ELSEVIER

Journal of Chromatography A, 912 (2001) 127–134

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Monitoring of isothiocyanates emanating from *Arabidopsis thaliana* upon paraquat spraying

Joeri Vercammen[a], Hai Pham-Tuan[a], Isabelle Arickx[b], Dominique Van der Straeten[b], Pat Sandra[a,*]

[a]*Department of Organic Chemistry, Ghent University, Krijgslaan 281, S4, B-9000 Gent, Belgium*

[b]*Department of Genetics, Ghent University, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium*

Received 18 September 2000; received in revised form 10 January 2001; accepted 11 January 2001

Abstract

Arabidopsis thaliana plants were sprayed with the superoxide-generating herbicide paraquat. The headspace of sprayed plants was characterized by a number of compounds, which were absent in the headspace of untreated plants. They were identified as isothiocyanates (ITCs) with 4-methylthiobutyl isothiocyanate as main compound. After identification, a GC-system, based on PDMS sorption, was used to continuously monitor the ITC emissions. The specificity of isothiocyanate emission was also determined by subjecting the *Arabidopsis thaliana* plants to in vitro mechanical wounding. Again, 4-methylthiobutyl isothiocyanate was the main component, but the emission profile was completely different since the compound was emitted immediately, i.e., during wounding itself. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Oxidative stress; *Arabidopsis thaliana*; Dynamic headspace sampling; Isothiocyanates; Paraquat; Organosulfur compounds

1. Introduction

A common feature in a number of stress conditions for plants, such as drought, temperature extremes and salinity is the elevation of the amount of active oxygen species (AOS), i.e., free radicals and other derivatives of oxygen, present in the plant. Plants are able to enzymatically detoxify low amounts of these AOS, which are inevitable by-products of photosynthesis. However, when under oxidative stress, the increase in AOS concentration is far above that normally experienced by the plant. Owing to this its defense mechanisms are overwhelmed which causes DNA and protein damage and lipid peroxidation. Deadly amounts of AOS differ from species to species, which is reflected in the species-specific differences in tolerance to oxidative stress. In order to improve the oxidative stress tolerance of economically important crops, knowledge of defense strategies and genetic correlation is needed [1].

The model plant, used in the majority of oxidative stress research is *Arabidopsis thaliana*. Extensive genetic and ecological literature is available on this small cruciferous plant, studied in classical genetic work for over 50 years. The plant, together with cabbages and radishes, is a member of the mustard family (Brassicacceae). *Arabidopsis thaliana* is a

*Corresponding author. Tel.: +32-9-2644-462; fax: +32-9-2644-998.

E-mail address: pat.sandra@rug.ac.be (P. Sandra).

0021-9673/01/$ – see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(01)00558-1

harmless weed with no economic value. It is characterized by a short generation time (seed-to-seed) of only 6–12 weeks (depending on the conditions), self-fertility and abundant seed production. At the same time its small size permits growth of a large number of individuals in a minimum of space [2]. Oxidative damage is commonly inflicted by means of the superoxide radical-generating quaternary ammonium herbicide paraquat. Paraquat is a redox active compound that captures electrons from the photosystem of the plant forming a stable bipyridyl cation radical, which in a next stage reacts with oxygen. This re-oxidizes the radical cations to the original diquaternary ammonium salts while catalytically producing very reactive superoxide radical anions [3].

We examined the volatile response of *Arabidopsis thaliana* to paraquat-mediated oxidative stress. An automated system to monitor the volatile emissions from living organisms, which we recently developed in our laboratory, was applied [4,5].

2. Experimental

2.1. Plant material

The *Arabidopsis thaliana* Columbia (Col-2) ecotype was purchased from the Nottingham Arabidopsis Stock Center (NASC). Plants were cultivated in a greenhouse located at the Department of Genetics of Ghent University, Belgium. For the accumulation experiment, 20 plants were grown on rock wool, while the monitoring experiments were performed with soil-grown plants. Temperature in the greenhouse was held at 22°C with 70% relative humidity. Light intensity was 30 μmol/m^2 s with a photoperiod of 16 h. For all experiments 5-week-old plants were used. The monitoring experiments were repeated 5 times. Typical experiments are shown.

