園学雑. (J. Japan. Soc. Hort. Sci.) 72 (4) : 268 - 274. 2003.

Identification of Active Substances in Chinese Chive and Rakkyo Plants Responsible for Breaking Bud Dormancy in Grape Cuttings

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Summary

The active substances in volatiles from two *Allium* species, Chinese chive (*A. tuberosum* Rottler) and rakkyo (*A. chinense* G. Don), responsible for breaking bud dormancy in grapevines, were investigated. Among several gas-chromatographic peaks, two major ones in Chinese chive and one in rakkyo were detected. Based on a comparison of the retention time with authentic chemicals and GC-MS analysis, the compounds were identified as methyl mercaptan (CH_3SH) and allyl mercaptan (CH_2=$CHCH_2SH$) in Chinese chive, and dimethyl disulfide (CH_3SSCH_3) in rakkyo. Dormant 'Kyoho' grape cuttings were exposed for different durations to these and related compounds at varying concentrations in late November, late December and mid January. Methyl mercaptan promoted budbreak in late December and mid January, although a lower concentration resulted in a uniform rate of budbreak. Allyl mercaptan was effective in late December but inhibitory in mid January. The effect of dimethyl disulfide seemed to vary with concentration, time and duration of exposure. In late November, 24-hr exposure to 99% dimethyl disulfide greatly promoted budbreak, whereas 12-hr exposure had a slight effect. Moreover, no budbreak was observed in cuttings exposed to 30% dimethyl disulfide for 12 or 24 hr. In late December, a 24-hr exposure to the 10% suspension of the same compound promoted budbreak but the same exposure to a 75% suspension inhibited it. Therefore, it is highly probable that these substances are effective for breaking dormancy of grapevine buds but their time course effects and optimum concentrations remain to be investigated in relation to the dormancy stages.

Key Words: active substances, *Allium* species, budbreak, grape cutting, sulfide compounds.

Introduction

Forcing budbreak of dormant grapevines in greenhouses is a common practice in Japan, so an efficient and uniform budbreak is desirable for stable berry production. Many investigations have been conducted to induce termination of bud dormancy in woody plants, including grapevines, through the application of chemicals, such as mineral oil and dinitro-*o*-cresol (DNOC) (Samish, 1954), calcium cyanamide ($CaCN_2$) (Iwasaki, 1980; Iwasaki and Weaver, 1977; Kuroi et al., 1963; Kuroi, 1985), hydrogen cyanamide (H_2CN_2) (Lin and Wang, 1985; Nir et al., 1988; Shulman et al., 1983; Williams, 1987; Zelleke and Kliewer, 1989), and growth regulators (Broome and Zimmerman, 1976; Iwasaki, 1980; Weaver et al., 1974).

Kubota et al. (1987, 2000) and Kubota and Miyamuki (1992) reported previously that freshly grated garlic (*Allium sativum* L.) paste and its volatiles are as effective as $CaCN_2$ suspension or H_2CN_2 solution in promoting budbreak of several grape cultivars, including 'Muscat of Alexandria'. Kubota et al. (1999a, 1999b) also observed that the active substances in garlic, responsible for breaking bud dormancy in grapevines, were volatile sulfide compounds with an allyl group (CH_2CHCH_2), particularly diallyl disulfide. Moreover, Kubota et al. (2002) observed that the volatile substance(s) in Chinese chive (*A. tuberosum* Rottler) and rakkyo (*A. chinense* G. Don), as well as paste and/or volatiles of other various *Allium* species, stimulated breaking dormancy in grapevines. However, the active substance(s) in Chinese chive and rakkyo plants responsible for this remain to be elucidated. Various sulfide compounds, including volatiles, have been identified in Chinese chive (Freeman and Whenham, 1975; Obata, 1961; Saghir et al., 1964) and rakkyo (Freeman and

Received; September 5, 2002. Accepted; February 13, 2003.
This work was supported in part by Grant-in-Aid for Scientific Research (No. 62560029) from The Ministry of Education, Science, Sports, and Culture, Japan.
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Whenham, 1975; Kojima, 1982; Saghir et al., 1964) as well as garlic (Freeman and Whenham, 1976; Kubota et al., 1999b, Saghir et al., 1964; Yu et al., 1989) and onion (Freeman and Whenham, 1976; Lancaster and Kakky, 1983; Sinha et al., 1992).

