obtained a diterpene sclareol together with its 13-epimer from the buds of *Salvia sclarea*. They found that the mixture of sclareol/13-epi-sclareol formed a eutectic in hexane, m.p. 95 °C, but that the 13-epimer could be separated by recrystallization from methanol to yield a crystalline solid, m.p. 129 to 130 °C.

Our data agree well with these findings and the analytical and spectral data of the compound of m.p. 127 to 128 °C are in complete accordance with its formulation as 13-epi-sclareol. The original substance, m.p. 94 to 99 °C, is therefore formulated as a mixture of sclareol and 13-epi-sclareol (Fig. 1) and this mixture was used in all subsequent experiments. Throughout the rest of this paper the mixture sclareol/13-epi-sclareol is referred to as 'sclareol'.

Concentration and distribution of 'sclareol' in tissues of Nicotiana glutinosa

During the isolation of the sclareol mixture, experiments had shown some variation in the amounts of diterpenes isolated from the tobacco leaves. The distribution of 'sclareol' within the plant and the effect of leaf age on its concentration was measured by gas–liquid chromatography. Typical chromatographic traces obtained from an extract of mature leaves and from a crystalline sample of 'sclareol' are shown in Fig. 2. This illustrates that 'sclareol' behaved as a single compound which was readily separated from other components in the extract. The concentrations of 'sclareol' in individual leaves, flower spike, stem and roots were measured. The results (Table 1) demonstrate that only trace amounts of these compounds were present in root tissues but there were large quantities in all the aerial parts of the plant. Although these results showed that highest concentrations were obtained from the upper, younger, parts of the plant, where levels exceeded 600 μg/g tissue, other experiments indicated that the level of 'sclareol' in all tissues increased as they matured.

The location of 'sclareol' within leaf tissues was also investigated. A single leaf was shaken successively in four 10 ml portions of ethyl acetate for 60 s. The leaf was then extracted in 10 ml benzene for 18 h. The ethyl acetate and benzene extracts were evaporated and the residue was redissolved in 1 ml ethyl acetate. The concentration of 'sclareol' was measured

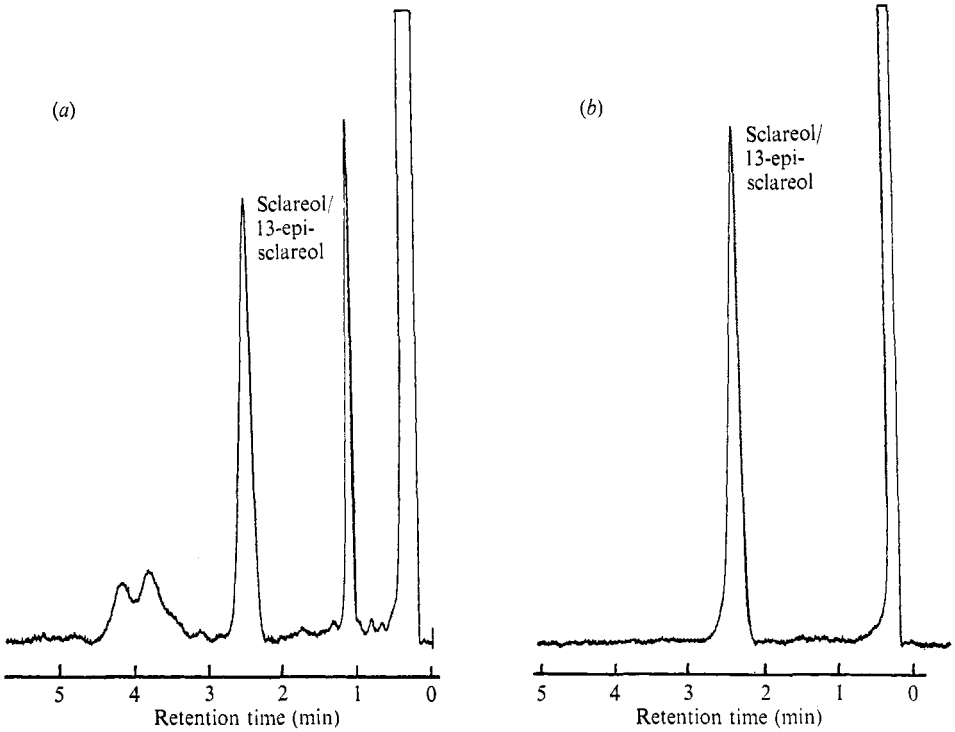

Fig. 2. Gas–liquid chromatographic analysis of 'sclareol'. (*a*) Two μl of an extract of *Nicotiana glutinosa* leaves (1 g) in ethyl acetate (1 ml). (*b*) Two μl of a solution of 'sclareol' in ethyl acetate (600 μg/ml).

