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1-Aminocyclopropane-1-carboxylic acid-independent ethylene production by disintegrated plant cells

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Abstract

The aim of the present paper was to describe nonenzymatic ethylene formation in disintegrated plant tissues. A variety of plant samples released ethylene in unphysiological conditions during incubation in hot alkali. The release of ethylene from intact pine needles and from pine needle homogenate was oxygen- and pH-dependent. Ethylene release during hydrolysis increased with incubation temperature both in acidic and alkalic conditions. The biosynthetic precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid, at physiological concentrations released only a minor amount of ethylene during hydrolysis. Exogenous 1-aminocyclopropane-1-carboxylic acid inhibited ethylene release from disintegrated tissues of different plants. Ethylene release was significantly promoted by the presence of Fe^{2+} and Cu^{2+} in incubation medium. Incubation of pine needles with hydrogen peroxide stimulated ethylene release during subsequent hydrolysis at the level of protein synthesis. The presented results suggest that plant tissues contain several substances that may contribute to ethylene production in severely stressed partially disintegrated plant tissues.

Key words: 1-aminocyclopropane-1-carboxylic acid; ethylene production; hydrolysis; metal ions; stress.
Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AOA, aminooxyacetic acid.

Introduction

In higher plants, the hormone ethylene is synthesized via formation of 1-aminocyclopropane-1-carboxylic acid (ACC) followed by enzymatic conversion to ethylene (Yang, Hoffman 1984). Induction of ethylene biosynthesis by a large variety of stress factors is a well-described phenomenon. However, ethylene biosynthesis is a characteristic of living cells, as disintegration of cells leads to immediate interruption of ethylene production (Elstner, Konze 1976).

On the other hand, it has been shown that ethylene can be formed nonenzymatically in severely stressed plant tissues, e.g., in needles of Norway spruce (Chen et al. 1990; Chen, Wellburn 1991) and in the aquatic plant *Spirodela oligorrhiza* upon membrane damage involving peroxidation of unsaturated fatty acids (Mattoo et al. 1986).

Production of ethylene proceeding through the regular methionine-ACC pathway has been suggested as an indicator of environmental stress damage in plants by a number of workers (Tingey et al. 1976; Castillo et al. 1987; Wolfenden et al. 1988). However, the use of stress ethylene evolution as a bioindicator for stress injury is considered to be limited because of unspecificity of the ethylene response (Saxe 1991). Alternatively, ethylene formation by an ACC-independent pathway from organic peroxides, observed in stressed Norway spruce needles has been suggested to have potential use in bioindication (Chen et al. 1990; Chen, Wellburn 1991). These results led to an interest in further investigating ethylene release from disintegrated plant tissues in relation to stress effects.

Previous experiments have shown that during alkaline hydrolysis of plant tissues a significant amount of ethylene is released (Kreitsbergs et al. 1989). However, the physiological significance of ethylene production in the present system is far from clear. Therefore, a series of experiments was initiated to further determine biochemical and physiological consequences of ethylene release from disintegrated plant tissues. The main objectives of the present paper were (i) to analyze optimal conditions of ethylene release during hydrolysis, (ii) to evaluate if ethylene production from disintegrated plant tissues is related to chemical breakdown of ACC and (iii) to search for possible effects of contaminating metals on nonenzymatic ethylene release.

Materials and methods

Plant material

Needle samples of *Pinus sylvestris* L., *Pinus nigra* L. and *Picea excelsa* L. were collected from two-year old shoots cut from 40 to 50 year old trees grown at the National Botanical Gardens of Latvia, Salaspils. Tissue samples of *Solanum tuberosum* L., *Lycopersicon esculentum* Mill., *Citrus lemon* L. *Beta vulgaris* L., *Phaseolus vulgaris* L., *Dryopteris carthusiana* (Vill) H.P.Fuchs, and *Equisetum*

hiemale L. were obtained from local sources and stored in polyethylene bags not more than 24 h before analysis. Seedlings of winter rye (*Secale cereale* L.) were grown in the laboratory in filter paper rolls.