The purpose of this study was to determine the active substance(s) in Chinese chive and rakkyo volatiles responsible for the breaking of bud dormancy in grape cuttings.

Materials and Methods

Identification of sulfur-containing compounds in Chinese chive and rakkyo volatiles

Fresh leaves of Chinese chive (*A. tuberosum* Rottler) and leaf sheaths of rakkyo (*A. chinense* G. Don) were chopped and grated into a paste. A 50-g sample of the paste was put into a 100 ml flask and sealed. A 200-μl gas aliquot was withdrawn from the head space gas of each and injected into a gas chromatograph (GC-9A, Shimadzu, Kyoto, Japan) for analysis of volatile sulfur-containing compounds. For comparison of retention times, volatiles of sulfur-containing standards, methyl mercaptan sodium salt, allyl mercaptan, ethyl mercaptan, *n*-propyl mercaptan, *n*-butyl mercaptan, and dimethyl mono- and di-sulfides, Tokyo Kasei Co., Ltd., Tokyo, Japan, were also injected. Conditions for gas chromatography were as follows: detector, FID; column, 3 mm × 3 m glass; packing, 25% 1,2,3,-Tris (2-cyanoethoxy) propane coated on Shimalite (AW-DMCS, 80/100 mesh) programmed at 40 ℃ for 5 min, then raised to 145 ℃ at 10 ℃ · min^{-1}; the carrier gas, N_2, was kept at a flow rate of 60 ml · min^{-1}. The volatile sulfur-containing substances in Chinese chive and rakkyo were further identified by GC-MS analysis (JMS-AUTOMASS 150, JEOL, Tokyo, Japan). Analytical conditions for GC-MS were as follows: ionizing voltage, 70 eV; ionizing current, 200 mA; scan range, 35 to 350 amu; scan speed, 0.45 sec/35 to 350 amu; detector gain, -800 V (max. -1.5 kV).

Effect of various volatile sulfide compounds on breaking dormancy in grape cuttings

Grapevines and general procedures

All plant materials were obtained from dormant grapevines (*Vitis labruscana* Bailey, cv. Kyoho) grown at the Research Farm, Okayama University, Japan. Cumulative chilling hours (CCH) were calculated, based on the duration of exposure of grapevines to 7.2 ℃ or lower, which ranged from 47 to 668 hr, depending on the experiment. Single-bud cuttings (10 cm long) were prepared from the 5th to the 9th nodes of canes and kept for 12 or 24 hr at room temperatures in a 5-liter desiccator containing the above various sulfide compounds; diallyl disulfide was obtained from Riken Chemical Industry Co., Ltd. (Kyoto, Japan). Unless otherwise stated, the following procedure was undertaken: immediately after treatment, the cuttings were mounted on a plastic foam plate, floated in a water bath, and placed in a growth chamber maintained at 25 ℃. Two or three replications of 10 cuttings were allocated to each treatment. Dormancy was considered broken when the buds became green. Data were evaluated by *t*-test (5% level) and expressed as means ± SE.

Effect of duration of exposure to three sulfide compounds and their concentrations (Expt. 1)

Cuttings of 'Kyoho' vines that were prepared in late November (47 CCH) were placed for 12 or 24 hr in a desiccator, containing 10 ml of authentic or 30% suspension of dimethyl mono- or di-sulfides, or diallyl disulfide, which is the active substance in garlic, responsible for breaking dormancy in grapevines (Kubota et al., 1999b). The proportions of active ingredients in each authentic sulfide compound were 99, 99 and 75%, the remaining contaminants were diallyl mono-, tri- and tetra-sulfides. Control cuttings were kept to the same period in a desiccator containing 10 ml of distilled water. Each treatment had two replications of 10 cuttings.

Effect of concentration of diallyl- and dimethyl-disulfides, and allyl- and methyl-mercaptans (Expt. 2)

Cuttings of 'Kyoho' vines, prepared in late December (356 CCH), were placed for 24 hr in a desiccator, containing 5 ml of 10% or 75% suspensions of methyl-mercaptan sodium salt, allyl mercaptan, dimethyl disulfide and diallyl disulfide. The control cuttings were exposed to 5 ml of distilled water. Three replications of 10 cuttings were allocated to each treatment.

