Stimulation of Germination of Teliospores of *Puccinia punctiformis* by Nonyl, Decyl, and Dodecyl Isothiocyanates and Related Volatile Compounds

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Teliospores of *Puccinia punctiformis* (Strauss) Roehl. are dormant, overwintering propagules of Canada thistle rust. This rust fungus is a prospective biocontrol agent for Canada thistle (*Cirsium arvense* L.), a noxious weed. Nonyl, decyl, and dodecyl isothiocyanates stimulated germination of these teliospores at dosages of 5–25, 5–100, and 5–1000 μL/L, respectively. In preliminary tests, maximum percentage germination was 55% for nonyl-NCS, 70% for decyl-NCS, and 85% for dodecyl-NCS at 21 days, as compared to 0% germination in the controls. Dodecyl-NCS was most effective and active over the greatest dosage range, 5–1000 μL/L. The nonyl, decyl, and dodecyl isothiocyanates are the first compounds of known structure to stimulate germination of teliospores of *P. punctiformis*. Although volatiles from onion and garlic stimulated low levels of germination, various allyl sulfide and isothiocyanate derivatives reported to occur in *Allium* spp. were not stimulatory at dosage levels of 10–1000 μL/L.

INTRODUCTION

Dormant, overwintering spores of fungi, such as teliospores of rust fungi and oospores of other fungi, usually germinate very slowly, and few compounds are known that hasten germination. Binder et al. (1977) identified 10 isomeric 13-carbon acetylenic hydrocarbons, obtained from safflower, as stimulators of teliospores of safflower rust, *Puccinia carthami* Cda. Many environmental factors have been investigated that might increase germination; these include temperature, washing or soaking to leach out inhibitors, repeated freezing and thawing, wetting and drying, or exposure to light. Biochemical factors, such as treatment with hydrolyzing enzymes, or pH changes, solvent treatments, and exposure to oxidizing chemicals, to ethylene, or to various types of plant volatiles sometimes have been effective in breaking dormancy. Hosoki et al. (1985, 1986) reported that volatiles from garlic and horseradish pastes broke dormancy in corms and buds of trees of certain species. Allyl sulfide, from garlic or horseradish, and allyl isothiocyanate, from horseradish, broke dormancy in corms of gladiolus, *Platycodon* tubers, and tree peonies.

In preliminary experiments, onion and garlic volatiles slightly stimulated germination of teliospores of *Puccinia punctiformis*. Thus, various naturally occurring allyl and isothiocyanate compounds known to occur in *Allium* spp. and some related compounds were tested for their ability to stimulate germination of teliospores of *P. punctiformis*, the causal agent of Canada thistle rust. Because the teliospore produces basidiospores which induce a systemic and devastating aecial infection of Canada thistle (*Cirsium arvense*), this rust disease is being studied as a potential biocontrol agent for this widespread noxious weed. Low germination rates of the teliospores may be a limiting factor in using this pathogen, hence our main objective was to find some way to increase spore germination.

Table I. Effect of Various Concentrations of Alkyl Isothiocyanates on Germination of Teliospores of *P. punctiformis* at 21 Days, 18 °C, in Darkness

	% germination at concn (μL/L)								
compound	0	5	10	25	50	100	250	500	1000
octyl-NCS	0		3	4	0	0	0	0	0
nonyl-NCS	0	53[a]	5[a]	13[a]	0	0	0	0	0
decyl-NCS	0	72[a]	73[a]	62[a]	31[a]	8[a]	0	0	0
dodecyl-NCS	0	63[a]	81[a]	84[a]	84[a]	86[a]	70[a]		
octadecyl-NCS	0	0	0	0	0	2	12	0	0
tertiary octyl-NCS	0		7	0	0	0	0	0	0

[a] Significantly different from 0 controls at $P = 0.01$ using Student's t-test.

MATERIALS AND METHODS

Teliospores used were collected from field-grown thistle plants in Montana and had been stored at 4 °C for over 3 years. Plant tissues containing volatile aroma compounds were chopped into ca. 0.5-cm pieces and weighed amounts placed in the outer rings of Conway diffusion cells with 1.0 mL of distilled water. Teliospores dispersed on 2.0 mL of 1% water agar in 2 cm diameter × 0.5 cm glass dishes were placed in the center wells. Cells were covered with ground glass plates and placed in the dark at 16 °C for 7–10 days, at which time germination was determined.

