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Short communication

Cell death associated release of volatile organic sulphur compounds with antioxidant properties in chemical-challenged tobacco BY-2 suspension cultured cells

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ABSTRACT

The production of volatile organic compounds (VOCs) during programmed cell death (PCD) is still insufficiently studied and their implication in the process is not well understood. The present study demonstrates that the release of VOSCs with presumed antioxidant capacity (methanethiol, dimethylsulfide and dimethyldisulfide) accompanies the cell death in chemical-stressed tobacco BY-2 suspension cultured cells. The cells were exposed to cell death inducers of biotic nature mastoparan (MP, wasp venom) and camptothecin (CPT, alkaloid), and to the abiotic stress agent $CdSO_4$. The VOCs emission was monitored by proton-transfer reaction mass spectrometry (PTR-MS). The three chemicals induced PCD expressing apoptotic-like phenotype. The identified VOSCs were emitted in response to MP and CPT but not in presence of Cd. The VOSCs production occurred within few hours after the administration of the elicitors, peaked up when 20–50 % of the cells were dead and further levelled off with cell death advancement. This suggests that VOSCs with antioxidant activity may contribute to alleviation of cell death-associated oxidative stress at medium severity of cell death in response to the stress factors of biotic origin. The findings provide novel information about cell death defence mechanisms in chemical-challenged BY-2 cells and show that PCD related VOSCs synthesis depends on the type of inducer.

1. Introduction

Volatile organic compounds (VOCs) represent a diversity of bio generated low molecular mass metabolites with high vapor pressure. They are products of normal physiological processes and play role in sensing of the environment, contribute to odor, serve as infochemicals at cell, tissue and organ level and in plant-to-plant communication, and are involved in defence against pathogens, insects and herbivorous (Pichersky et al., 2006; Baldwin, 2010; Holopainen and Gershenzon, 2010; Jürgens and Viljoen, 2010; Scala et al., 2013; Jesus et al., 2016; Erb, 2018). Some volatile organic sulphur compounds (VOSCs) are ascribed to possess antioxidant capacity (Karsten et al., 1996; Bentley and Chasteen, 2004). Among them dimethylsulfoniopropionate (DMSP), dimethylsulfide (DMS), dimethyldisulfide (DMDS) and methanethiol (MT) are suggested to effectively scavenge reactive oxygen species (ROS) and under conditions of severe physiological stress to potentiate the survival rate of algal and other plant cells (Sunda et al., 2002). The formation of these VOCs is interconnected. Methanethiol is obtained from DMSP by demethylation/demethiolation; DMDS is an oxidation product of MT. Basically DMS is formed through the cleavage of DMSP

Abbreviations: CPT, camptothecin; CLSM, Confocal laser scanning microscopy; DMS, dimethyl sulfide; DMDS, dimethyldisulfide; GLVs, green leaf volatiles; MP, mastoparan; MT, methanethiol; PCD, programmed cell death; PLD, phospholipase D; ROS, reactive oxygen species; VOCs, volatile organic compounds; VOSCs, volatile organic sulphur compounds

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catalyzed by DMSP-lyases, an enzyme family widely distributed in bacteria, algae and some plants. DMS can be also a product of MT transformation through a simple methyl transfer reaction (Stefels, 2000; Bentley and Chasteen, 2004; Bullock et al., 2017; Lei et al., 2018). Sunda et al. (2002) and Franklin et al. (2010) suggested that the enzymatic cleavage of DMSP may be a mechanism to generate ROS-scavenging antioxidants and at stress conditions may operate as antioxidant system supplementary to the general antioxidant enzymes. The green leaf volatiles (GLVs), a class of VOCs are other molecules with defense and potential antioxidant properties produced at stress impacts of biotic and abiotic origin (Zhang et al., 2008; Piesik et al., 2011; Scala et al., 2013; Matsui and Koeduka, 2016; Nawrocka et al., 2018; Shi et al., 2018).

