COMMUNICATIONS

Effect of Nonanol and Related Fungal Spore Stimulators on Germination of Pollen of Several *Pinus* Species

Germination rate of pollen of *Pinus echinata*, *Pinus rigida*, *Pinus sylvestris*, and *Pinus strobus* was stimulated by 10 to 50 μL/L of nonanol incorporated in 2% water agar. A related compound, nonanal, and 6-methyl-5-hepten-2-one were previously reported to be endogenous stimulators of germination of uredospores of *Puccinia graminis* var. *tritici* (stem rust of wheat) and other rust spores. At 25 μL/L concentrations, octyl thiocyanate, octyl cyanide, citral, rhodinol, safrol, and geraniol stimulated germination of pollen of *P. strobus* at 24 h. Nonylamine inhibited germination. Nonanol was most effective of the 7- to 11-C linear alcohols in stimulating germination of pollen of *P. strobus*. Pollen tube length (*P. strobus*) was stimulated about 23% by 25 μL/L of nonanol in 48 h at 22 °C.

Nonanal, methylheptenone, and other related naturally occurring flavor compounds have previously been reported to stimulate germination of various fungal spores (French and Weintraub, 1957; French, 1961; French and Gallimore, 1971; Rines et al., 1974). In a recent survey made to assess the extent of the stimulator effect among rusts and smuts, 21 species of fungal spores, representing five genera (*Puccinia*, *Coleosporium*, *Uromyces*, *Melampsora*, and *Ustilago*), were stimulated by nonanal, nonanol, methylheptenone, β-ionone, or related compounds (French et al., 1975b). An extension of the survey to nonfungal propagules revealed pine pollen to be among those responding to stimulators. The purpose of this communication is to report stimulation of germination of pollen of several species of *Pinus* by nonanol and related compounds. This may be the first reported stimulation of a growth function of a higher plant by nonanol.

MATERIALS AND METHODS

Staminate cones were collected from several species of pine 1 or more days before use. Cones were allowed to dry overnight on sieves, and the pollen was collected after sieving. Pollen was blown from a microcyclone device into a 50-L glass jar or dispersed by carbon dioxide pistol into a 1.8-m settling tower and allowed to fall on 5-cm plastic petri plates containing 2% water agar with or without suspensions of test compounds.

For long-term spore density–concentration studies, pollen was weighed to the nearest tenth milligram and distributed on agar plates at three levels of pollen grain concentration. Pollen concentration per 90× microscopic field was determined for 20 fields and averaged to give an estimate of the number of pollen grains per field. Pollen was germinated in darkness at 20–22 °C and counted at 24-h intervals up to 7 days. Two hundred pollen grains on each plate (single concentration) were counted by three observers, averaged, and expressed as percent germination ± standard error.

In short-term experiments, dosage levels were made up in triplicate, 200 pollen grains were counted for each plate by two observers, and the results were averaged. Spore levels were approximately four to seven pollen grains/90× microscopic field for chemical studies. Compounds were diluted with redistilled methanol in experiments requiring concentrations below 10 μL/L. Appropriate methanol controls were included in such experiments. At the end of the incubation period at 22 °C, germination was terminated by placing the open plates in formaldehyde vapor.

Pollen tube lengths were measured using a calibrated optical micrometer. Pollen was grown on the usual agar medium with and without 25 μL/L of nonanol, four replications of each, for 48 h at 22 °C. Two observers measured 25 pollen tube lengths per plate and results were averaged.