Effect of five mercaptan homologues and diallyl disulfide (Expt. 3)

Six sets of 20 cuttings prepared from 'Kyoho' vines in mid January (668 CCH) were placed for 24 hr in a desiccator containing 5 ml each of 10% aqueous suspension of mercaptan homologues, allyl-, ethyl-, *n*-butyl- and *n*-propyl-mercaptans, and methyl mercaptan sodium salt. For comparison, 10% suspension of diallyl disulfide was also tested. Two replications of 10 cuttings were allocated to each treatment. Control cuttings were placed for 24 hr in a desiccator containing 5 ml of distilled water.

Results

Identification of sulfur-containing compounds in Chinese chive and rakkyo volatiles

Although several sulfur-containing compounds were detected in both *Allium* species, only two (A and B) and one (C) major peaks were observed in Chinese chive and rakkyo, respectively (Fig. 1). Comparison of the retention times of the three major peaks with those of

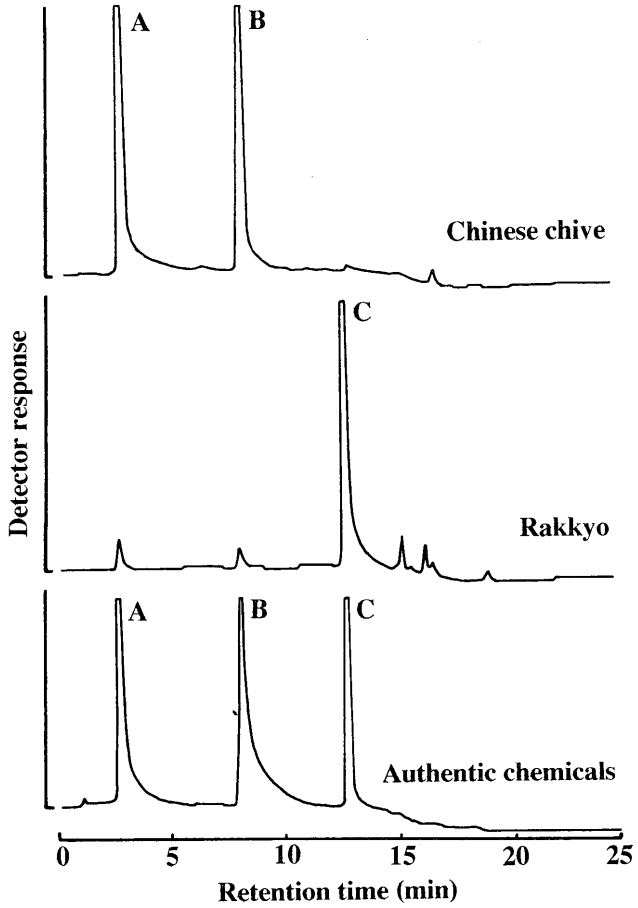

Fig. 1. Gas chromatogram tracing of volatiles of Chinese chive (upper) and rakkyo (center) plants, and authentic chemicals (lower). Peaks A, methyl mercaptan; B, allyl mercaptan; C, dimethyl disulfide.