The model systems of cell and tissue cultures can be useful tools to study VOCs production. For example, in tobacco BY-2 suspension cultured cells. Nagashima et al. (2019) found that VOCs from the class of sesquiterpenes can induce the expression of stress-responsive genes (such as genes encoding for proteins in jasmonic acid signaling) and VOCs involved in the communication between neighboring plants at herbivore attack. In cell culture of white cedar (*Cupressus lusitanica*), treated with yeast extract, De Alwis et al. (2009) detected volatile monoterpenes with antioxidant ability. In suspension cultured rice cells challenged with cell wall hydrolysate of the fungus *Magnaporthe oryzae*, a causal agent of rice blast, the released GLVs 1-octanol and 1-decanol exerted fungistatic effects (Forlani et al., 2011). Hexanal isomers have been produced from *in vitro* cultured apple (*Malus pumila* var. *domestica*) microplants in response to infection with the bacterium *Erwinia amylovora* (a causal agent of fire blight) whereby the growth and migration of bacterium and the damage of the plants have been suppressed (Cellini et al., 2018).

Nevertheless the large amount of information on plant programmed cell death (PCD), the possible involvement of VOCs with antioxidant properties in the regulation of PCD associated oxidative stress remains still elusive. The PCD is evolutionary conserved genetically coded process of cellular suicide that is a part of normal development and occurs in response to stressful environmental signals. It is characterized by specific morphological, biochemical and molecular determinants (van Doorn et al., 2011). Although the suspension cultured cells derived from various higher plants are efficient experimental models for elucidating the mechanisms of PCD (Cimini et al., 2018), PCD related VOCs formation is reported mainly in few works with algae. Zuo et al. (2012, 2015) established that acetic acid-treated suspension cells of unicellular alga *Chlamidomonas reinhardtii* undergo PCD accompanied with increased activity of caspase-3-like protease (a hallmark of PCD) and ROS accumulation. The process was associated with quickly occurring emission of oxygenated VOCs (aldehydes, ketones and esters). The level of ROS that were not scavenged by the antioxidant enzyme system (involving superoxide dismutase, peroxidase and catalase) decreased concomitant with the elevation of the amount of VOCs. The authors suggested that ROS might be scavenged by VOCs, indicative of a role of volatiles in the alleviation of oxidative stress during PCD. A release of VOSCs with antioxidant properties (DMS and MT; unpublished data), ROS accumulation, emission of the hormone ethylene (a VOC) together with enhanced activity of caspase-1-like protease and DNA fragmentation (PCD markers) were observed in *C. reinhardtii* expressing PCD phenotype at treatment with the wasp venom mastoparan (MP) (Yordanova et al., 2010, 2013).

In our previous research and by other authors the chemicals MP, an activator of G-proteins and downstream lipid signaling (Higashijima et al., 1988), camptothecin (CPT), alkaloid from the tree *Camptotheca acuminata*, an inhibitor of DNA repairing enzyme topoisomerase 1 and PCD inducer in tumor cells (Li et al., 2017), and the heavy metal cadmium (Cd) were established as potent PCD and oxidative stress inducers in tomato and tobacco suspension cultured cells (de Jong et al., 2000; Fojtova et al., 2002; Kuthanova et al., 2004; Yakimova et al., 2006; Iakimova et al., 2008, 2013, 2019; Jiang et al., 2019). The mentioned findings suggested a cross-talk between ROS, ethylene and lipid-derived signals toward cell death execution at MP, CPT and Cd toxicity. The present study was motivated by the intriguing question whether the toxic chemical agents MP, CPT and Cd may induce PCD associated production of VOCs in tobacco BY-2 suspension cultured cells The results suggest involvement of VOSCs with presumed antioxidant properties in the alleviation of cell death response to the elicitors of biotic origin.

2. Material and methods

2.1. Cell culture

Tobacco (*Nicotiana tabacum* L.) suspension cells, line Bright Yellow -2 (BY-2) were cultured in medium based on the protocol of Nagata and Kumagai (1999), essentially as described in Iakimova et al. (2019). The suspension was kept in sterile closed 100 mL flasks on a horizontal rotary shaker (120 rpm) at 25 °C, in the dark, and sub cultured every 5 days by 4:100 dilution. Cell density was maintained at 5×10^4 cells mL^{-1}.