2.2. Chemicals

Paraquat (1,1-dimethyl-4,4-bipyridylium dichloride) was obtained from Sigma (Bornem, Belgium). A solution of paraquat in water (1 mg/ml) was used to spray the plants. Water was purified with a Milli-Q installation (Millipore, Bedford, MA, USA). N50 grade air was purchased from L'Air Liquide (Schelle, Belgium). 4-Methylthiobutyl isothiocyanate was synthesized from methyl-thiobutanol, which was converted under mild conditions into the corresponding amine using the method proposed by Mitsunobu et al. [6]. Reaction of this intermediate with thiophosgene produced the ITC.

2.3. Automated dynamic sampling unit

The dynamic sampling system (Fig. 1) is built around a Gerstel on-line TDS-G unit (Mülheim a/d Ruhr, Germany). This system basically consisted of two programmable temperature vaporization (PTV) injectors placed in series. The first injector (1) is the actual thermodesorption system (TDS) in which the sampling tube is placed. A standard glass desorption tube (177 mm L×4 mm I.D.×6 mm O.D.) filled with 100% polydimethylsiloxane (PDMS) particles (Ger-

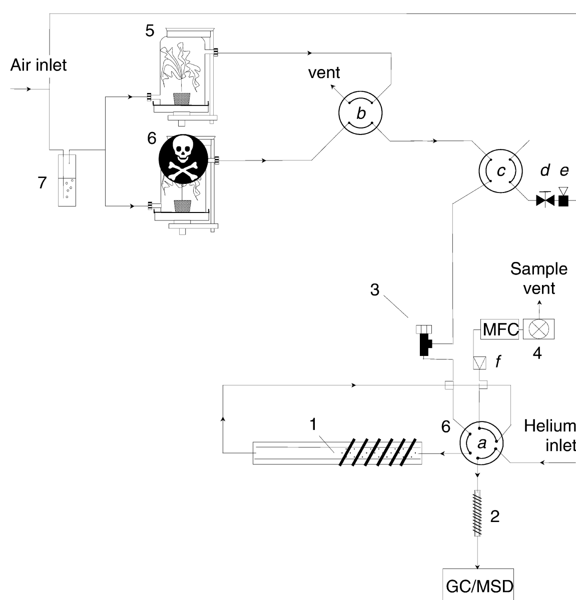

Fig. 1. Schematic drawing of the set-up for automated dynamic headspace sampling of living organisms. (1) Desorption tube filled with PDMS, (2) cryofocusing device, (3) heated T-piece placed in sampling line, (4) vacuum pump, (5) bulb with stressed plant, (6) bulb with stressed plant, (7) H_2O bubbler. Following valves control the sampling cycle: (a) six-port rotary valve for sorption/desorption, (b) four-port rotary valve for bulb-selection, (c) four-port rotary valve for purging, (d) open/close valve, (e) needle valve, (f) solenoid valve for purge gas vent (MFC, mass flow controller).

stel) was used as enrichment device. The metal plugs, which normally hold the material in place, were replaced by PDMS plugs, made by 3 cm×0.3 mm I.D. knotted Silastic® tubing (Dow Corning, Midland, MI, USA), to minimize activity. The second PTV is a CIS-4 (Gerstel) used for cryofocusing and fast injection of desorbed analytes (2). Automatic switching between on-line sampling and desorption is achieved by means of a six-port rotary valve (a), heated by a cartridge heater to 300°C, and located in-between both injectors. A sampling line with separately heated T-piece (3) and a vacuum pump (4) complete the system. All connections are made with 530-μm I.D. fused-silica coated metal tubing, which is held at 270°C. Full control and programming of the unit is achieved via the Gerstel Master Software.

Each sampling/desorption cycle starts with a 5-min TDS equilibration time at the TDS initial temperature. When completed the vacuum pump is activated, sucking the headspace through the sampling line over the packed bed. During the sampling period the TDS is held at its initial temperature. Four minutes before the end of this period the CIS-4 PTV injector is cooled down with liquid nitrogen. After completion of the sampling cycle the pump stops and the six-port valve is rotated into the desorption position. Consequently, the carrier gas is led over the bed in a direction opposite to the sampling flow. Here ends the sampling step and starts the thermodesorption step.