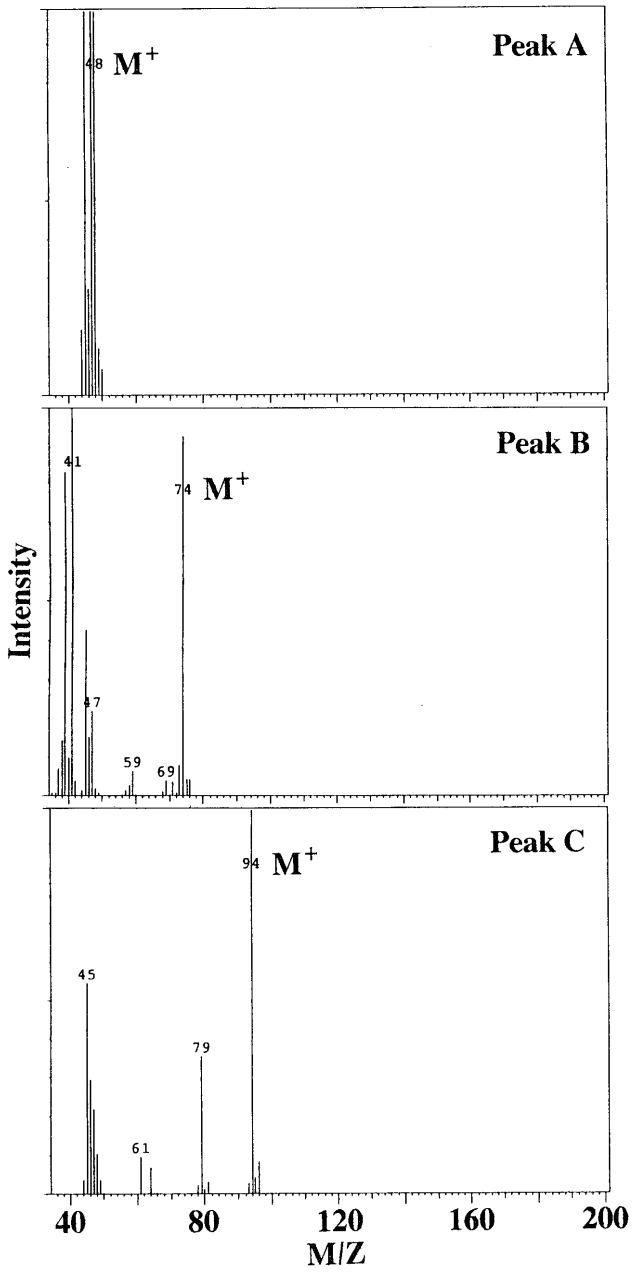

Fig. 2. Mass spectra of peaks A (upper), B (center) and C (lower) in the gas chromatogram (Fig. 1). Peaks A, methyl mercaptan (CH_3SH); B, allyl mercaptan (CH_2=$CHCH_2SH$); C, dimethyl disulfide (CH_3SSCH_3).

authentic samples, showed that the two peaks in Chinese chive, and one peak in rakkyo corresponded to methyl mercaptan (CH_3SH) and allyl mercaptan (CH_2=$CHCH_2$ SH), and dimethyl disulfide (CH_3SSCH_3), with molecular weights of 48 and 74, and 94, respectively. This was confirmed by GC-MS analysis (Fig. 2).

Effect of various volatile sulfide compounds on breaking dormancy in grape cuttings

Expt. 1

The exposure of cuttings to volatiles from the authentic solutions or those of 30% suspensions of diallyl disulfide, dimethyl sulfide and dimethyl disulfide revealed that in the 12-hr treatment (Fig. 3, upper) both 30% suspension of diallyl disulfide and authentic dimethyl sulfide (e. g. 99%) significantly promoted budbreak (5% level, t-test), which began 13 days after treatment. Budbreak in the control cuttings occurred after 28 days. Cuttings treated with diallyl disulfide exhibited a more uniform budbreak than did those treated with dimethyl sulfide, irrespective of the concentration. No significant difference in sprout promotion was observed among 75% authentic diallyl disulfide and 99% dimethyl disulfide, and the control, although the final percentages of budbreak of the cuttings treated with diallyl disulfide and dimethyl disulfide were higher than that of the control. Budbreak was not observed in any of the cuttings treated with 30% dimethyl disulfide.

In the 24-hr treatment (Fig. 3, lower), budbreak was significantly (5% level, t-test) accelerated by 30% suspensions of diallyl disulfide, followed by 99% dimethyl disulfide and 75% diallyl disulfide. No budbreak was observed in the cuttings treated with either 99%