2.2. Chemical treatments

Three days after subculture, 5 mL of the suspension cells were subjected to treatments with MP, CPT and $CdSO_4$. The chemicals were tested in concentrations of 1, 5 and 10 μM MP and CPT and 50, 100 μM and 1 mM $CdSO_4$. In the present work, only the effects on cell death of 5 μM MP, 5 μM CPT and 100 μM $CdSO_4$ that exerted the most pronounced effect on VOCs production are shown.

2.3. Cell death evaluation

The cell death was determined at regular intervals in time period of 56 h and is presented as percentage of dead cells from the total number of cells counted in 3 randomly chosen non-overlapping microscope fields, each containing at least 100 cells, as described in Iakimova et al. (2019). The living cells were distinguished as positive to fluorescein diacetate (FDA; a dye permeable in the vital cells and, when cleaved by esterases, producing green fluorescence emitting fluorescein detectable at 495/520 nm excitation/emission wavelength). The dead cells were detected by staining with propidium iodide (PI; permeable in the dead cells only and emitting red fluorescence at excitation/emission 530/625 nm wavelength). Additionally the dead cells were identified by features of PCD such as protoplast shrinkage and separation from cell wall, and nucleus condensation. Cellular morphology was identified by using confocal laser scanning microscopy (CLSM) system (Leica-Microsystems GmbH, Mannheim, Germany) mounted on an inverted Leica DM IRE2 microscope and by using the Leica Confocal software for imaging as described in Yordanova et al. (2013).

2.4. PTR-MS analysis

The profile of VOCs released from chemical-treated cells was monitored by headspace PTR-MS analysis. Sterile medium and untreated suspension were used for comparison. The PTR-MS technique allows online detection of trace amounts of compounds with proton affinity higher than water with a time resolution of 1 s per compound. The analysis relies on chemical ionization, which is a soft ionization method with few or no ion fragments in the mass spectra. Primary H_3O^+ ions are produced in a discharge in water vapor and helium (carrier) gas and the trace gases emitted from the sample are ionized by proton-transfer reactions with H_3O + ions (Steeghs et al., 2007a). Briefly, as previously described also by Farneti et al. (2012), the PTR-MS consisted of an ion source in which H_3O^+ ions are produced, a drift tube for trace gases ionization by PTR with H_3O^+ ions, a collision dissociation chamber, a quadruple mass filter, and a secondary electron multiplier. The drift

tube had a pressure of 2.08 mbar and was heated at about 55 °C. The mass number of detected ions is given by the molecular mass of the substance plus the mass of the single proton mH (mRH + in atomic mass units, amu). Mass spectrometric data were collected over a mass range of *m/z* (mass/charge) from approximately 20–140. Tightly closed glass cuvettes (30 mL) containing 10 mL of BY-2 cell suspension were used for each tested chemical. The cuvettes were continuously flushed with clean air. Before each measurement the flow was stopped for 20 min allowing accumulation of compounds in the headspace, thereafter cuvettes were flushed again. Five µM MP, 5 µM CPT or 100 µM $CdSO_4$ were injected into the samples after 20 h stabilization of the cultures. To avoid possible cross contamination from one measurement to the next, between the consecutive measurements the system was flushed with clean air. In preliminary experiments with the PTR-MS in scan mode, it was observed that only a limited number of masses showed appreciable changes in abundance following chemical treatment of the cells. In subsequent experiments, the PTR-MS was set in monitoring mode to study the dynamics of only the *m/z* channels of these more abundant masses. Presented values are average of 4 independent sets of experiments with one cuvette per treatment.

2.5. Chemicals

Mastoparan and $CdSO_4$ were dissolved in distilled water. Stock solution of CPT was prepared in DMSO and the chemical was applied in 0.1 % v/v final solvent concentration in the cell culture; FDA was dissolved in 70 % (v/v) acetone (0.002 % final concentration). In the final concentrations DMSO and acetone were tested alone and did not affect the cell viability. All chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

2.6. Statistical analysis

Data were processed by Student's t test, one-way analysis of variance (ANOVA) at probability level $p \leq 0.05$ (IBM SPSS Statistics). The presented values for cell death are means with standard error ($SE_{(n-1)}$) of one sample per treatment in each of 4 independent experiments. In the graph the significance of the differences is indicated with letters. For PTR-MS data the value of LSD at $\rho \leq 0.05$ in the figure caption is shown and additionally the differences at specific time points are indicated with different letters.