Table 1. *Concentration of 'sclareol' in tissues of Nicotiana glutinosa*

Tissue*	Concentration (μg/g tissue)	Tissue*	Concentration (μg/g tissue)
Roots	1	Leaf 10	195
Leaf 1	19	Leaf 11	264
Leaf 2	20	Leaf 12	274
Leaf 3	52	Leaf 13	391
Leaf 4	43	Leaf 14	366
Leaf 5	90	Leaf 15	619
Leaf 6	100	Leaf 16	606
Leaf 7	162	Leaf 17	537
Leaf 8	359	Flowerbuds	632
Leaf 9	319	Stem	408

* Leaf 1 was the oldest leaf, leaf 17 the youngest. Leaves 1, 2, 3 and 4 were in part chlorotic, leaves 5 to 14 were dark green and fully expanded, and leaves 15 to 17 were also dark green but were not fully expanded.

by gas–liquid chromatography. The results (Table 2) demonstrate that most 'sclareol' was present in the first ethyl acetate solution, very little being detected in the subsequent ethyl acetate extracts or in the benzene extract of leaf tissues. When leaves were examined with a binocular microscope they were found to be covered with numerous hairs bearing droplets of liquid. These droplets dispersed when ethyl acetate was placed on the surface of the leaf. In other experiments, a weighed glass slide was pressed on to a leaf without bruising it and

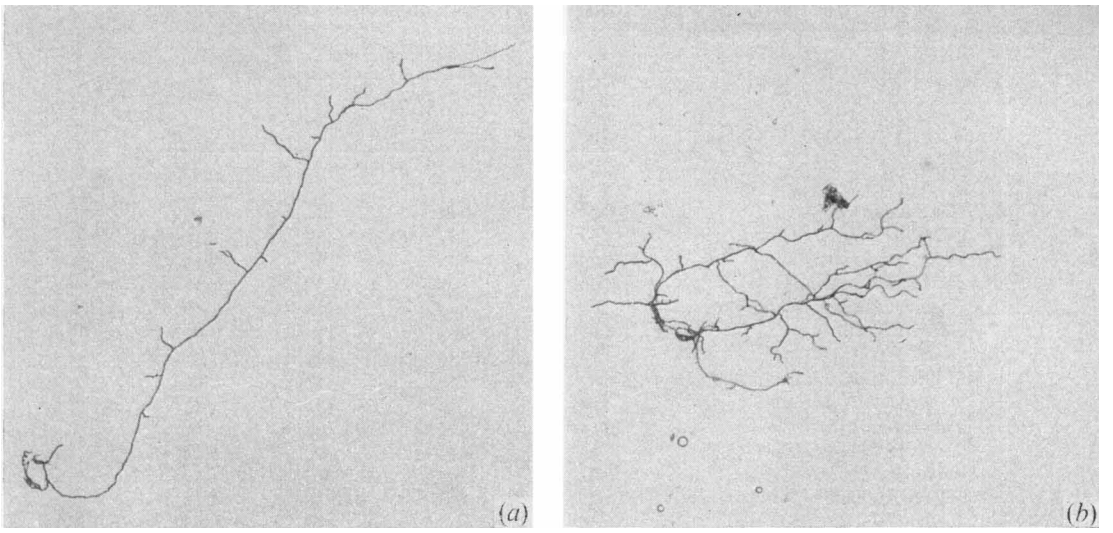

Fig. 3. Effect of 'sclareol' on germ-tube growth of *Alternaria longipes*. Spores were distributed on to agar and the plates incubated for 24 h. (*a*) Czapek Dox agar. (*b*) Czapek Dox agar incorporating 20 μg 'sclareol'/ml. Both agars contained 0·5 % (w/v) dimethylsulphoxide.