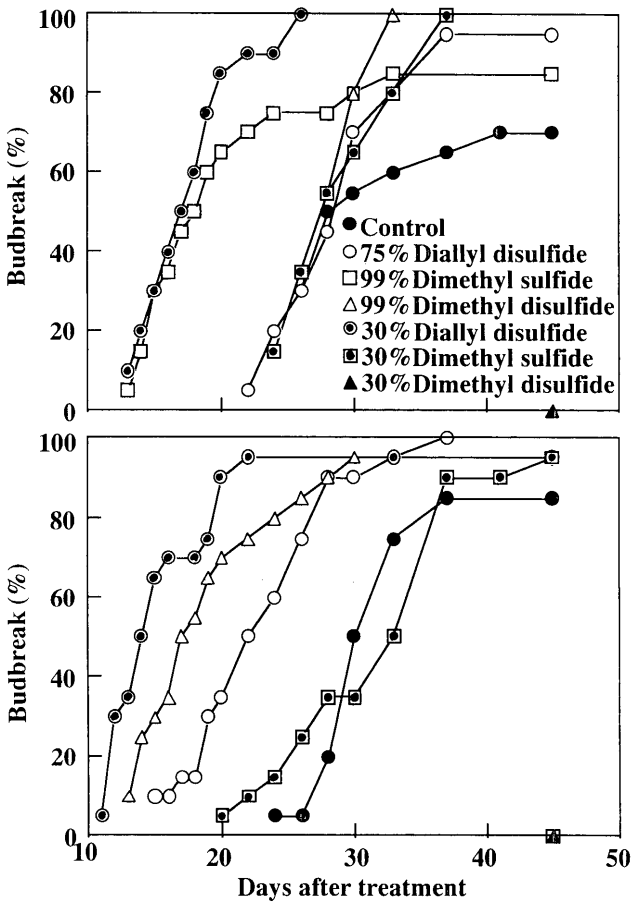

Fig. 3. Effect of exposure for 12 (upper) or 24 (lower) hr to volatiles of authentic solutions or 30% suspensions of diallyl disulfide, dimethyl sulfide and dimethyl disulfide on budbreak of single-bud cuttings of 'Kyoho' grapevine (treatment in late November).

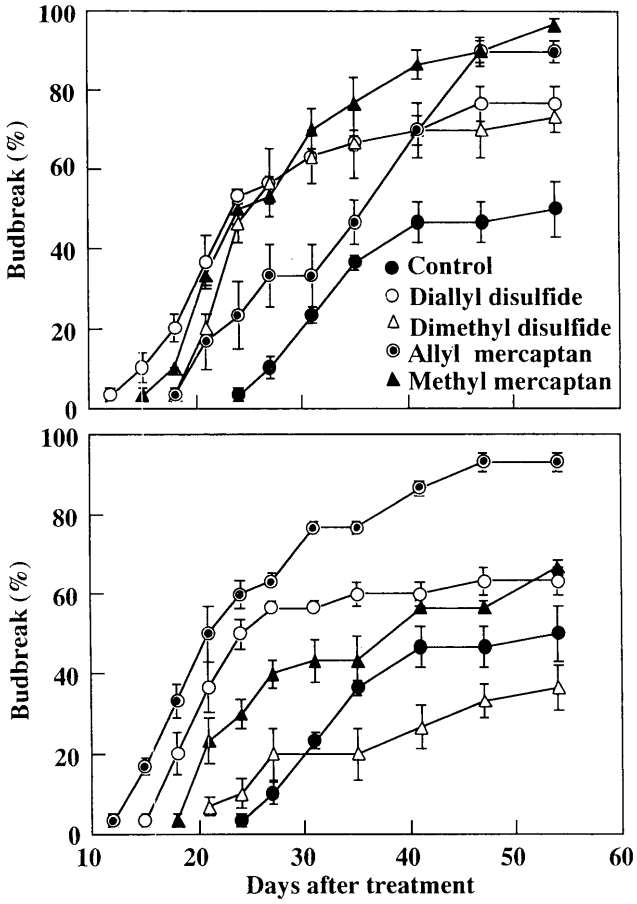

Fig. 4. Effect of exposure for 24 hr to volatiles of 10% (upper) or 75% (lower) suspensions of diallyl disulfide, dimethyl disulfide, allyl mercaptan and methyl mercaptan sodium salt on budbreak of single-bud cuttings of 'Kyoho' grapevine (treatment in late December). Vertical bars denote the SE (n = 3).

dimethyl sulfide or 30% suspension of dimethyl disulfide.

Expt. 2

Treatments with volatiles of 10% suspensions of diallyl disulfide, dimethyl disulfide, allyl mercaptan and methyl mercaptan sodium salt (Fig. 4, upper) accelerated significantly budbreak (5% level, *t*-test), although the final percentage of budbreak of the cuttings treated with diallyl and dimethyl disulfides was slightly lower.

Budbreak in cuttings, treated with volatiles of 75% allyl mercaptan, diallyl disulfide, and methyl mercaptan sodium salt, was initiated at 12, 15 and 18 days (significant at 5% level by *t*-test), respectively, whereas the first budbreak occurred 24 days after treatment in the control (Fig. 4, lower). The final percentage of budbreak of cuttings treated with volatiles of 75% dimethyl disulfide was less than that of the control cuttings.