3. Results and discussion

3.1. Cell death induction and morphological characterization

The stress agents MP, CPT and $CdSO_4$ were selected on the base of their previously established potency to induce cell death in tomato and tobacco cell cultures (refer to division 1). The treatments with any of the tested compounds led to cell death, progressing up to 100 % within about 56 h with similar cell death dynamics. Overall, cell death magnitude in response to the three chemicals was between 15–35 % after 14 h and between 30 and 55 % after 28 h (Fig. 1).

Microscopic observations revealed that the dead cells expressed morphological features of apoptotic-like PCD. The living cells appeared FDA positive with intact (FDA negative) vacuole and diffuse nucleus (Fig. 2 A); the dead cells were with PI positive condensed nucleus (Fig. 2 B) and shrunken protoplast retracted from cell wall (Fig. 2 C). Dead cells showing these PCD features were abundantly found in all treated cultures irrespective the inducing chemical.

3.2. Stress-induced VOCs production

To study whether the cell death response of BY-2 cells to MP, CPT and Cd is associated with production of VOCs, PTR-MS analysis of headspace composition was employed. In preliminary experiments it

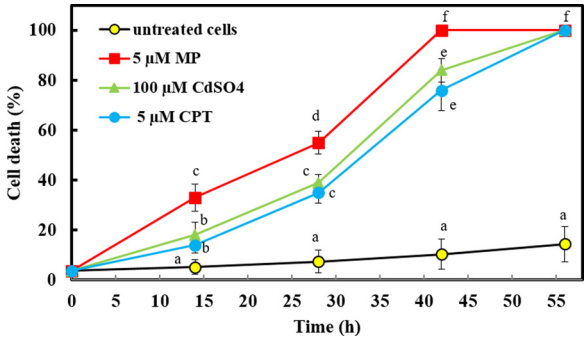

Fig. 1. Dynamics of cell death in tobacco BY-2 suspension cultured cells exposed to the chemical stressors MP, $CdSO_4$ and CPT. Error bars show $SE_{(n-1)}$. Data indicated with different letters are statistically different.

was observed that only ion masses *m/z* 49, *m/z* 63, and *m/z* 95 showed appreciable changes in abundance after the treatments of the cells with MP and CPT. Minor signals, just above noise level, were detected at masses 65, 67, 77, 79, 81 and 97. These latter masses could not directly be linked to specific volatile compounds. No significant changes in any of these signals were observed from Cd-treated suspension. In subsequent experiments only the *m/z* channels 49, 63 and 95 were monitored allowing for more frequent sampling. Three major VOSCs were identified: MT (CH_4S; *m/z* 49), DMS ($(CH_3)_2S$; *m/z* 63) and DMDS ($C_2H_6S_2$; *m/z* 95).

Under the conditions of our experiments, signals expected to correlate with other antioxidant VOCs such as monoterpenes (*e.g.* ion mass *m/z* 137) or hexenals (*m/z* 99) (Steeghs et al., 2007b; Misztal et al., 2012) were not detected although they have been documented in other cases of PCD in suspension cultured cells. An example for PCD related release in suspension cells is the work of Liu et al. (2015) which reports that oxidative stress induced by treatment with H_2O_2 of the suspension cultured cells of the medicinal plant *Aquilaria sinensis* has induced PCD (caspase 1- and caspase-3-like activity, gene expression of metacaspases and release of cytochrome c), associated with gene expression and emission of volatile sesquiterpenes. As another example, inoculation of grapevine (*Vitis venifera*) cultured suspension cells with oomycete *Plasmopara viticola,* the causal agent of downy mildew, that induces HR-like cell death response has been shown to stimulate the synthesis of volatile mono- and sesquiterpene in the resistant genotype (Algarra Alarcon et al., 2015).