Table 2. *Distribution of 'sclareol' in leaf tissues of Nicotiana glutinosa*

A leaf (0·5 g) was placed in four successive 10 ml portions of ethyl acetate for 60 s (extracts 1 to 4 respectively) and in 10 ml benzene for 18 h (extract 5). The weight of 'sclareol' in these extracts was measured by gas–liquid chromatography.

Extract	Weight of 'sclareol' (μg)
1	621·0
2	13·8
3	1·4
4	1·4
5	2·8

then removed. The amount of liquid transferred to the glass surface was obtained by reweighing, and the fluid was then dissolved in ethyl acetate. Analysis of the fluid by gas–liquid chromatography showed that the leaf droplets contained about 10% (w/w) of sclareol.

Antifungal activity of sclareol/13-epi-sclareol

Activity on silica gel. Quantities of crystalline 'sclareol' were dissolved in ether and applied to silica gel layers, as 1 cm origins. The plates were developed in hexane and acetone (3:1) and assayed for antifungal activity against *Cladosporium cucumerinum*. The smallest amount which caused inhibition of growth was 100 μg.

Activity against germination of fungal spores. By means of the microscope slide assay, it was found that 'sclareol' at concentrations up to 500 μg/ml did not prevent the germination in Czapek Dox liquid medium of spores of *Alternaria brassicicola*, *Alternaria longipes*, *Aspergillus niger*, *Botrytis cinerea*, *Cladosporium cucumerinum or Colletotrichum lindemuthianum*. Repetitions of these tests at pH 4·0, pH 6·8 or in media supplemented with 15 % (w/v) ethanol or 0·5 % (w/v) dimethylsulphoxide also revealed no inhibition of spore germination.

Table 3. *The effect of 'sclareol' on the linear mycelial growth rate of several fungi*

	Linear growth rate* (%)			
	Concn of 'sclareol' (μg/ml)			
	0†	5	20	100
Alternaria longipes	142	88‡	34‡	20‡
Sclerotinia fructigena	150	94‡	38‡	24‡
A. brassicicola	105	83‡	39‡	45‡
Rhizoctonia solani	106	89‡	43‡	38‡
Cladosporium cucumerinum	83	58‡	51‡	40‡
Botrytis fabae	117	90‡	47‡	41‡
A. alternata	112	115	84‡	41‡
Colletotrichum lindemuthianum	107	67‡	56‡	43‡
Aspergillus niger	116	87‡	58‡	47‡
Fusarium oxysporum	115	97‡	59‡	48‡
Glomerella cingulata	91	94‡	60‡	51‡
Stemphylium botryosum	143	120	70‡	55‡
Alternaria sp.	87	87‡	68‡	63‡
Colletotrichum lagenarium	105	83‡	71‡	63‡
A. brassicae	122	101	87‡	66‡
A. solani	100	101	95	74‡
Septoria nodorum	104	98	103	95
Phytophthora cinnamomi	125	119	118	101

* Growth rates are expressed as a percentage of the rate on Czapek Dox agar containing 0·5 % dimethylsulphoxide. Mean of three replicates.

† Growth rate on agar lacking dimethylsulphoxide.

‡ Growth rate significantly less ($P = 5$ %) than on agar lacking 'sclareol'.

Table 4. *Effect of 'sclareol' on mycelial growth of Cladosporium cucumerinum and Alternaria longipes in liquid medium*

Assays were carried out in 100 ml Czapek Dox liquid medium containing 0·5 % dimethylsulphoxide. The weights of mycelium were measured after 10 days. Values presented are the means of two replicates.

Concn of 'sclareol' (μg/ml)	Weight of mycelium (mg ± S.E.M.)	
	Alternaria longipes	*Cladosporium cucumerinum*
0	75·2 ± 0·05	163
10	86·5 ± 14·2	184 ± 9·9
25	79·3 ± 7·7	165 ± 7·2
100	84·3 ± 21·8	165 ± 0·5
500	82·5 ± 9·9	139 ± 6·9

Further experiments were carried out in which spores were placed on agar containing 0·5 % (w/v) dimethylsulphoxide and incorporating 'sclareol'. As above, the germination of spores of *Cladosporium cucumerinum*, *Alternaria brassicicola* and *A. longipes* was not affected. However, when germinated spores of both species of Alternaria were incubated further, it was observed that the colonies produced on agar incorporating 'sclareol' (> 10 μg/ml) expanded slowly and the agar surface became covered with discrete colonies (1 to 3 mm diam). In the absence of 'sclareol' colonies expanded rapidly and covered the entire agar surface. This effect was examined with a microscope and is illustrated in Fig. 3. On agar alone, both *A. brassicicola* and *A. longipes* produced hyphal growth which was predominately at the apex, with very little extension of the lateral branches. Apical growth was reduced in the presence of 'sclareol' but branch development was greatly enhanced.