RESULTS

1-Nonanol stimulated the rate of germination of pollen of *P. rigida* Mill. and *P. echinata* Mill. (Figure 1). Pollen germination increased without nonanol with increasing numbers of pollen grains over the range of 3, 38, and 68 pollen grains/90× microscopic field for *P. rigida* and 4, 33, and 84 pollen grains/90× field for *P. echinata*. Percent germination increased above that of controls at all pollen levels for *P. rigida* and *P. echinata*, with 10 to 50 μL/L of nonanol. With increasing time, up to 7 days, control germination values approached those of the nonanol-stimulated values more rapidly when larger numbers of pollen grains were placed on agar because of the self-stimulation effect. Nonanol was more effective at earlier germination times and when fewer pollen grains were present. Nonanol at 25 μL/L was most stimulatory to pollen of *P. rigida* at a level of 3.7 pollen grains/90× field. Percent germination generally increased with increases in pollen grain levels, both in controls and in the nonanol-treated samples. Germination responses of pollen of *P. echinata* were similar to *P. rigida*, except 100 μL/L of nonanol tended to suppress germination of low spore levels of *P. rigida*. Other experiments showed a doubling of germination of *P. sylvestris* L. pollen, relative to controls, by 10 μL/L of nonanol at 15 and 72 pollen grains/90× field at 2 days.

Three levels of *P. rigida* pollen from the current season responded to nonanol as in previous years (Table I). At 1 day, germination values at 33.2 grains/field were significantly different from controls with 25 and 50 μL/L nonanol. At 61.5 grains/field, values at 10, 25, 50 and 100 μL/L of nonanol were significantly different from controls. At 2 days, 1.6 grains/field, and at the higher pollen grain levels, all germination values higher than controls were significant. Germination was slightly repressed from that

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Table I. Effect of Nonanol on Germination of Three Levels of Pine Pollen (*Pinus rigida*) at 1 and 2 days, 22 °C

concn of nonanol, μL/L	% germination[a] pollen grains/90× microscopic field[b] 1.6 ± 0.11 SE	33.2 ± 1.26 SE	61.5 ± 1.43 SE
1 day			
0	0	0	1
10	0	1	3**
25	1	4**	7**
50	1	4**	11**
100	0	0	0**
2 days			
0	7	14	13
10	27**[c]	43**	57**
25	43**	60**	74**
50	23**	70**	77**
100	0**	0**	10

[a] Average of 400 pollen grains per each of three replicates. [b] Average of ten 90× microscopic fields per each of three replicates. [c] Significantly different from control at 0.01 level (**).

Table II. Effect of Various Spore Germination Stimulators at 25 μL/L on Germination of Pollen of *Pinus strobus* on 2% Water Agar

compound	% germination[a] in 24 h at 22 °C
control	30
nonanol	68**[b]
nonanal	41
octyl thiocyanate	61*
nonyl amine	0**
octyl cyanide	54*
nonyl mercaptan	39
citral	57*
rhodinol	56*
safrol	53*
geraniol	59*
α-pinene	15
β-pinene	18
terpineol	34
linalool	26

[a] Average of 400 pollen grains per each of three replicates. [b] Significantly different from control at 0.05 level (*). Significantly different from control at 0.01 level (**).

of controls at 61.5 grains/field by 100 μL/L of nonanol at 2 days and completely inhibited by this amount of nonanol at all other pollen levels and at 1 day.

Pollen of *P. strobus* was treated with nonanol, nonanal, and with other volatile chemicals found in flavors and fragrances, some of which are also found in turpentines (Furia and Bellanca, 1975; Guenther, 1952b), and known for their ability to stimulate fungal spore germination. Nonanol was much more active (68% germination at 25 μL/L) than nonanal (41%) (Table II). In addition to nonanol, several other chemicals with a variety of chemical structures stimulated pollen germination at 25 μL/L, including octyl thiocyanate, octyl cyanide, citral, rhodinol, safrol, and geraniol. Nonylamine was inhibitory; α- and β-pinenes and terpineol were not stimulatory. In other tests, the compounds β-ionone and 6-methyl-5-hepten-2-one, particularly effective with certain fungal spores, and limonene, another constituent of turpentines, were all inactive over the concentration range of 10 to 100 μL/L.