An increase of VOSCs was observed starting 2.5–10 h after exposure of the cells to MP or CPT. In non-treated cells the masses corresponding to MT, DMS and DMDS were negligible throughout the experiment. In MP-treated cells VOSCs were more abundant than in CPT-treated cells. After approximately 28 h in MP-treated cells MT reached the highest concentration of approximately 0.5 mg m^{-3}; in CPT-treated cells a concentration of approximately 0.35 mg m^{-3} was measured 15 h post-treatment (Fig. 3 A); DMS reached a maximum of over 7.0 mg m^{-3} after 28 h in MP-treated cells and over 4.0 mg m^{-3} after 18 h in CPT-treated cells (Fig. 3 B); DMDS peaked up to 0.9 mg m^{-3} 10 h after MP and up to 0.7 mg m^{-3} 18 h post CPT treatment. A second peak of DMDS emanation was observed about 28 h post-treatment with MP (Fig. 3 C). Following the peaks, in the remaining time span of the experiments the concentrations of volatiles in the headspace levelled off or descended but never to the basic levels (Fig. 3). The trend of accumulation of the volatiles paralleled the dynamics of cell death progression.

Recently we have established that in tobacco BY-2 cells substances with ROS scavenging ability could effectively abolish MP-induced cell death (Iakimova et al., 2019). This indicates that ROS production is an important intermediate in cell death in this system. In the current trial the maximum of VOSCs emission coincided with the medium severity of cell death post MP and CPT treatments. Similarly, in experiments with MP-treated *C. reinhardtii*, DMS and MT production coincided with cell

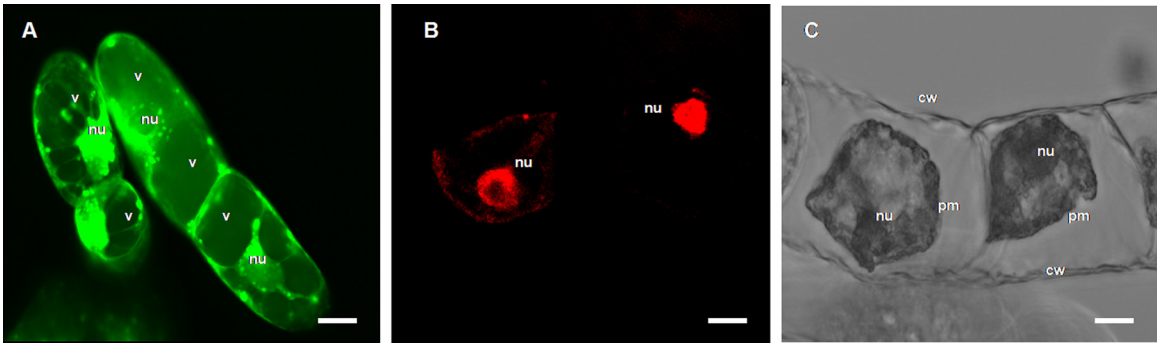

Fig. 2. Representative CLSM images of living and dead tobacco BY-2 suspension cultured cells.
(A) Living FDA positive cells with diffuse nucleus and intact vacuole. (B) Dead cells with PI positive nucleus. (C) Dead cells with compact nucleus, shrunken protoplast and plasma membrane retracted from cell wall. Scale bars 25 μm. Cw, cell wall; nu, nucleus; pm, plasma membrane; v, vacuole.

death of intermediate severity and remained abundant for the duration of the experiment (unpublished data). Bearing in mind the potential antioxidant capacity of identified VOSCs, the results suggest that in response to applied biotic cell death inducers, at relatively medium long duration of the stress, VOSCs may contribute to cell death mitigation by operating as antioxidant factors. With the advancement of stress impact they might be insufficient to cope with the oxidative stress. In addition to their potential antioxidant capacity these molecules may also serve as cell death warning messengers sent from the dying to the living cells in the population, as suggested for their role in MP- and acetic acid-treated *C. reinhardtii* (Zuo et al., 2012; Yordanova et al., 2013). Production of DMS, MT and DMSP is documented in various algal systems, *e.g.* the diatom *Thalassiosira pseudonana*, marine coccolithophore *Emiliania huxleyi* (a phytoplankton), the green alga *Ulva lactuca* (sea lettuce) and in antarctic green algae of *Ulothrix* spp. experiencing stressful physiological conditions or in presence of stress factors of abiotic nature that can stimulate excessive ROS generation (Rijssel and Gieskes, 2002; Sunda et al., 2002; Ross and Van Alstyne, 2007; Loreto and Schnitzler, 2010; Kameyama et al., 2011; Kerrison et al., 2012). Our data suggest that these VOSCs may play a role in the regulation of the cell death associated oxidative state. To test a possible function of DMS in cell death, experiments involving a pharmacological approach with an inhibitor of DMSP lyase were undertaken. DMSP lyase belongs to a family of metalloenzymes and the metal chelator EDTA is reported to partially inhibit its activity *in vitro* and thereby the formation of DMS (Nishiguchi et al., 1995; Mohapatra et al., 2013; Brummett et al., 2015). Addition of 10–100 μM EDTA to MP- and CPT- treated tobacco BY-2 cells resulted in approximately 20–30 % increase of cell death whereas EDTA alone did not induce such an effect (Supplemental Fig. S1). Assuming a partial blockage of DMSP lyase activity by the chelator, these data suggest that a lowered production of DMS may increase the cell death and indicates that DMS may at least potentially participate in alleviation of cell death through its presumed antioxidant capacity. Final proof should come from experiments where the headspace of chemical-treated cell cultures