The TDS was connected, via a combination of valves (b,c), to two identical glass bulbs (20 cm height×15 cm I.D.). Both sample containers were placed over the target plants and tightly pressed onto separate glass plates (25×25 cm) by means of clamps [7]. One bulb served as reference unit (5), while in the other bulb plants were brought under stress (6). Valve b was used to achieve run-to-run switching between reference and target unit to correct for changes in external parameters during the course of analyses and was controlled by the GC instrument. Valve c, controlled by the Gerstel unit, permitted post-sampling purging to remove trapped moisture. The purge flow of dry N50 air was directed towards the tube by opening of valve e and was regulated using needle valve d. The flow left the system through valve f. Both four-port rotary valves b and c also guaranteed continuous flow of humidified N50 air (7) throughout the total monitoring process to avoid accumulation of volatiles. For more details about the total system we refer to Refs. [4,5].

The headspace was sampled for 20 min at 50 ml/min, while the PDMS-tube was held at 20°C. When sampling was completed, moisture was removed by flushing with dry N50 air for 5 min at 500 ml/min. Before heating the TDS, residual air was removed by flushing the system with carrier gas for 1 min at 100 ml/min. Afterwards the TDS was heated from 20°C to 225°C at 60°C/min, with a final time of 5 min. Compounds were swept towards the CIS-4 cryotrap, which was held at −150°C, at 100 ml/min. During desorption the GC split valve was open. Before injection, the system was held at −150°C for an additional 30 s to allow the flow inside the injector to stabilize after closure of the GC split valve. Finally, the CIS-4 was heated at 12°C/s to 350°C (5 min). A splitless time of 2.5 min was used in all experiments.

2.4. Capillary GC–MS

The TDS-G was mounted on top of an HP 6890 GC (Agilent Technologies, Little Falls, DE, USA) connected to an HP 5973 MSD, which was equipped with a Wiley database. Full scan spectra were taken between 50 and 250 amu at 7 scans/s for compound identification. During the monitoring experiments the MSD was used in the SIM-mode (m/z 72) to achieve highest sensitivity. In all experiments an HP-5MS capillary column (30 m L×250 μm I.D.×0.25 μm d_f) was used. The oven was programmed from 35 to 280°C at 10°C/min with initial and final time being 5 min.

3. Results and discussion

3.1. Identification of stress-related volatiles

Stress-related volatiles were determined by comparing the headspace profile of the target plant before and after spraying with paraquat after overnight accumulation. The experiment was carried out by placing 20 *Arabidopsis thaliana* plants, grown on

rock wool, underneath the airtight glass bulb. The paraquat solution was sprayed onto the plants until all leafs were covered with very small droplets. This corresponds to ca. 1 mg paraquat per plant. In order to accelerate the effect of the paraquat action, four TL-lamps were closely positioned towards the bulb. The same plants were used for the blank as well as for the paraquat experiment. Fig. 2 represents a selected part of the headspace profiles obtained after overnight accumulation of the plants without (A) and with paraquat spraying (B). One main peak appeared after the oxidative stress treatment. The mass spectrum of this compound is also given in Fig. 2.

Screening the Wiley database on this spectrum, indicated *n*-butyl and isobutyl ITC as prime hits. The poor qualification between library and compound spectra of only 45 and 30%, respectively, necessitated, however, a more precise identification. The most straightforward method to achieve this is to inject a standard solution into the system without changing any of the chromatographic parameters. This was done for *n*-butyl ITC, which was injected into the sampling line through a separately heated T-piece (250°C) placed on top of the TDS (3 in Fig. 1). The significant retention time mismatch between *n*-butyl and thus also isobutyl ITC, and the unknown component eliminated as a result both database suggestions. The general view of the unknown spectrum and especially the presence of the peak at *m*/*z* 72 still suggested the presence of an ITC functionality.