For incubation treatments, 0.5 g of freshly detached pine needles were placed in 10 mL glass bottles containing 1 mL of the appropriate media. H_2O_2 was used at a concentration of 1 M. The final concentrations in the medium for inhibitors were as follows: 2 mM aminooxyacetic acid (AOA), 5 mM $CoCl_2$, 0.1 mM cycloheximide. Bottles were illuminated with fluorescent lamps (photon flux density 350 μmol m^{-2} s^{-1}) throughout the incubation period. After appropriate intervals of time, needles were collected, frozen in liquid N_2 and stored at –25 °C until analysis.

Measurement of ethylene formation

For determination of ethylene release during hydrolysis, tissue samples (about 0.5 g fresh weight) or 1 mL of appropriate liquid media (tissue extracts or sample solutions) were placed in 50 mL screw capped borosilicate tubes. After adjusting the volume to 5 mL with water, 5 mL of appropriate solution (HCl or NaOH) was used to adjust pH. For alkaline hydrolysis, 6% NaOH (w/v) in saturated Na_2SO_4 was added, giving pH 12.5. Typically tubes were incubated at 65 °C for 5 h.

Amount of ethylene and other hydrocarbon gases was measured on a Chrom 5 gas chromatograph (Czech Republic) equipped with a glass column filled with activated Al_2O_3 and a flame ionization detector. Helium was used as a carrier gas. Oven temperature was 80 °C, temperature of the detector 110 °C. Gas peaks were identified by the retention times of standard gas samples (AGA-Gas, Latvia).

Extraction and chromatography

Potato tuber extracts were prepared by incubating ground tuber tissue (100 g) in 100 mL of 90 % ethanol for 1 h. The homogenate was filtered through a glass filter, and the residue was extracted with an additional 50 mL of ethanol for 0.5 h. Pooled extracts were evaporated to a volume of 50 mL, filtered and used for analysis.

For extraction of ethylene-releasing substances from pine needle tissue, 15 g of two-year-old pine needles were homogenized in a commercial blender and extracted with 50 mL of deionized H_2O for 1 h. After filtration on a glass filter, the homogenate was centrifuged at 10 000 g_n for 12 min. The clear supernatant (8 mL) was loaded onto a Sephadex G-15 column (12 × 150 mm) equilibrated with H_2O. Fractions were eluted sequentially with H_2O, 0.5 M K-Na phosphate buffer, pH 7.0, and K-Na phosphate buffer, pH 10. Fractions of 5 mL were collected at a flow rate of 30 mL h^{-1} and subjected to alkaline hydrolysis. As a reference ACC (10 ng in 5 mL H_2O) was chromatographed at the same conditions.

Individual experiments were repeated three or four times when results were similar. Only data from the representative experiment are shown.

Results

In vitro *conditions of ethylene production by disintegrated plant cells*

The experiments revealed that a great variety of plant tissues

Table 1. Ethylene production from intact tissues and ethylene release during alakaline hydrolysis (65 °C, pH 12.5) of various plant tissues. Data are means from three to five samples ±SE

Plant species	Plant material	Ethylene production (nmol h^{-1} g^{-1} FM)	Ethylene release (nmol h^{-1} g^{-1} FM)
Pinus sylvestris	green needles	0.060 ± 0.010	0.42 ± 0.03
	extract from green needles	-	0.14 ± 0.01
	brown needles	0.018 ± 0.003	3.90 ± 0.25
Pinus nigra	green needles	0.070 ± 0.005	0.61 ± 0.04
	brown needles	0.059 ± 0.004	5.80 ± 0.30
Picea excelsa	green needles	0.027 ± 0.003	0.12 ± 0.04
Driopteris carthusiana		0.068 ± 0.009	0.63 ± 0.03
Equisetum hiemale		0.021 ± 0.003	0.09 ± 0.01
Solanum tuberosum	tubers	n.d.	0.04 ± 0.01
	etiolated sprouts	n.d.	0.06 ± 0.01
	leaves	n.d.	0.25 ± 0.01
Lycpersicon esculentum	fruits	n.d.	0.93 ± 0.04
Citrus lemon	pulp	n.d.	0.06 ± 0.01
	peel	n.d.	0.07 ± 0.01
	seeds	n.d.	0.14 ± 0.01
Beta vulgaris	root	n.d.	0.19 ± 0.01
	shoot	n.d.	0.10 ± 0.02
Secale cereale	4-day-old seedlings	n.d.	0.15 ± 0.01

