S-Propenylcysteine Sulfoxide in Exudates of Onion Roots and Its Possible Decompartmentalization in Root Cells by Bacteria into Attractant of the Onion Maggot, *Hylemya antiqua* (Diptera: Anthomyiidae)¹

Toshiaki IKESHOJI

*Applied Entomology Laboratory, Faculty of Agriculture, University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan*

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The contents of S-propenylcysteine sulfoxide (PCSO) in roots and exudates of aseptic onion seedlings, and the total root bacteria counts in septic seedlings were all higher in cultivars that are susceptible to the onion maggot, *Hylemya antiqua* MEIGEN, than in the resistant ones. In addition, more propylthio oviposition attractants were generated in the susceptible cultivars. In aseptic soil where bacterial metabolism was impossible, PCSO accumulated. *In vitro* metabolism of the PCSO extract of onion by bacteria produced more methylthio compounds than propylthio compounds, which was in contrast to alliinase metabolism in chopped onions. Since intact septic onion seedlings generated these compounds in intermediate proportions, it is suggested that both bacterial metabolism of exudates in soil and activation of alliinase by bacterial decompartmentalization are simultaneously taking place in the onion rhizosphere.

INTRODUCTION

Involvement of soil microorganisms in oviposition attraction of the onion maggot, *Hylemya antiqua* MEIGEN, to onion bulbs and seedlings is now well established. For instance, decaying bulbs attract more oviposition than intact bulbs (DINDONIS and MILLER, 1980; ISHIKAWA et al., 1981), because the former generate more propylthio compounds during metabolism of the precursor, S-propenylcysteine sulfoxide and also generate non-sulfur attractants such as ethanol and ethyl acetate (IKESHOJI et al., 1980, 1981) and 2-phenylethanol (ISHIKAWA et al., 1983).

In intact onion seedlings, on the other hand, COLEY-SMITH and KING (1969) could not detect any sulfide compounds and speculated that the precursors are exuded by roots and then converted to sulfides by soil bacteria. HOUGH et al. (1981) proposed alternative hypothesis that bacteria may cause the release of volatiles by decompartmentalizing alliinase and the precursors within the plant itself, but rejected this hypothesis because only bacterial activity on the autoclaved medium of nutrient agar plus ground onion seedlings enhanced oviposition.

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Meanwhile, IKESHOJI et al. (1982) reported that the bulbing onion seedlings of maggot-susceptible cultivars showed higher activity of alliinase and hence produced more sulfur compounds than did resistant cultivars. IKESHOJI and MATSUMOTO (1982) also detected higher numbers of bacteria in the seedling roots of susceptible cultivars than in resistant ones. These results indicated the need for detailed investigation of the role of bacteria in the root ecosystem with reference to attraction of the onion maggot and the susceptibility of different cultivars.

MATERIALS AND METHODS

Onion cultivars. Seeds of 11 commercial cultivars were obtained from various sources and referred to by numbers as follows: (1) Takii 10 and (8) Takii 80-02, Takii Co., Kyoto, Japan; (9) Autumn Spice, Bukerfield Co., Vancouver; (2) Early Yellow Globe (EYG) and (5) Ailsa Craig, Dominion Seed House, Ontario; (4) Tamarack, (6) Mirage, (7) White Lisbon, (10) Yellow Globe Danvers (YGD) and (11) Michigan Yellow Globe (MYG), Stokes Seeds, Ontario; and (3) 12KROP, Dr. J. E. RAHE, Simon Fraser Univ.

Culture of seedlings in petri dishes. A 13.5 cm-diam petri dish was filled with washed clean silica sand and covered with a plexiglass plate which had 32, 1.5 cm-diam holes drilled 2.5 cm apart in a grid. Forty milliliters of a nutrient solution [0.33 g Ca (NO₃)₂, 0.04 g MgSO₄, 0.06 g KNO₃, 0.06 g KH₂PO₄, 0.0003 g Fe₂ (SO₄)₃, 0.0001 g ZnSO₄ and 0.0001 g H₃BO₃ in 1 l of distilled water], and 40 ml of field soil infusion were poured into the sand. Seeds of a test cultivar of Autumn Spice (the standard) were deposited singly in alternate holes in the grid, and the dish was incubated at 20°C and a 16L-8D photoperiod. The sand was moistened with the nutrient solution as it dried.