Many members of the Brassicacceae family are characterized by the presence of so-called glucosinolates in their tissues [8]. Glucosinolates have been detected in all organs of the plant and are located within the vacuole of the cell. The hydrolysis of glucosinolates is catalyzed by endogenous β-thioglucosidases, the myrosinases, which are localized in the "myrosin" cells scattered throughout most plant tissues. Within these cells the enzyme is stored inside myrosin grains. When tissues are disrupted substrate and enzyme are associated, hydrolyzing the glucosinolates and liberating their unstable aglycones, which rearrange to yield a variety of volatile catabolites, such as ITCs, thiocyanates (TCs), nitriles (CNs), oxazolidinethiones (OZTs) and epithionitriles (ETCNs) [9,10]. Glucosinolates themselves are rather inactive in fungal bioassays, but their volatile products on the contrary are characterized by a wide-ranging biological activity, including interactions with fungal and microbial pathogens and insects [11]. Especially the ITCs are highly active, suggesting a function in plant defense [12,13]. With the help of published data it was rather simple to correlate the unknown spectrum with 4-

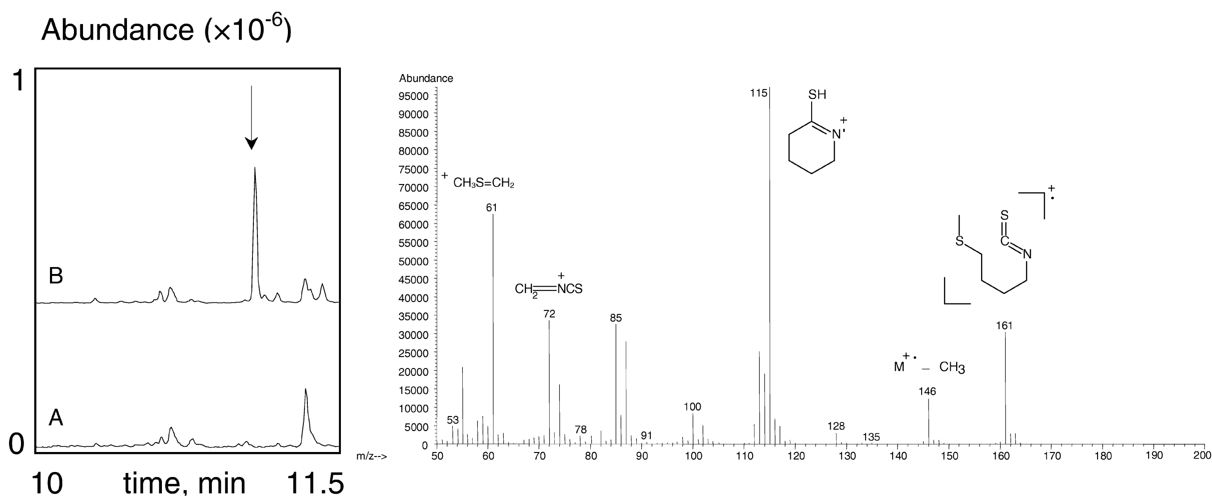

Fig. 2. Relevant parts of full scan chromatograms of 20 *Arabidopsis thaliana* plants after overnight accumulation. (A) Blank experiment and (B) paraquat experiment. The mass spectrum of the stress-related emittant, in a later stage identified as 4-methylthiobutyl ITC, is also given.

methylthiobutyl ITC [14]. Identification became totally positive after analysis of synthesized 4-methylthiobutyl ITC.

Since the presence of other glucosinolates and volatile aglycones is well documented the TIC after paraquat spraying was extracted at different m/z ions to determine the presence of other aglycones. Only the extraction of ion m/z 72 produced a chromatogram with several abundant peaks. Extraction of other ions, characteristic for TCs, CNs, OZTs and ETCNs, produced no detectable peaks with exception of benzyl cyanide [15]. The extracted ion chromatograms (m/z 72) of both the blank and the paraquat experiment are depicted in Fig. 3. Compounds were identified with help of the fragmentation patterns taken from Ref. [14] and are listed in Table 1.

The presence of ethyl ITC and, to a lesser extent, benzyl cyanide in the blank experiment (Fig. 3A, compounds 1 and 2) was possibly a consequence of the normal ageing process of the plant, which involves cell destruction. The absence of hydrolysis products other than ITCs demonstrated the importance of using near real-life sampling methods like dynamic headspace sampling.