Table 2. Release of hydrocarbon gases (nmol h^{-1} g^{-1} FM) from homogenate of pine needles in an atmosphere or argon at 65 °C (pH 12.5) during 2 h. Data are means from four samples ±SE. (*) indicates statistically significant ($p < 0.005$) difference from the controls

Atmosphere	Ethylene	Ethane	Propylene	Propane
Air (control)	0.45 ± 0.03	0.11 ± 0.04	0.23 ± 0.02	0.84 ± 0.05
Argone	0.20 ± 0.01	0.08 ± 0.01	0.23 ± 0.06	0.67 ± 0.02
Argone (% from control)	44*	73	100	56*

released ethylene in unphysiological conditions during incubation in hot alkali (Table 1). The rate of ethylene production during hydrolysis varied between different tissues of the particular species analyzed, and in relation to the physiological stage of the tissues. In particular, brown needles of conifers released proportionally higher amounts of ethylene during hydrolysis as the green ones.

During alkaline hydrolysis of pine needle tissue homogenate, different amounts of hydrocarbon gases were produced, including both saturated (ethane and propane) and unsaturated (ethylene and propylene) types (Table 2). To determine if the release of gases requires oxygen, some samples were hydrolyzed in an argon atmosphere. The release of ethylene and propane under these conditions seemed to be an oxygen-dependent process, at least in part, in contrast to the formation of ethane and propylene (Table 2).

The kinetics of ethylene and ethane evolution from intact pine needles during hydrolysis showed that ethane release stopped after 3 h and ethylene release after 5 h of hydrolysis (Fig. 1) indicating that the process of breakdown of the respective precursor substances was completed within the particular period of time.

Experiments on hydrolysis of intact pine needles in

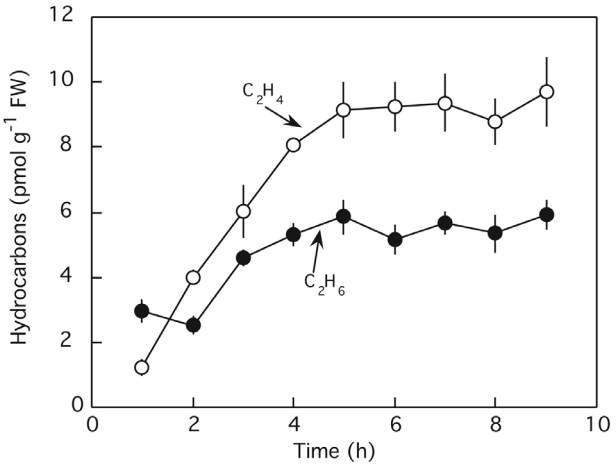

Fig. 1. Time course of ethylene and ethane release during hydrolysis of intact pine needles. Intact pine needles were hydrolysed at 65 °C, pH 12.5. Data are means from five samples ±SE.

media with different pH values showed that ethylene release depended on pH, with significantly higher intensity of ethylene formation both at extremely acidic and alkalic media, and a minimum rate at pH 9 to 10 (Fig. 2A). At pH values near physiological levels for pine needles (pH 3.9 to 4.3 for pine needle homogenate, data not shown), release of ethylene was relatively high. In contrast to ethylene release from the intact needles, pH-dependent ethylene release during hydrolysis of pine needle extracts was associated with different characteristics (Fig. 2B). At acidic and neutral values of pH (3.5 to 7.0) ethylene release did not occur as a function of pH. However, above pH 7, the intensity of ethylene release clearly increased with increase of alkalinity. The present results suggest that several different substances with different pH-breakdown relationships contribute to ethylene release during hydrolysis. Additional support for this hypothesis was obtained in chromatography