Culture of seedlings in flasks. Three hundred and fifty seeds were sterilized in 0.1% HgCl₂ solution for 10 min, rinsed briefly in 70% ethanol and washed with sterilized water 4 times. They were spread on the bottom of a sterilized 6 l conical flask which had two outlets for ventilation, and covered with 250 ml of sterilized silica sand. The sand was moistened with 150 ml of the nutrient solution. The flask was ventilated at 40 ml/min with clean air which had passed through a 20 cm column of 20-40 mesh activated charcoal and a sterilized absorbent cotton column of the same length and was incubated as above. A distilled water column was inserted between the flask and the cotton column when moisture was needed for seedlings. Germination rate of seeds decreased moderately following sterilization. Septic cultures of seedlings in flasks were grown in the same manner but with an initial addition of 75 ml of soil infusion and 75 ml of the nutrient solution.

Bioassay of oviposition attractancy of seedlings. When seedlings in a petri dish grew 2 to 5 cm high in 1-2 weeks after germination, the dish was placed for 30 min to 1 hr in a 30×45×60 cm cage containing 100 to 200 adult *H. antiqua*.

Eggs laid inside a sand hole were counted under a dissecting microscope. Oviposition around 13 to 26 seedlings (40-80%)/dish was ideal. At oviposition frequencies below or above, the dish was discarded. A ratio of the number of test seedlings around which eggs were laid against that of Autumn Spice was calculated for each choice test. Seven or eight tests are made for each cultivar and the average ratios of different cultivars were compared with the NEWMAN KEULS test.

In vitro metabolism of PCSO by bacteria. PCSO was isolated from onion bulbs by ion-exchange chromatography (CARSON et al., 1966). Purity of the isolate was checked by an automatic amino acid analyzer. The isolate consisted of 56.7% PCSO, 22.0% *r*-glutamyl methionine and the remaining 21.3% common amino acids. Although highly pure PCSO could not be obtained due to a mechanical failure at the final stage of isolation, >50% purity was suitable for the present objectives. Five species of bacteria were isolated from healthy onion roots and labelled as A, B, C, D and E. *Bacillus subtilis* was obtained from a colony showing antagonism to onion white rot, *Sclerotium cepivorum* (WONG, 1981).

Five milliliters of medium containing nutrient broth (8 g/l) and 0.1% PCSO in a test tube was inoculated with a bacterium and allowed to stand in darkness at room temperature until bioassay for oviposition. For bioassay, a portion of the inoculated medium was diluted 100-fold with distilled water. One hundred microliters of the diluted culture (which corresponded to 1 μ g of PCSO) was injected into the sand as above. The injection was alternated with the diluted, uninoculated control medium. A 3 cm-long yellow straw was stuck into the sand in each hole to increase attraction of gravid flies (HARRIS and MILLER, 1982). Twenty milliliters each of the inoculates with bacteria C, D and *B. subtilis*, which showed a high attractancy for oviposition, were also made in a 150 ml flask and incubated until chemical analysis of the head space volatiles with GC and GC-MS. Each bioassay was replicated once or twice, but the chemical analysis was done only once.

Bacterial counts in onion roots. About 30, 1 to 2-week-old seedlings were washed in tap water and their root crowns with roots (hereafter called roots) were removed with a forceps. The roots were weighed, washed again in sterilized distilled water and ground with mortar and pestle. The ground tissue was suspended in 50 ml of sterilized water which was further diluted 10- and 50-fold. The total bacteria counts of these suspensions were made with Millipore Samplers (Total-Count Sampler, Millipore Corp., Mass.) by incubating at 35°C for 24 hr. The bacterial colonies on the samplers were counted 4 to 6 times for each test.