Other glucosinolate degradation products, especially CNs and TCs, were in the past only detected owing to the experimental hydrolysis conditions, such as pH and temperature [9].

Table 1
Compounds identified in the headspace of *A. thaliana* after overnight accumulation

No.	Compound
1	Ethyl ITC
2	Benzyl CN
3	Pentyl ITC
4	Methylpentyl ITC
5	Hexyl ITC
6	Heptyl ITC
7	3-Methylthiopropyl ITC
8	4-Methylthiobutyl ITC
9	Phenylethyl ITC
*	Cyclic siloxane (PDMS)

3.2. Continuous monitoring of ITC-emissions

To increase the reliability and physiological relevance of the acquired results the oxidative treatment was repeated under continuous flow conditions. Dynamic headspace collection of volatile glucosinolate catabolites was recently performed by Doughty

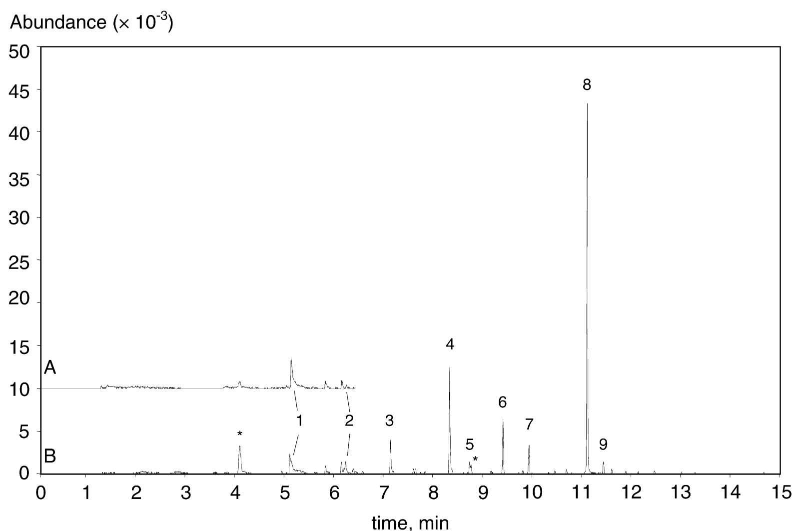

Fig. 3. Extracted ion chromatograms (m/z 72) of the TICs acquired after (A) blank accumulation experiment (no peaks detected after 6.5 min) and (B) paraquat accumulation experiment. Compounds are enlisted in Table 1.

et al. [16]. The use of adsorptive enrichment in combination with long sampling times (64 h at 700 ml/min) renders, however, their results questionable. For reasons stated elsewhere, sorption with PDMS is far more favorable compared to enrichment onto an adsorbent [7,17]. In the monitoring experiments eight soil-grown plants were randomly split into two separate groups. One group was sprayed with the paraquat solution (ca. 1 mg per plant) and both groups were separately enclosed in cleaned bulbs immediately upon arrival in the laboratory. Both bulbs were directly fed with a flow of 50 ml/min of humidified N50 air. Continuity of this flow before, during and after sampling was guaranteed by the incorporation of two four-port rotary valves. After enrichment of the headspace of one bulb (20 min at 50 ml/min) the PDMS sampling tube was flushed with 2.5 l of dry N50 air (5 min at 500 ml/min). This volume was high enough for complete removal of sorbed moisture, which was indispensable to avoid water introduction into the analytical system. Interest was primarily focused on the qualitative emission of the 4-methylthiobutyl ITC, which was the main contributor in total ITC content of *Arabidopsis thaliana* in the accumulation experiments. The compound was characterized by a retention slightly higher than *n*-tetradecane, which produced a theoretical breakthrough volume of 150 l at 500 ml/min. This breakthrough volume was used as safe indication of the experimental volume [5]. The response profiles of 4-methylthiobutyl ITC as function of time for unsprayed and sprayed *Arabidopsis thaliana*, analyzed consecutively, are given in Fig. 4.