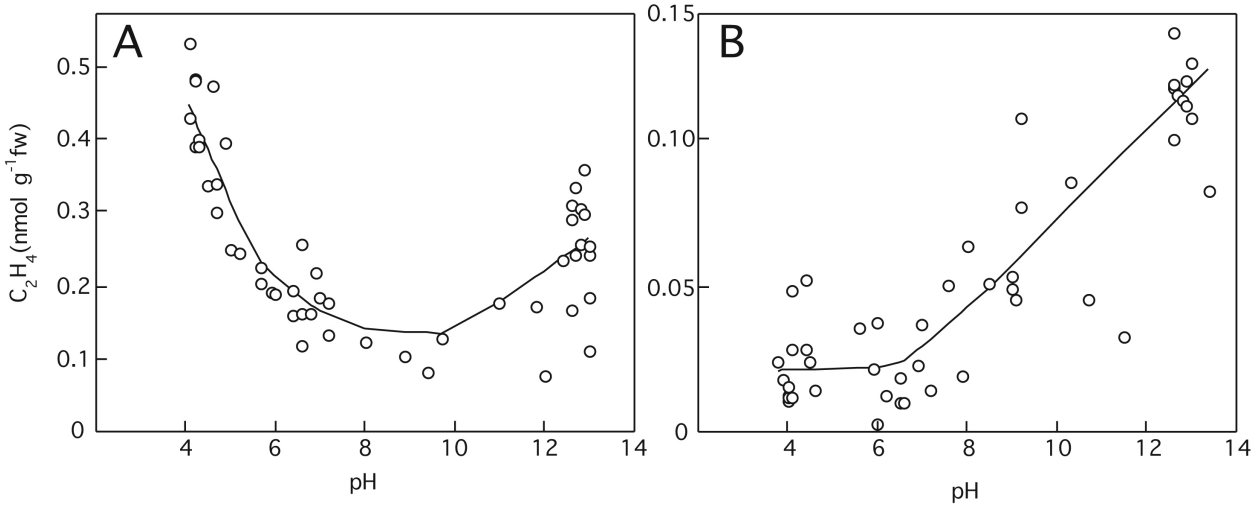

Fig. 2. pH dependence of ethylene release during hydrolysis of intact pine neeldes (A) and pine needle water extract (B). Hydrolysis was performed at 65 °C for 5 h. Each circle represents one individual sample.

Table 3. Relative amounts (%) of ethylene released at different pH during hydrolysis of different fractions of water extract from pine needles after chromatography on a DEAE cellulose column

pH	H_2O	7% Na_2SO_4	15% Na_2SO_4 0.6 M HCl	15% Na_2SO_4 1% NaOH
4.4	26	29	100	22
6.8	31	19	21	19
8.8	90	12	18	42
12.5	100	100	27	100

Table 4. Ethylene release from extract of *Pinus sylvestris* L. needles in relation to incubation temperature. Hydrolysis was performed at pH 4.2 and pH 13.2 for 4 h. Data are means from threesamples for each data point ±SE

Temperature	pH 4.2		pH 13.2	
(°C)	(nmol mL^{-1})	%	(nmol mL^{-1})	%
18	0.005 ± 0.001	10	0.02 ± 0.00	9
40	0.020 ± 0.001	40	0.10 ± 0.01	45
50	0.034 ± 0.003	68	0.16 ± 0.01	73
65	0.050 ± 0.003	100	0.22 ± 0.02	100

experiments, in which pine needle extracts were separated on a DEAE cellulose column with different eluents (Table 3). Fractions eluted either with water, neutral 7% solution of Na_2SO_4 or alkalic 15% solution of Na_2SO_4 produced maximum ethylene at pH 12.5. In contrast, the fraction eluted with acidic 15% solution of Na_2SO_4 produced maximum ethylene at the most acidic value of pH used (pH 4.4).