PCSO and amino acid analyses of roots and exudates in soil. After weighing and heating in a microwave oven 30 or 50 roots were ground with mortar and pestle and suspended in 2 ml of 70% ethanol with a few drops of 10% trichloro acetic acid to deproteinize them. For collection of the root exudates of septic and aseptic seedlings, the cultured sand from which all traces of plant tissues and ungerminated seeds were eliminated was transferred onto a filter paper in a glass funnel and washed with 350 ml of 70% ethanol. The washed liquid was concentrated to 2 ml *in vacuo*. The concentrates of these root and soil extracts were analyzed with a Beckman amino acid analyzer Model 119, equipped with a 0.9 $\times$ 56 cm column of PA-28 resin. The column was eluted at a flow rate of 50 ml/hr with 0.2 N Na-citrate buffer at pH 3.25 and then pH 4.25. Only acidic and neutral fractions were analyzed. PCSO and *r*-glutamyl methionine were located on the chromatograms according to MATIKKALA and VIRTANEN (1967), and by analysis of the acid hydrolysates of some samples. These compounds were, however, quantified in terms of glutamic acid which showed an average colour intensity of the ninhydrin reaction with common amino acids. All chemical analyses were done once or twice for each test.

GC and GC-MS analyses of the volatiles of seedlings and bacterial metabolites. After flushing of the accumulated volatiles in the head space of culture flask containing seedlings or

Fig. 1. Relationship between the oviposition ratio (R) of onion cultivars, and $\log_{10}$ of PCSO (Pr), and amino acid contents (Ar) and the bacteria counts (Br) in the roots in petri dish cultures. See text for the cultivar numbers.

Fig. 2. Relationship between the oviposition ratio (R) of onion cultivars, and $\log_{10}$ of PCSO content (Pr) and the quantity of propylthio compounds (Sh) in the volatiles of seedlings in septic and aseptic flask cultures.

bacterial inoculate with clean air, an activated charcoal column (20–40 mesh, 0.4×8 cm) was attached to an outlet of the culture flask and suctioned at a flow rate of 40 ml/min for 24 hr. The column was eluted with 1 or 2 ml of CS_2 and the eluates were stored in a deep freezer until the following chemical analyses. A Hewlett-Packard 5830A gas chromatograph with a 18850A terminal was used with a $45 \text{ m} \times 1.5 \text{ mm}$ ID capillary column of Superrox-4 and a FID. The GC temperature program ran from 70 to 240°C at $4^\circ\text{C}/\text{min}$. Mass spectra were obtained with a Hewlett-Packard 5985 GC-MS system with the same column. Identifications of the isolated compounds according to their mass fragmentation patterns were confirmed by reference to the spectra compiled in the EPA/NIH Spectrum Data Base (HELLER and MILNE, 1978).

RESULTS

Experiments in the petri dish culture

Attractancy of onion seedlings

When oviposition ratios of the onion flies on the seedlings of 11 cultivars were determined by the choice test in petri dishes, the average ratio of Takii 10 was 0.71, significantly lower than the ratios of other cultivars (NEUMAN KEULS test, $p < 0.05$). Although no significant differences were observed among the average ratios of other cultivars due to the large variations among the replicates, their ratios were taken as the probable indexes of susceptibility levels of cultivars to the onion fly. They were as

Fig. 3. Relationship between the quantity of propylthio compounds in the volatiles of septic seedlings, and the PCSO content in septic soil.

follows in a descending order: (2) EYG 0.80, (3) 12KROP 0.90, (4) Tamarack 0.91, (5) Ailsa Craig 0.92, (6) Mirage 0.94, (7) White Lisbon 0.97, (8) Takii 80-02 0.97, (9) Autumn Spice 1.00, (10) YGD 1.01 and (11) MYG 1.09.

PCSO and amino acid contents of roots

The PCSO contents varied from $2.19 \mu\text{mol/g}$ wet weight of roots in Takii 10 to $8.54 \mu\text{mol/g}$ in MYG, whereas the total amino acid content was lowest in EYG at $52.22 \mu\text{mol/g}$ and highest in Autumn Spice at $148.88 \mu\text{mol/g}$. There were significant correlations between the oviposition ratios of the cultivars (R) and the log PCSO contents (Pr), and between the ratios (R) and the log amino acid contents (Ar), $r=0.732$ ($p<0.05$, $n=11$) and $r=0.599$ ($p<0.10$, $n=11$), respectively (Fig. 1).