Of the linear alcohols containing 1 to 12 carbons, those containing up to six carbons were inactive at 25 μL/L. Significant activity was observed in the 7- to 11-C alcohols

Table III. Effect of Concentrations of C-7 to C-12 Linear Alcohols on Germination of Pollen of *Pinus strobus* on 2% Water Agar at 22 °C for 24 h

compound	% germination[a] in response to concentration 0	1	5	10	25	50	100 μL/L
heptanol		18	19	31	31*	34*	37*
octanol		27	30	37*	46**	40*	39**
nonanol		38*[c]	35*	47**	47**	50**	25
decanol		36*	39*	46**	39*	4**	2**
undecanol		33*	32	33	27	8**	0**
dodecanol		29	31	33	33	20	17
control	22						
methanol controls[b]					18	26	

[a] Average of 400 pollen grains per each of three replicates. [b] (Redistilled), diluant for 1 and 5 μL/L concentrations. [c] Significantly different from control 0.05 level (*). Significantly different from control at 0.01 level (**).

(Table III). Significant activity at the 0.05 level of probability was observed in the 7- to 11-carbon alcohols; activity at the 0.01 level of probability was observed with octanol, nonanol, and decanol. Nonanol was most active of all (47 to 50% at 10 to 50 μL/L or 0.56 to 2.8 × 10^{-4} M). The 8-, 9-, and 10-C alcohols were most active of the 7–11-C compounds. Nonanol showed significant activity over the greatest concentration range. In other experiments dodecanol sometimes showed weak activity.

In addition to the stimulation of germination, an increase in the length of the pollen tubes of *P. strobus* was observed. After 48 h at 22 °C, the average of 200 measurements of pollen tube length was 25.9 μm (±1.31 SE) with 25 μL/L of nonanol, which compared to 21.0 μm (±1.28 SE) for controls was an increase of 23%. The difference between means was significant at the 0.01 probability level.

DISCUSSION

Pine pollen in this geographic area is available during a brief period in May or June, thus the number of experiments which can be performed with fresh pollen is limited. The stimulatory effect of nonanol on pine pollen germination was first observed in this laboratory in 1973, and the effect has been confirmed in several types of experiments each season since then. As with our fungal spore studies, the primary objective of this research has been to determine the existence of biological activity. The experimental approach has been empirical because nonanol and the related flavor chemicals studied are only slightly soluble in water and are volatile; hence exact dosages are difficult to determine or maintain.

Like the uredospores of many species of rust fungi, the pollen of several species of *Pinus* was stimulated to germinate by nonanol and a group of related compounds having a variety of chemical substituents. Pollen of *P. strobus*, like the uredospores of *Puccinia graminis* var. *tritici* (French, 1961, 1973; French and Gallimore, 1971) was most responsive to the 9-C alcohol, nonanol, of the 7- to 11-C active alcohols studied and was much more responsive to the alcohol, nonanol, than the 9-C aldehyde, nonanal. As with rust spores, the response to stimulators was not completely specific as to chemical structures, since compounds such as geraniol (branched, unsaturated, isoprenic), octyl cyanide (nitrile), and safrol (cyclic) were active.

While germ tube length of uredospores may be somewhat repressed at stimulatory concentrations of nonanol (French and Gallimore, 1971), growth in length of pollen