This experiment was repeated 5 times to determine the reproducibility in emission start and end, general emission profile and maximal emitted amount. The timing of maximum emission was quite consistent in all experiments and appeared around day 2, i.e., 36 h after spraying. The long emission period of approximately 15 h, suggests a slow oxidative-driven digestion of plant interior. In order to determine the amount of 4-methylthiobutyl ITC, enriched after each analysis, liquid standards were prepared containing the compound in concentrations ranging from 200 to 2 ppb in *n*-hexane. Gaseous standards should be the first choice since in this case maximum agreement between sampling and quantitation experi-

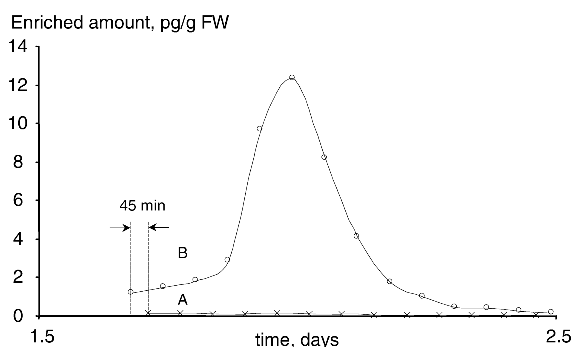

Fig. 4. Response profiles of four *Arabidopsis thaliana* plants (SIM at m/z 72 for 4-methylthiobutyl ITC). (A) untreated reference plants and (B) paraquat-sprayed plants. Plants were sprayed at 12.00 h (=0.5 days) (FW, fresh weight).

ments is achieved. Liquid standards, however, are more easily prepared and reproducibly injected. It has, nevertheless, to be kept in mind that accurate quantitation is only possible when solutions are cold injected. If not, syringe discrimination, producing inaccurate results, is unavoidable.

For this reason the heated T-piece was not used, but injections were made at the front end of the PDMS-tube, directly onto the Silastic® plug. After injection, the tube was sampled for 2 min at 100 ml/min and purged for 5 min at 500 ml/min. The reproducibility of injection for six repeated analyses was less than 3% RSD. The attained calibration graph was used to get an idea of enriched emittant concentration. Detected amounts are expressed as pg/g of fresh weight (FW) to correct for differences in total quantity of plant material, and varied from 10 to 20 pg. FW of intact plants was determined directly after the monitoring experiment was finished. Besides 4-methylthiobutyl ITC also other components were detected, but as expected from the accumulation experiment, in much smaller amounts. Other compounds were methylpentyl ITC, heptyl ITC and 3-methylthiopropyl ITC and showed an analogous emission profile, regarding emission start and end, as 4-methylthiobutyl ITC.

In a next series of experiments the ITC-emission from *Arabidopsis thaliana* after in vitro wounding, was determined. Therefore, four plants were placed inside a glass bulb, which was equipped with an extra glass screwthread connection (Sovirel no. 42), sealed by means of a plastic nut and thick PTFE-

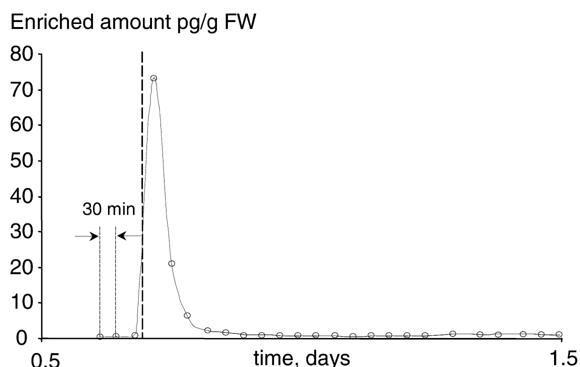

Fig. 5. Response profile of four in vitro wounded *A. thaliana* plants by means of endoscopic pliers (SIM at m/z 72 for 4-methylthiobutyl ITC). Vertical line denotes the time of wound infliction.

lined septum. After a stabilization period of 4 h the thick septum was pierced with long endoscopic pliers. Each leaf (approx. 10 in total) was pressed twice in between the pliers without actually destructing the leaves. ITCs were in this experiment also positively detected as depicted in the emission of 4-methylthiobutyl ITC shown in Fig. 5. The major difference compared with the paraquat experiment is the instant emission of the compound already during the wounding period itself. The subsequent analysis (only 30 min later) was characterized by a steep decrease in response of ca. 60%. This experiment was repeated 3 times on 5-week-old plants within 6 months, without any difference in general 4-methylthiobutyl ITC emission profile.