The ratios were also significantly correlated with the following individual amino acids: asparatic acid ($r=0.635$), serine ($r=0.778$), glutamic acid ($r=0.615$), glycine ($r=0.681$), *i*-leucine ($r=0.674$), glutamine plus asparagine ($r=0.673$) and tyrosine ($r=0.723$) ($p<0.05$), and valine ($r=0.550$) ($p<0.10$).

Bacteria counts in roots

The bacteria count (Br) was lowest at $4.8 \times 10^6/\text{g}$ in Takii 10 and highest at $11.2 \times 10^6/\text{g}$ in YGD. The log counts were again significantly correlated with the oviposition ratios (R) ($p<0.10$, $r=0.566$, $n=11$) (Fig. 1). The correlation was, however, non-significant between the bacteria counts (Br) and the PCSO contents (Pr) or the amino acid contents (Ar).

Experiments in the flask culture

Septic culture

The quantity of PCSO in soil (Pss) varied from 1.1 pmol/g wet weight of whole standing seedlings in YGD to 3.9 pmol/g in Mirage (Fig. 2). The correlation between the PCSO quantities of 8 tested cultivars and the oviposition ratios (R) was not significant. Meanwhile, the larger quantity of propylthio compounds including MeSSPr and PrSSPr (the principal attractants) was generated by the more susceptible seedlings:

Fig. 4. Typical gas chromatograms of the volatiles of onion seedlings and bacterial incubates of PCSO medium. (A) Takii 80-02 in septic soil, (B) Autumn Spice in aseptic soil, (C) Incubate of bacteria C after 14 days and (D) Incubate of *B. subtilis* without PCSO. The broken arrows indicate absences of the corresponding sulfur compounds. MeSSMe, dimethyl disulfide. MeSSPr, methyl propyl disulfide. PrSSPr, dipropyl disulfide. MeSSPe, methyl propenyl disulfide. MeSSSPe, methyl propenyl trisulfide. MeNCS, methyl isothiocyanate. EtPr, ethyl propionate.

0.2 μg (1.3 pmol in terms of PrSSPr)/g seedlings in 24 hr in EYG to 22.9 μg (153 pmol)/g, 24 hr in Takii 80-20. The correlation coefficient between the quantities of propylthio compounds (Sh) and the oviposition ratios (R) was 0.629 ($n=9$) and was significant ($p<0.05$) (Fig. 3). In addition, the quantity of propylthio compounds was significantly correlated ($r=0.559$, $p<0.10$, $n=8$) with the PCSO content in septic soil. Therefore, the more PCSO was present in the septic soil, the more attractant was generated, which in turn attracted more oviposition.

Aseptic culture

The bacteria counts of aseptic roots of various cultivars varied from $1 \times 10^3/\text{g}$ to $8.7 \times 10^3/\text{g}$ wet weight of roots. These counts were approximately 1,000 times lower than the counts in the septic cultures. The oviposition ratio for the septic cultures in

Fig. 5. Production of disulfides by the bacteria A-E and the oviposition attractancy of incubates in various incubation days. S=*B. subtilis* and (S)=*B. subtilis* without PCSO. The dotted lines indicate 95% confidential limits of the oviposition ratio 1.

petri dishes (R) was significantly correlated with the PCSO content in the aseptic soil (Pas) ($r=0.654$, $p<0.05$, $n=9$) (Fig. 3). (No oviposition experiments were done under aseptic conditions.) However, generation of propylthio compounds in the aseptic culture was minimal: less than $0.1 \mu\text{g/g}$ wet weight of whole standing seedlings in 24 hr in each of EYG, 12KROP, Autumn Spice and MYG. Takii 10 was an exception which produced $1.1 \mu\text{g/g}$. In this case, the roots were contaminated with 100 times more bacteria than in the average aseptic counts.

Typical chromatograms of Takii 80-02 in the septic culture (A) and that of Autumn Spice in the aseptic culture (B) are shown in Fig. 4. There were few traces of sulfur compounds in the latter.

In brief, the log quantity of PCSO in the aseptic soil was linearly proportional to the attractancy of onion seedlings, but generation of the attractants was almost nil without rhizobacteria.