Stimulatory activity was measured by placing the teliospores on agar solutions or suspensions of compounds. Test compounds were measured in a 1-μL Hamilton syringe, and the chemicals were vigorously mixed with 5.0 mL of liquid 1% water agar and poured into 5-cm plastic Petri plates. The usual test range was 0, 10, 25, 50, 100, 250, 500, and 1000 μL/L. Plates were kept at 18 °C in the dark. Teliospores were suspended in isopentane (2-methylbutane), and 0.2 mL was pipetted to the agar surface. Isopentane evaporated in less than 2 min and dispersed the spores evenly over the plates. Teliospores were in isopentane for less than 15 min. Percent germination was determined microscopically (100×) at 7-day intervals, 8 × 100 counts per Petri plate. Active compounds were retested, and 4 × 100 counts were made on each of three replicates at each concentration of compound. Statistical significance of means com-

Table II. Volatile, Naturally Occurring Aroma Compounds Related to Those in Onion/Garlic Inactive in Stimulating Germination of Teliospores of *P. punctiformis* over the Concentration Range 0–1000 μL/L

compound	natural occurrence	reference
(A) isothiocyanates		
2-phenethyl-NCS	rutabaga, turnip	Ju et al. (1982)
benzyl-NCS	papaya	Tang (1974), Tang and Szed (1972), el-Tayeb et al. (1974)
allyl-NCS	cabbage	West et al. (1977)
(B) allyl derivatives		
allyl-NCS	*Allium* sp.—onion, garlic, leek, chives	Sahgir et al. (1964)
allyl sulfide	same	
allyl disulfide	same	
allyl methyl sulfide	same	
methyl disulfide	same	
propyl disulfide	same	

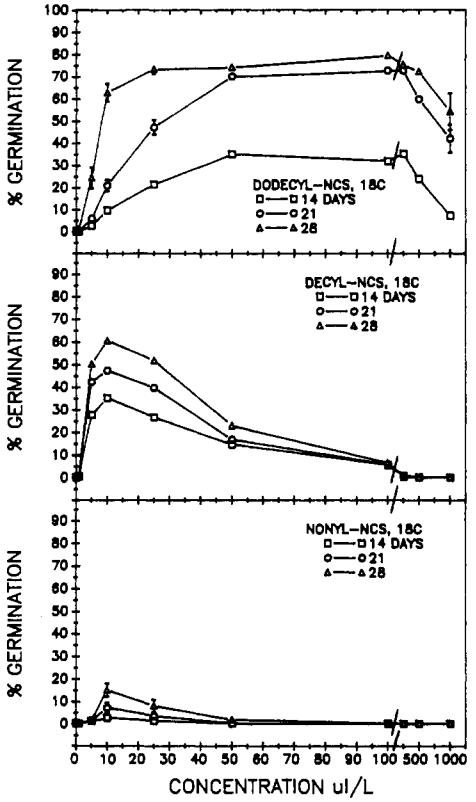

Figure 1. Effect of 5–1000 μL/L nonyl-NCS, decyl-NCS, and dodecyl-NCS on germination of teliospores of *P. punctiformis*, 18 °C, in darkness. Vertical bars represent 95% confidence limits.

pared to control value was determined by using Student's *t*-test at $P = 0.01$, or Waller's grouping, performed on arcsin transformed data, $\alpha = 0.01$.

Since these active compounds are volatile, it was desirable to know if the teliospores would respond to the vapor phase and how long an exposure time was required to activate the germination process. To accomplish this, 10 μL of compound was placed on 1-cm filter paper disks placed inside the lids of plastic Petri plates. Teliospores, dispersed on 1% agar, were exposed to the compounds for 0, 0.5, 1, 2, 4, 16, and 24 h. Germination counts were made on three plates of each treatment, as described above.

Isothiocyanate derivatives were obtained from Fairfield Chemical Co. (Blythewood, SC 29016). Allyl and related derivatives were obtained from Aldrich Chemical Co. (Milwaukee, WI 53233).