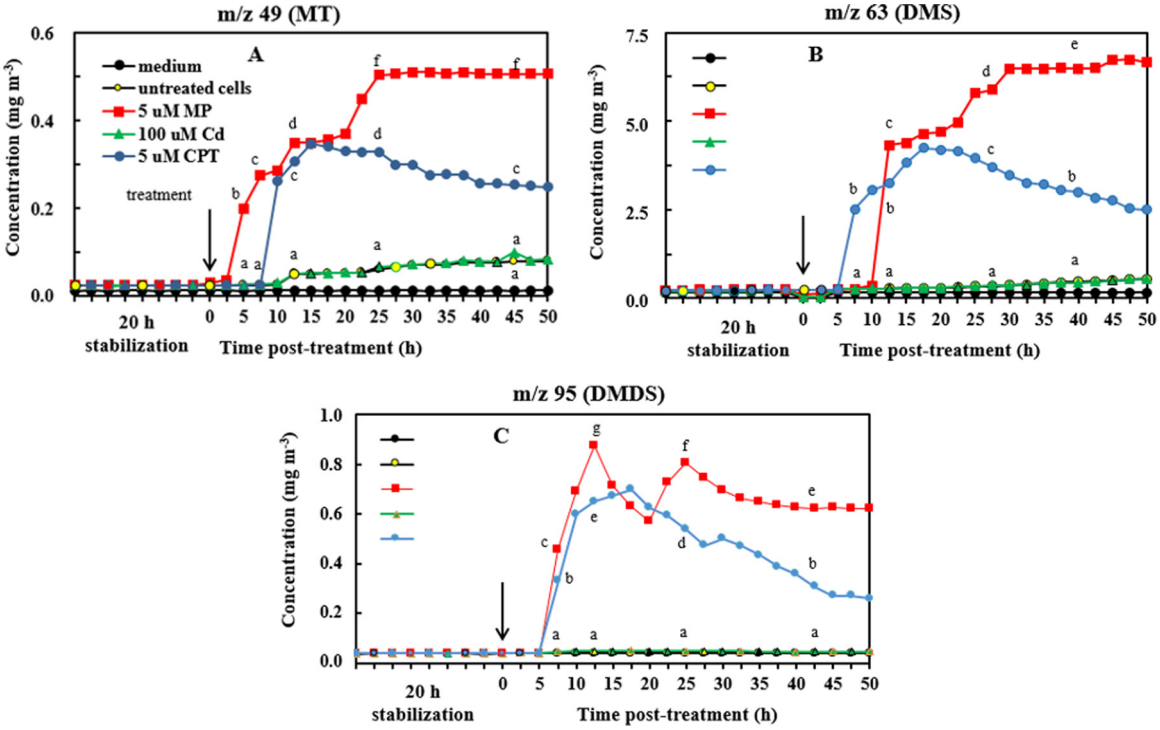

Fig. 3. Dynamics of VOSCs production in MP-, CPT- and $CdSO_4$-treated tobacco BY-2 suspension cultured cells.
Methanethiol, MT (A), Dimethylsulfide, DMS (B), Dimethyldisulphide, DMDS (C). Time period for cell suspension stabilization before treatments was 20 h. Time point 0 applies to the start of the chemical treatment and is indicated with an arrow. The legend in (A) applies also to (B) and (C). LSD values at $\rho \leq 0.05$ for data in panels A, B and C are 0.017, 0.044 and 0.13, respectively. Different letters indicate significant differences between treatments at selected time points.

be spiked with appropriate amounts of the identified VOSCs.