Expt. 3

When cuttings of 'Kyoho' vines were exposed for 24 hr to volatiles from 10% suspensions of mercaptan homologues and diallyl disulfide, diallyl disulfide, *n*-propyl mercaptan, methyl mercaptan sodium salt and ethyl mercaptan promoted budbreak significantly (5% level, *t*-test), whereas treatments with methyl mercaptan sodium salt and ethyl mercaptan resulted in a slower rate of budbreak thereafter. Allyl mercaptan inhibited budbreak significantly (Fig. 5).

Discussion

Sulfides that have been identified in garlic include as diallyl mono-, di- and tri-sulfides (Kubota et al., 1999b; Yu et al., 1989), and in onion as diallyl thiosulfonate and propyl methanethiosulfonate (Sinha et al., 1992). In this study, an attempt was made to identify the sulfur-containing compounds in Chinese chive and rakkyo volatiles, which are responsible for breaking bud dormancy in grapevines (Kubota et al., 2002). The two main volatile substances detected in Chinese chive and one in rakkyo by gas chromatography were identified as methyl mercaptan and allyl mercaptan and dimethyl disulfide, respectively, by GC-MS analysis. This find-

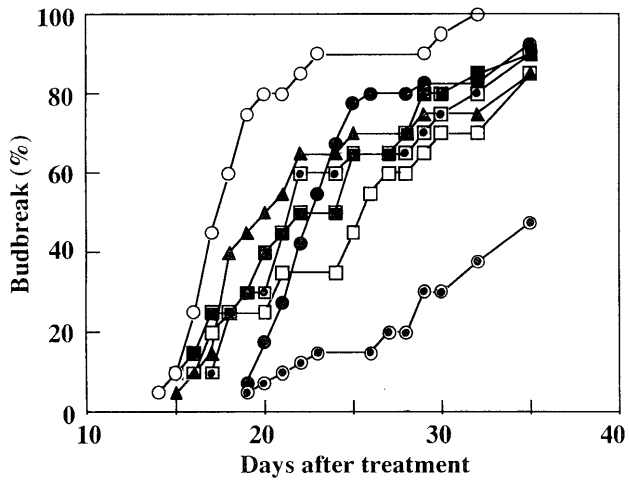

Fig. 5. Effect of exposure for 24 hr to volatiles of 10% mercaptan homologues and diallyl disulfide on budbreak of single-bud cuttings of 'Kyoho' grapevine (treatment in mid January). ● ; control, ○ ; diallyl disulfide, ◉ ; allyl mercaptan, ▲ ; methyl mercaptan sodium salt, □ ; ethyl mercaptan, ⊡ ; *n*-butyl mercaptan, ■ ; *n*-propyl mercaptan.

ing agrees with the results of Saghir et al. (1964) and Freeman and Whenham (1975), who observed that the main sulfide compound in rakkyo, based on a series of gas and thin-layer chromatographic and ultraviolet spectrophotometric methods, was a substance with a methyl radical, including dimethyl disulfide. Saghir et al. (1964) reported the same compounds in Chinese chive and rakkyo volatiles. However, Freeman and Whenham (1975) reported that the substance had a 2-propenyl radical, whereas the other had a methyl radical. Our findings are in agreement with the latter because the allyl and methyl mercaptans identified in this study have 2-propenyl and methyl radicals, respectively.

Effects of exposure to methyl and allyl mercaptans and dimethyl disulfide and their related sulfide compounds on budbreak of single-bud cuttings of 'Kyoho' grapevines were investigated in late November, late December and mid January. When the cuttings were exposed for 24 hr to volatiles of 75% allyl mercaptan, methyl mercaptan and diallyl disulfide in late December, allyl mercaptan was most effective in promoting budbreak, followed by diallyl disulfide and methyl mercaptan. However, when exposed to 10% suspensions, diallyl disulfide was most effective, followed by methyl mercaptan and allyl mercaptan. Note that the lower concentration resulted in a uniform rate of budbreak for both allyl and methyl mercaptans. The mid-January treatment with volatiles of 10% methyl mercaptan was effective in promoting the budbreak as well as diallyl disufide, but allyl mercaptan was not; it lowered the budbreak percentage. The reasons for different responses by buds to the volatiles of allyl mercaptan between the two experiments are not known, but the results seem to indicate that the deeper the dormancy, the more pronounced the effect on budbreak. Hosoki et al. (1985, 1986) reported that several sulfide compounds, including methyl mercaptan, were effective in breaking the dormancy of corms, tubers and trees. However, they did not test allyl mercaptan, which was as effective as methyl mercaptan in the breaking of bud dormancy of grape cuttings, but the response was influenced by the concentration and duration of exposure. These observations indicate that several volatile sulfur-containing compounds such as allyl mercaptan and methyl mercaptan in Chinese chive promote budbreak in grapevines. That *n*-propyl mercaptan is effective in breaking bud dormancy of grape suggests that there are mercaptans other than methyl and allyl mercaptans in Chinese chive that stimulate budbreak.