RESULTS

Plant Tissue Volatiles. Of various aromatic plant tissues tested, such as onion, garlic, geranium leaf, and orange peel, only onion and garlic stimulated teliospore germination. Volatile emanations from these tissues stimulated teliospore germination at 7 days, 18 °C, in darkness, as follows: chopped onion (10 mg), 15%* germination; chopped garlic (10 mg), 27%* germination; distilled water control, 0% germination (*significant at $P = 0.01$ using Student's *t*-test).

Positive responses with these tissues led to a study of the stimulatory activity of a group of isothiocyanate and allyl derivatives, some of which are known to occur in onion and garlic.

Direct Tests with Authentic Compounds. Of the linear, saturated alkyl-NCS derivatives available, only the nonyl, decyl, and dodecyl isothiocyanates significantly increased germination. No activity was found in the C_4, C_5, C_6, and C_7 derivatives for up to 21 days. A maximum of 4% stimulation at 25 μL/L was observed with *n*-octyl-NCS at 21 days (Table I). At 10 μL/L tertiary octyl-NCS there was 7% germination at 21 days. At 250 μL/L, octadecyl-NCS stimulated a maximum of 12%, also at 21 days. Other isothiocyanates tested, phenethyl-NCS, benzyl-NCS, and allyl-NCS (Table IIA), were inactive.

Nonyl-NCS was the first known compound found to be active. In replicated tests, low germination was observed with this compound at 14 days, and maximum germination was 15% at 10 μL/L at 28 days (Figure 1). No activity was observed above 25 μL/L. Control germination was 0%.

Decyl-NCS stimulated over a concentration range of 5–100 μL/L. Maximum germination at 10 μL/L was 35% at 14 days, 47% at 21 days, and 60% at 28 days. Germination dropped to 5% at 100 μL/L and to 0% at concentrations above 100 μL/L (Figure 1).

Stimulation by dodecyl-NCS was 35% from 50 to 250 μL/L at 14 days. Maximum germination increased to 70% at 21 days. Germination reached 80% at 250 μL/L at 28 days (Figure 1). In general, percent germination increased significantly with dosage to a maximum (Table III) with nonyl-NCS and decyl-NCS and to a high plateau with dodecyl-NCS. Only dodecyl-NCS was as active as the stimulatory control, 50 μL/L of a hexane extract of steam-distilled thistle roots (French et al., 1988). Of the three compounds, dodecyl-NCS was most effective and active over a wide concentration range, from 5 to 1000 μL/L. Mixtures of the C_9-, C_{10}-, and C_{12}-NCS compounds in a 1:1:1 ratio were less effective than any of the compounds alone (data not shown). It should be noted that nonyl-NCS was more active in earlier germination tests, such as those in Table I. After several months, a slight discoloration was observed in the compound, a volatile oily liquid, along with an appreciable loss of activity.

Volatile Tests of Compounds. The teliospores were stimulated by vapors from the C_9-, C_{10}-, and C_{12}-NCS compounds (Figure 2). Decyl-NCS was the most active of the three in diffusion tests but required an 8-h diffusion time for maximum significant stimulation. Nonyl-NCS reached maximum activity in 2 h but was the least effective of the three, as it was in the direct tests. Dodecyl-NCS was moderately active but was the slowest to diffuse. Since

Table III. Effect of 1–1000 μL/L Concentrations of C_9-NCS, C_{10}-NCS, and C_{12}-NCS on Germination of Teliospores of *P. punctiformis* at 21 Days, 18 °C, Compared to 50 μL/L Hexane Extract* of Steam-Distilled Thistle Roots

	% germination[a]		
concn, μL/L	C_9-NCS	C_{10}-NCS	C_{12}-NCS
0 control	0.0 c	0.0 g	0.0 f
1	0.1 c	0.5 f	0.0 f
5	0.9 c	42.2 bc	5.8 e
10	9.9 b	47.3 b	20.9 d
25	0.3 c	40.5 c	47.1 bc
50	0.0 c	16.6 d	70.0 a
100	0.0 c	5.6 e	72.6 a
250	0.0 c	0.9 f	72.6 a
500	0.0 c	0.0 g	59.7 ab
1000	0.0 c	0.0 g	41.9 c
stim control*	63.5 a	63.4 a	63.1 a

[a] Means within a column followed by the same letters are not significantly different as determined by Waller's grouping, performed on arcsin transformed data, $\alpha = 0.01$.