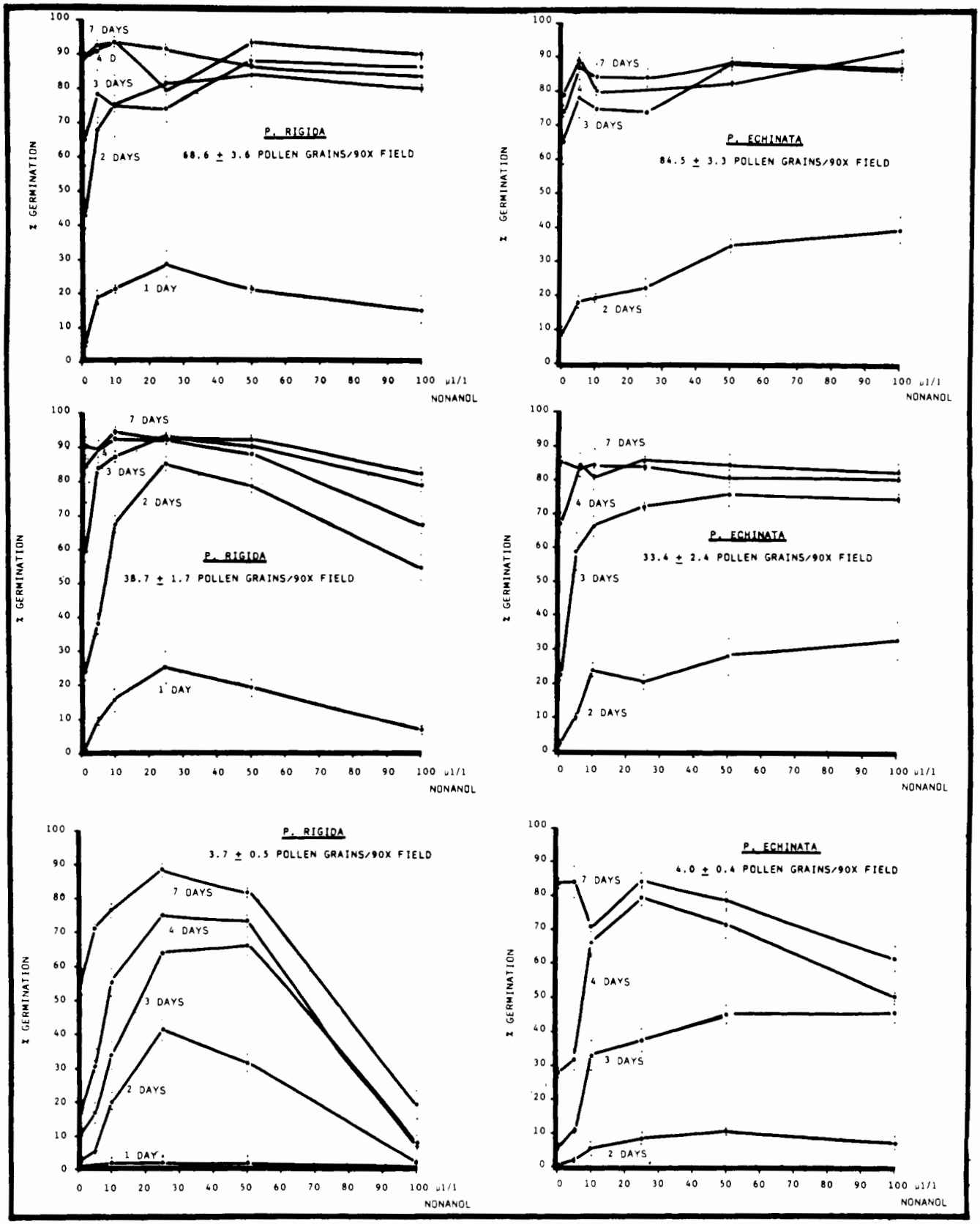

Figure 1. Time course of percent germination ± standard error of pollen of *P. rigida* (left) and *P. echinata* (right) on 2% water agar, in response to 5–100 μL/L of nonanol, at three pollen levels.

tubes of *Pinus* appeared to be enhanced by nonanol. Stimulation of the growth of certain fungal species by these compounds has been observed. Fries (1960, 1961) reported stimulated mycelial growth of *Stereum sanguinolentum* and other fungi by nonanal and to a lesser extent by nonanol. In several cases, the mycelium grew toward a source of nonanal placed in a cup in the petri dish.