Bacterial metabolism of PCSO in vitro

When the bacterial incubates of the PCSO medium were tested for oviposition against the non-inoculated medium, the oviposition ratios began to increase in 10 days and peaked at 3-fold in 17 days with bacteria C, D and E, while the ratios were only 1.7-fold with bacteria A and B (Fig. 5). On the other hand, the ratio of *B. subtilis* peaked earlier in 10 days. The incubate of *B. subtilis* without PCSO in the medium showed no attractancy throughout the experimental period. The volatiles of incubates of three bacteria C, D and *B. subtilis* were quantitatively analyzed by GC. Propylthio- and non-propylthio compounds shown by the hatched and open bars, respectively, were minimum at the beginning of inoculation, but substantially increased in 14 days, then decreased slightly in 21 days. Thus, the increase of attractancy and the generation of attractants were more or less synchronized.

Typical gas chromatograms are shown in Fig. 4. Chromatogram C shows the

Fig. 6. Profiles of sulfur compounds found in the volatiles of bacterial incubates, intact seedlings and chopped onions. —: bacterial metabolism ($n=8$), ----: onion seedlings ($n=11$),: chopped onions ($n=3$).

incubate of bacteria C after 14 days, which produced 49.8 μg of MeSSMe, 52.5 μg of MeSSPr, 1.6 μg of MeSSPe, 17.8 μg of PrSSPr plus MeSSMe, 3.0 μg of PrSSPe and a trace of MrSSSPr in a 24 hr period. On the contrary, the sulfur compounds were totally absent in chromatogram C of *B. subtilis* without PCSO. In addition to ethylene chlorides and aromatics which were impurities of the CS_2 solvent, 4-methyl-2-pentanone, decane, hendecane, dodecane and pentadecane were detected. But the origin and bioactivity of these compounds is unknown. Ethyl propionate was also eluted at 40 sec prior to PrSSPr in all bacterial incubates.

Profiles of sulfur compounds in the volatiles of bacterial incubates, intact seedlings and chopped onion bulbs

The bacteria on the PCSO extract generated an average 46% of MeSSMe against the total amount of sulfur compounds, 32% of MeSSPr and 21% of PrSSPr and very little of MeSSPe and PrSSPe (Fig. 6). On the other hand, according to the previous quantitative analyses (ISHIKAWA et al. 1978; IKESHOJI et al., 1980) in which loss of volatile MeSSMe was carefully avoided, chopped onions produced 6% of MeSSMe, 23% of MeSSPr and 65% of PrSSPr. The quantity of these sulfur compounds in the volatiles of intact seedlings in septic culture was an intermediate between the above two analytical results: 47% of MeSSMe, 27% of MeSSPr and 24% of PrSSPr.

DISCUSSION

Various characteristics of onion cultivars in relation to the oviposition attractancy

In the previous work (IKESHOJI et al., 1982) the PCSO content of onion roots of

various cultivars was inversely proportional to the onion maggot. This relationship was presumably due to the higher decomposition rate of PCSO in the susceptible cultivars which have occurred during the storage period between harvest to chemical analysis since alliinase was more active in the susceptible cultivars than in the resistant ones.

In the present work, therefore, the seedlings were analyzed immediately after harvest. In addition, the more accurate analytical procedure using an automatic amino acid analyzer was adopted instead of the previous indirect measurement of PCSO by TLC and densitometry. As the result, a good correlation was obtained between PCSO content and the oviposition ratios for different cultivars. This result is more reasonable in reference to the fact that the volatiles of susceptible seedlings include more attractants than those of resistant ones.

The amino acid contents showed no consistent tendency in regard to the susceptibility of various cultivars in the previous work (IKESHOJI et al., 1982). In the present work, the total amino acid as well as individual amino acid content were significantly correlated with the susceptibility levels of cultivars. Among them, serine, valine and glutamic acid are directly involved in the biosynthesis of alkylcysteine sulfoxides. The PCSO residue in the aseptic soil was also significantly correlated with the attractancy of cultivars. These findings suggest that PCSO and some amino acids are passively diffused out of roots into the rhizosphere according to their concentration gradients.