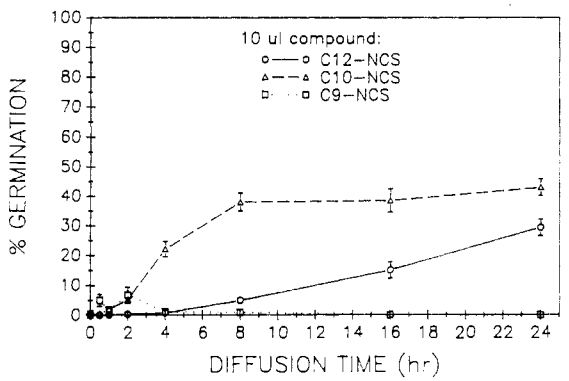

Figure 2. Effect of diffusion of volatiles from 10 μL of nonyl-NCS, decyl-NCS, and dodecyl-NCS (21–24 °C) on germination of teliospores of *P. punctiformis* at 28 days, 18 °C, in darkness. Vertical bars represent 95% confidence limits.

diffusion is related to molecular weight, nonyl-NCS (M_r 187) would diffuse most rapidly, followed by decyl-NCS (M_r 201) and dodecyl-NCS (M_r 229).

Tests of Allyl Derivatives. The naturally occurring allyl derivatives tested (Table IIB) were inactive. Some compounds were tested down to 0.1 μL/L, but all were inactive on teliospores of *P. punctiformis*. Allylurea, allylcyclopentanone, allyl glycidyl ether, and octyl thiocyanate, in which the sulfur, rather than the nitrogen, atom is connected to the alkyl chain, also were inactive.

DISCUSSION

In earlier research at this laboratory β-ionone (French et al., 1977), *n*-nonanal (French, 1985), benzonitrile (French, 1984), methyl salicylate (French et al., 1986), and some related compounds were found to be selectively active stimulators of germination of several species of rust urediniospores and two species of weed seed. None of these compounds stimulated teliospores of *P. punctiformis*. Another compound, 5-methyl-2-hexanone, stimulated urediniospores (French, 1983) but not the teliospores of *P. punctiformis*. Of all the compounds we have tested, including the sulfur derivatives, only the nonyl, decyl, and dodecyl isothiocyanates stimulated the germination of teliospores of *P. punctiformis*. Of these, dodecyl-NCS was the most active, it was active over the widest concentration range, and it showed the least inhibition.

As yet we have found no evidence of the natural occurrence of nonyl, decyl, or dodecyl isothiocyanates. Related compounds, the butenyl, pentenyl, and hexenyl isothiocyanates, have been reported to occur as degradation products of glucosinolates in germinating rape seeds (*Brassica napus* L.) by Kondo et al. (1985).

The positive response of teliospores to vapor exposure indicates that the stimulatory compounds do not have to be present throughout the entire germination period, 7, 14, 21, or 28 days, to be effective. If the teliospores of this pathogen were to be used in a biocontrol procedure for Canada thistle, pretreatment of spores with a stimulator could be an important technique for securing successful infection.

In previously reported research (French et al., 1987) we found that inoculation of root buds with teliospores was effective in causing systemic infection in thistle shoots. Thistle roots produce an unidentified endogenous stimulator of teliospore germination which acts more quickly than the active isothiocyanates. Concentrated hexane extracts of steam-distilled thistle roots (French et al., 1988) stimulated germination greater than 40% from 5 to 100 μL/L in 7 days, compared to a maximum of 2% at 5 μL/L of decyl-NCS, also at 7 days, the earliest germination observed with any of the isothiocyanate compounds.

The C_9, C_{10}, and C_{12} isothiocyanates are the first known compounds found to stimulate the germination of teliospores of *P. punctiformis*, and this may be the first report of their biological activity. Possessing the isothiocyanate functional group, they represent a unique new chemical class of fungal spore germination stimulators. While there is no indication of their natural occurrence, and they are not quite so rapid in action as the endogenous stimulatory material in thistle roots, they are of known structure and are commercially available. Hence, they may be useful for increasing the germination of teliospores of the Canada thistle rust in biocontrol procedures, and they may be found to stimulate other dormant propagules. Although allyl isothiocyanate and the other sulfur derivatives were inactive on teliospores of *P. punctiformis*, they may be found useful on other dormant fungal spores or other propagules.

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