Contribution of GLVs in relation to retardation of senescence (a form of PCD) has been documented, for example, in accordance with delayed senescence of postharvest apple and mango fruit (Corbo et al., 2000; Jincy et al., 2017). Some VOCs may play a role in hypersensitive response (HR; a form of PCD involved in plant defense against agents of biotic origin) by interacting with lipid-associated signaling pathways. (Qi et al., 2011). Phospholipase D signaling downstream of G-proteins was suggested to induce excess of ROS and cell death in MP-, CPT-, Fumonisin B1 (a toxin from the fungus *Fusarium moniliforme*)- and Cd-treated tomato suspension cells (Iakimova et al., 2013). In the present study with tobacco BY-2 cells sulphur containing VOCs were found only in response to MP and CPT. Interestingly, Cd did not cause a release of the identified volatiles. This implies that in our experimental model these compounds may presumably exclusively contribute to or be associated with PCD induced by biotic elicitors.

Here and in the previous study with BY-2 cells (Iakimova et al., 2019) Cd stress induced ROS-dependent PCD in a manner similar to MP and CPT. Some reports indicate that Cd effect on VOCs production may differ from the effect of other heavy metals. For example, in maize plants Zn and Cu promoted the production of volatile terpenes. The exposure to Cd, however, did not stimulate their emission both in maize and in *in vitro* grown plantlets of oilseed rape (*Brassica napus*) (Bibbiani et al., 2018; Durenne et al., 2018). Jesus et al. (2016) observed that in *Martianthus leucocephalus*, the release of VOCs from the class of essential oils was provoked by Zn but not by Cu and Cd. Our results suggest that in Cd-induced PCD, for managing with the oxidative stress tobacco BY-2 cells may engage mechanisms not involving the identified antioxidant VOSCs.

Although biotic and heavy metal stresses can commonly induce redox imbalance, it is suggested that ROS detoxification in either case may rely on different enzymatic and non-enzymatic factors (Mithöfer et al., 2004). Depending on the necessity of defense, the type of heavy metal, the physiological state and the plant species the synthesis of VOCs with different function and chemical structure may be required (Mithöfer et al., 2004; Pichersky et al., 2006; Erb, 2018). Our findings suggest that in cultured BY-2 cells DMS, DMDS, MT may play a role mainly in biotic stress-induced cell death. In wider scale, however, it should be taken into consideration that during PCD the generation of VOCs with antioxidant potential may differ depending on the type of biotic stress agent, the plant species and the specificity of cell death defense mechanisms.

4. Conclusions

The present study demonstrates that PCD in tobacco BY-2 suspension cultured cells exposed to the cell death elicitors of biotic origin MP and CPT is accompanied by the emission of VOSCs with presumed antioxidant capacity, identified as MT, DMS and DMDS. The VOSCs accumulation paralleled the cell death dynamics and peaked at medium cell death strength. These observations suggest that the detected metabolites may contribute to alleviation of cell death, possibly by controlling the severity of oxidative stress. No VOCs production was recorded at Cd stress which implies that the identified molecules might not contribute to or be associated with Cd-induced cell death in tobacco BY-2 cells. To the best of our knowledge, this is the first report on the involvement of VOSCs in PCD of BY-2 cells. It provides guiding information about PCD-related VOSCs production in model system of suspension cultured cells derived from higher plants. The findings can be a platform for further studies on VOCs functions in plant PCD.

5. Author contributions

ETI did the pharmacological and microscopy cell death analyses, overall data collection, interpretation and discussion, and drafted the manuscript. JPY carried out PTR-MS measurements and data processing. SMC, FJMH and EJW contributed to funding acquisition. SMC and FJMH assisted in the experimental settings and PTR-MS assay. EJW conceived, designed and supervised the study, and edited the manuscript.

Declaration of Competing Interest

There are no commercial links or other connections for each of the authors that could pose a conflict of interest in any way.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2020.153223.

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