In the septic culture in flasks, however, the attractancy was not significantly correlated with either PCSO or amino acid quantity in soil. This discrepancy between the aseptic and septic cultures should result because rhizobacteria would have consumed these nutrient compounds in the septic soil and the residues in soil are the result of balance between the amounts diffused out from the roots and the bacterial consumption. Here, both diffusion as well as consumption rates are dependent on the population size and species of rhizobacteria.

IKESHOJI and MATSUMOTO (1982) found a significant correlation between the bacteria counts of bulbing onion roots and the susceptibility of various cultivars in the field. The same result was obtained in the present septic culture of seedlings in petri dishes. Therefore, it may be inferred that the susceptible seedlings exude more PCSO and amino acids into the soil and so support more bacteria than the resistant ones do. But, the quantities of these compounds in soil and the bacteria counts failed to show any significant correlation. Other factors such as soil moisture may be more important in regulation of the population size of rhizobacteria.

Attractancy of various cultivars was significantly correlated with the quantity of sulfur compounds in the volatiles of bulbing seedlings in the previous work (IKESHOJI et al., 1982). This result was reconfirmed by the present septic culture in flasks. The septic cultures of various cultivars generated 0.2 to 23 $\mu\text{g/g}$ (1.3 pmol/g to 153 pmol/g) of propylthio compounds in 24 hr in metabolism of the exudated PCSO. As the result, only 1.2 pmol/g to 3.9 pmol/g of PCSO remained in the septic soil. This PCSO residue in the septic soil could significantly be correlated ($p < 0.10$) with the generation of propylthio compounds, but not directly with the attractancy of onion cultivars. In comparison, as much as 5.5 pmol/g to 53 pmol/g of PCSO remained in the aseptic soil and generation of sulfur compounds was almost nil. Since rhizobacteria are well known to double the quantity of root exudates under septic conditions (BOWEN and ROVIRA, 1976), the original quantity of PCSO, that already metabolized

plus the amount remaining, in the septic root exudate would have been much higher than in the aseptic exudate.

Bacterial metabolism and possible decompartmentalization of PCSO in onion roots

Involvement of rhizobacteria in the generation of propylthio compounds in intact onion seedlings is now apparent (DINDONIS and MILLER, 1980; HOUGH et al., 1981; IKESHOJI and MATSUMOTO, 1982). KING and COLEY-SMITH (1969) demonstrated that 6 out of 10 bacteria tested metabolized allyl- and *n*-propylcysteines and their sulfoxides in nutrient broth. The bacteria generated A11SH, A11SA11, A11SSA11, and PrSH and PrSSPr from the above substrates, respectively. In the present work, when PCSO and *r*-glutamyl methylcysteine extracted from onion bulbs were used as the substrate, the three bacteria generated substantial quantities of MeSSMe, MeSSPr and PrSSPr.

The profile of these sulfur compounds seems to be of importance and interest. The bacteria generated more methylthio and less propylthio compounds, in spite of the larger quantity of PCSO and smaller quantity of *r*-glutamyl methylcysteine included in the medium. In this regard, most research on the bacterial metabolism in the past has been done with S-methylcysteine sulfoxide instead of PCSO (KING and COLEY-SMITH, 1969). These facts may imply that bacterial S-alkylcysteinase metabolizes S-methylcysteine and its sulfoxide more readily than PCSO. On the other hand, chopped onions produce predominantly MeSSPr and PrSSPr. This is simply because the affinity of alliinase (S-alkylcysteinase of onion) to S-propyl derivatives is 3.2 times higher than that to methyl derivatives (WHITAKER, 1976).

Meanwhile, the intact seedlings under the septic condition generated an intermediate proportions of these sulfur compounds between the above two sources. Therefore, it could be hypothesized that the precursors both passively diffused out and actively leaked by rhizobacteria are metabolized to methylthio compounds mostly by the bacteria. The bacteria, at the same time, inflict on root epithelium a mechanical damage by the bacteria. The bacteria, at the same time, inflict on root epithelium a mechanical damage which activates (decompartmentalizes) alliinase to produce propylthio compounds. In support of this hypothesis, there is a microscopic evidence for perforation of the root epidermal cells of 3 graminaceous plants by non-pathogenic bacteria (OLD and NICOLSON, 1975).

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