The self-stimulating effect of pollen has been recognized for many years, and has been discussed in reviews by Linskens (1964) and Rosen (1968). The self-stimulating effect of pine pollen was evident in these studies, but a stimulatory effect of nonanol on germination rate was

observed at all three levels of pollen grain concentration with *P. rigida* and *P. echinata*. Calcium and/or boron have been associated with the self-stimulation effect in several plant species (Rosen, 1968). The relationship of nonanol or related compounds to the endogenous self-stimulating agent of pine pollen is not known.

Some species of fungal spores have been found to respond only to certain specific chemical structures (French et al., 1975a,b). Uredospores of *Uromyces phaseoli*, for example, respond only to methyl ketones, particularly β-ionone (French et al., 1977), while those of *P. graminis* var. *tritici* respond to certain alcohols, aldehydes, and ketones, particularly nonanol and nonanal. Perhaps pollen of other genera of plants may be found to respond to specific chemical germination stimulators.

The mechanism of action of stimulating germination of rust spores may be that of overcoming endogenous inhibitors, identified by Macko et al. (1970, 1971) in uredospores. Methyl ferulate was found in *P. graminis* var. *tritici* and methyl 3,4-dimethoxycinnamate in *U. phaseoli* which the appropriate chemical stimulators appear to neutralize in inducing germination. Other biochemical activity has been observed with certain germination stimulators. Sinohara (1973) has shown that 1-octanol is the most effective of the 1- to 8-carbon alcohols in inducing de novo glucose dehydrogenase synthesis in dormant spores of *Aspergillus oryzae*. He postulates a mechanism of action in which octanol expands the endoplasmic reticulum, permitting synthesis of the enzyme. Feofilova and Arbuzov (1975) have shown β-ionone to stimulate de novo synthesis of carotogenic enzymes in *Blakeslea trispora*.

In many angiosperms, flowering is accompanied by production of a scent, often composed of fungal spore stimulating compounds, such as rhodinol, geraniol, and nonanal in the rose, or ionone in acacia and boronia flowers (Guenther, 1952a; Furia and Bellanca, 1975). Similar volatile compounds occur in gymnosperms and in various turpentines made from *Pinus* species (Guenther, 1952b; Linskens, 1964; Furia and Bellanca, 1975), in which pinenes, limonenes, and even small amounts of 8-, 9-, and 10-carbon *n*-aldehydes have been reported. A great variety of such compounds have previously been shown to stimulate fungal spore germination. Perhaps some of them stimulate pollen germination in vivo and could be effective in overcoming endogenously inhibited pollen tube growth.

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Enhanced Elimination of Kepone-^{14}C in Rats Fed Liquid Paraffin

Twelve male rats were fed a diet containing 3.3 ppm Kepone-^{14}C for 3 days. After 1 day on the control diet, six animals received a ration containing 8% light liquid paraffin for 24 days. The remaining six animals served as control. Paraffin-treated rats excreted significantly more radioactivity with feces (61%) compared to controls (52%) and had significantly lower concentrations of radioactivity in 14 of 18 tissues analyzed. Excretion of radioactivity with urine was of minor importance (0.5–0.6%) in both groups.

Kepone, decachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta[*cd*]pentalen-2-one, is a persistent insecticide used mainly against ants and cockroaches (Martin, 1971). In recent years great quantities of Kepone have been released into the environment of the Virginia–Maryland coastal region due to an industrial mismanagement. Seventy of 150 occupationally exposed workers had symptoms of Kepone poisoning and high levels of Kepone in blood (Sterrett and Boss, 1977). In the Netherlands Kepone was a trace component in the fly ash of a municipal incinerator (Lahaniatis et al., 1977). Previous studies in our laboratory showed paraffin feeding to considerably enhance the elimination of hexachlorobenzene (HCB) in rats (Richter et al., 1977). In the present study we have investigated the influence of paraffin treatment upon the elimination of Kepone-^{14}C in rats.

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