Dimethyl disulfide, which was the predominant component in the rakkyo volatiles, was also effective in breaking the bud dormancy of grape cuttings. However, its effect varied widely depending on the time, concentration and duration of the treatment. For instance, the exposure to volatiles of the authentic dimethyl disulfide (a. i. 99%) was effective in the 24-hr treatment in late November, whereas it was ineffective in the 12-hr-treatment. When exposed to 30% suspension, no budbreak was observed, irrespective of the duration of exposure (Fig. 4), whereas 10% dimethyl disulfide accelerated budbreak of 'Kyoho' cuttings exposed for 24 hr in late December; the 75% dimethyl disulfide sample inhibited it as did the standard dimethyl sulfide volatile. The exposure of cuttings to these volatile chemicals was carried out at different stages of dormancy, so that their varied responses need further investigation. Hosoki et al. (1986) suggested that dimethyl disulfide is one of the most effective dormancy-breaking chemicals, as it is not phytotoxic to gladiolus corms and tree peony. There may be different bases why different plants respond to chemicals. For example, gibberellic acid, a plant growth regulator, increased the percentage of budbreak in peach (Donoho and Walker, 1957), but decreasesed the emergence rate in *Vitis vinifera* (Weaver, 1959).

Based on our observations, we conclude that the active substances in Chinese chive and rakkyo volatiles, responsible for the breaking of bud dormancy in grapevines, are methyl and allyl mercaptans and dimethyl disulfide. However, the effects of these substances on the budbreak of grape cuttings varied widely among the experiments compared to the diallyl disulfide, the predominant substance in garlic, responsible for the breaking of bud dormancy in grapevines (Kubota et al., 1999a, 1999b, 2000). Therefore, further investigations are required to determine the optimum concentration and duration of exposure of these compounds in relation to the dormancy stages.

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ニラとラッキョウに含まれるブドウの芽の休眠打破に有効な物質の同定

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摘　要

アリウム属植物のニラとラッキョウについて，ブドウの芽の休眠打破に有効な成分を検討した．ニラとラッキョウの揮発性物質をガスクロマトグラフィで分析したところ，いずれにもいくつかのピークが検出されたが，大きなピークはニラでは2つ，ラッキョウでは1つであった．標品との保持時間の比較およびGC-MSによる解析の結果，ニラの2つのピークはメチルメルカプタン(CH_3SH)とアリルメルカプタン($CH_2=CHCH_2SH$)，ラッキョウのピークは2硫化ジメチル(CH_3SSCH_3)であった．これらの化合物およびこれらに関連する化合物について，休眠期(11月下旬，12月下旬あるいは1月中旬)の‘巨峰’の挿し穂を気浴処理し，休眠打破効果を調査した．メチルメルカプタンは12月下旬，1月中旬処理ともに発芽を促進したが，発芽の揃いは低濃度で優れた．アリルメルカプタンは，12月下旬処理では発芽を促したが，1月中旬処理では逆に抑制した．2硫化ジメチルの効果は処理の濃度，時期および時間によって異なった．すなわち，11月処理の場合，99%では24時間処理で発芽が著しく促進されたのに対し，12時間処理の効果は小さく，また30%では12時間，24時間処理ともに全く発芽しなかった．12月に10および75%の2硫化ジメチルで24時間気浴処理した場合，10%では発芽が促進されたが，75%では抑制された．以上より，ブドウの芽の休眠打破に有効なニラおよびラッキョウに含まれる揮発性物質は，それぞれメチルメルカプタンとアリルメルカプタンおよび2硫化ジメチルと推察されたが，休眠打破に有効な処理の時間や濃度については休眠の深さとの関連でさらに検討する必要があると思われた．