4. Conclusion

Paraquat-mediated oxidative treatment of *Arabidopsis thaliana* initiated the accumulation of a number of isothiocyanates in the headspace, with as main compound 4-methylthiobutyl isothiocyanate. Since accumulation of compounds is not the proper method to obtain physiologically relevant results, continuous monitoring experiments, with constant headspace refreshment, were initiated. The response profile as function of time for 4-methylthiobutyl isothiocyanate reached a maximum 36 h after paraquat spraying. In order to determine the specificity of isothiocyanate emission *Arabidopsis thaliana* was also subjected to in vitro mechanical wounding. Again, 4-methylthiobutyl isothiocyanate was the main component but the emission started immediately, i.e., during wounding itself. This contribution mainly emphasizes the analytical set-up. In depth studies on the quantitative aspects of ITC emission under different stress conditions are presently carried out.

Acknowledgements

We thank Ghent University for supporting this work through grant GOA 12051898. JV gratefully acknowledges the Flemish Institute for the Promotion of Scientific and Technological Research in the Industry (IWT), Flanders, Belgium for a study grant. DVDS is a research director and IA is a research assistant of the Fund for Scientific Research, Flanders. We also thank W. Van Caeneghem for technical support and J. Goeman for synthesizing 4-methylthiobutyl ITC.

References

[1] P. Bolwell, Curr. Opin. Plant Biol. 2 (1999) 287.
[2] M. Van Lijsebettens, N. Terryn, M. Van Montagu, in: T.E. Creighton (Ed.), Encyclopedia of Molecular Biology, Wiley, New York, 1999, p. 190.
[3] A. Calderbank, P. Slade, in: P.C. Kearney, D.D. Kaufman (Eds.), Herbicides. Chemistry, Degradation and Mode of Action, Marcel Dekker, New York, 1976, p. 501.
[4] H. Pham-Tuan, J. Vercammen, C. Devos, P. Sandra, J. Chromatogr. A 868 (2000) 249.
[5] J. Vercammen, H. Pham-Tuan, P. Sandra, in: P. Sandra, A.J. Rackstraw (Eds.), CD Rom (paper D41) of the 23rd International Symposium on Capillary Chromatography, Riva del Garda, June 2000, I.O.P.M.S. vzw, Kortrijk, Belgium.
[6] O. Mitsunobu, M. Wada, T. Sano, J. Am. Chem. Soc. 94 (1972) 679.
[7] J. Vercammen, E. Baltussen, T. Sandra, F. David, P. Sandra, J. High Resolut. Chromatogr. 23 (2000) 547.
[8] B.A. Halkier, L. Du, Trends Plant Sci. 2 (1997) 425.
[9] R.A. Cole, Phytochemistry 15 (1975) 759.
[10] A.J. Duncan, in: J.P.F. D'Mello, C.M. Duffus, J.H. Duffus (Eds.), Toxic Substances in Crop Plants, Royal Society of Chemistry, Cambridge, 1991, p. 126.
[11] L. Buchwaldt, J.K. Nielsen, H. Sørensen, in: H. Sørensen (Ed.), Advances in the Production and Utilization of Cruciferous Crops, M. Nijhoff & W. Jung, Dordrecht, 1985, p. 260.

[12] J.F. Angus, P.A. Gardner, J.A. Kirkegaard, J.M. Desmarchelier, Plant Soil 162 (1994) 107.
[13] U. Smolinska, G.R. Knudsen, M.J. Morra, V. Borek, Plant Dis. 81 (1997) 288.
[14] A. Kjær, Acta Chem. Scand. 17 (1963) 2143.
[15] G.F. Spencer, M.E. Daxenbichler, J. Sci. Food Agric. 31 (1980) 359.
[16] K.J. Doughty, M.M. Blight, C.H. Bock, J.K. Fieldsend, J.A. Pickett, Phytochemistry 43 (1996) 371.
[17] E. Baltussen, F. David, P. Sandra, H.-G. Janssen, C.A. Cramers, J. Chromatogr. A 864 (1999) 345.