Activity against growth of fungal mycelium. The effect of 'sclareol' on the growth of mycelium on Czapek Dox agar was also assessed. The results (Table 3) demonstrate that the radial growth rates of 16 species of fungi were significantly reduced by 20 μg 'sclareol'/ml agar. *Phytophthora cinnamomi* and *Septoria nodorum* were unaffected. The effect of 'sclareol' on the mycelial growth of *Cladosporium cucumerinum* and *Alternaria longipes* in Czapek Dox liquid is shown in Table 4. These results show that no major reduction in the weight of mycelia was produced after incubation for 10 days.

DISCUSSION

Extracts of *N. glutinosa* produced several areas of fungal growth inhibition on silica gel plates when assayed by means of *Cladosporium cucumerinum*. The material responsible for the major inhibitory area was identified as a mixture of sclareol and 13-epi-sclareol. Although this mixture inhibited *C. cucumerinum* on silica plates it did not prevent spore germination on glass slides or reduce the amount of mycelia produced in liquid media. However, as discussed below, it did inhibit the radial growth of mycelial colonies on agar. These results indicate that fungitoxicity on silica plates should be interpreted with caution and be investigated further in other *in vitro* assays.

'Sclareol' was isolated from the flower buds of *Salvia sclarea* but no fungitoxic activity was reported (Popa & Lazurevskii, 1963). Results obtained with *N. glutinosa* show that these compounds can be extracted from all aerial parts of the plant. Subsequent experiments indicate that 'sclareol' is located at high concentrations in the liquid droplets which occur on the leaf surface.

The observations that, on agar, low concentrations of these diterpenes reduce the hyphal extension of a wide range of fungal species is of interest since the loss of apical dominance resembles a hormonal effect. The lateral branches, which were present but short in normal hyphae, had extended considerably on agar containing 'sclareol'. This increased branching and loss of apical growth explains the inhibition of hyphal extension across agar surfaces. Similar morphological effects have been reported for other compounds. For instance, at high concentrations (50 to 100 mg/ml), L-sorbose caused *Neurospora crassa* to branch profusely (Trinci & Collinge, 1973), and the antibiotics cytochalasin A and B (12·5 to 100 μg/ml) caused branching at the edges of developing colonies of *Botrytis cinerea* (Betina, Mičeková & Nemec, 1972). However, the action of griseofulvin is most comparable with the effects reported here since this compound, at a concentration of 0·1 μg/ml, has been shown to cause excessive branching and curling of hyphae of *Botrytis allii*. In the presence of higher concentrations (10 μg/ml) hyphae failed to develop. Subsequently, griseofulvin was found to affect many fungi but it had no effect on Oomycetes (Brian, 1949). In the present paper 'sclareol' inhibited a wide range of fungi, and investigations with *Alternaria brassicicola* and *A. longipes* revealed that this arose from a greater degree of branching. Thus, these results suggest that 'sclareol', like griseofulvin, affects the growth-regulatory systems of fungi.

Finally it is necessary to emphasize that 'sclareol' was present on the surface of leaves of *N. glutinosa* at concentrations which are capable of influencing the growth of many species of fungi. These could include the saprophytic flora and also species pathogenic to *N. glutinosa*. It has not been possible to infect leaves of *N. glutinosa*, *N. clevelandii* or *N. tabacum* cultivar White Burley with the isolate of *A. longipes* used in this work and therefore no conclusions can be drawn regarding a role for these compounds *in vivo*. However, studies on the significance of sclareol and of glutinosone, an antifungal sesquiterpene isolated from

virus-infected *N. glutinosa* (Burden, Bailey & Vincent, 1974), in resistance to attack by fungi are continuing in this laboratory.

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