Ethylene release during hydrolysis, both in acidic and alkalic conditions, increased with incubation temperature (Table 4). However, even at physiologically normal temperatures and pH (18 °C and 4.2, respectively), the intensity of ethylene release was significant (10% of the ethylene release at pH 4.2 and 65 °C or 2 % of that at pH 13.2 and 65°C).

ACC as a possible precursor of nonenzymatic ethylene production

ACC, as the biosynthetic precursor of ethylene, can be easily degraded in different chemical systems leading to release of ethylene (Stegink, Siedow 1986). It is thus logical to propose that in our system ACC may contribute to ethylene formation during hydrolysis of disintegrated plant tissues. However, only a very small amount of ethylene was detected during hydrolysis of 0.05 nmol ACC at pH 12.5 (data not shown), which is equal to the level of ACC in 1 g of non-stressed pine needles (Ievinsh, Tillberg 1995). The amount of ACC equal to that which is present in 1 g of H_2O_2-treated pine needles (25 nmol; Ievinsh, Tillberg 1995) released less than one half of the ethylene evolved during alkaline degradation of control needles (data not shown). Moreover, at pH characteristic for pine needle tissues, ethylene release from ACC decreased by about 100-times.

Table 5. The effect of 0.4 mM ACC on ethylene release (nmol h^{-1} g^{-1} FM) from tissue homogenates during alkaline hydrolysis. Hydrolysis was performed at pH 12.5 (65 °C) for 1 h. Data are means from five samples ±SE

	Bean leaves	Rye leaves	Potato tubers
Plant material (1 g)	0.82 ± 0.01	0.33 ± 0.04	0.46 ± 0.01
Plant material (1 g) + ACC (0.4 mM)	0.15 ± 0.01	0.22 ± 0.01	0.26 ± 0.01
ACC (0.4 mM)	0.29 ± 0.01	0.30 ± 0.01	0.34 ± 0.00

More evidence against a major contribution of ACC to ethylene release from disintegrated plant tissues came from experiments in which ACC was added exogenously. When up to 0.4 mM ACC was added to homogenate from bean leaves, ethylene formation during alkaline hydrolysis was inhibited by 82% (Table 5). The same phenomenon was observed in experiments with rye leaf homogenate and potato tuber homogenate, although at a lesser degree. It is interesting to note that the same amount of ACC produced more ethylene during hydrolysis, than that released from tissue homogenates plus ACC (Table 5). Consequently, not only did ACC inhibited the breakdown of appropriate endogenous precursors of ethylene, but also tissue homogenate inhibited ethylene release from ACC.

Gel filtration chromatography on a Sephadex G-15 column of pine needle extracts resolved four peaks of ethylene releasing compounds (data not shown). Only the first peak contained amino acids. This peak also involved substance(s) that released ethane, propane and propylene. When a sample of 25 nmol of ACC was separated in the same system, with subsequent alkaline hydrolysis of eluted fractions, the ethylene release clearly coincided with the first fraction of the pine needle homogenate (data not shown). Similarly, during chromatography on DEAE cellulose, ACC was eluted with water in the first fraction (data not shown).

Metal ions as possible activators of nonenzymatic ethylene formation

As contamination of plant tissues with metal ions may affect ethylene release from disintegrated tissues, we tested the effect of several metals on ethylene production from plant material during hydrolysis, as well as the effect of the metal ions on chemical breakdown of ACC.

Ethylene production from plant material during alkaline hydrolysis was activated by the presence of Fe^{2+} in the incubation medium (Table 6). Formation of other hydrocarbon gases, such as ethane and propane, was enhanced also by Fe^{2+} during hydrolysis . Effect of Fe^{2+} was significantly higher in youngest needles both for ethylene and ethane production. However, the most pronounced effect of Fe^{2+} on propane formation was found in two-year-old needles.

Cu^{2+} appeared to activate ethylene release from pine

Table 6. The effect of Fe^{2+} on hydrocarbon release from intact pine needles of different age during alkaline hydrolysis. Data are fold increase due to 2 mM Fe^{2+} addition. Hydrolysis was performed at 65 °C, pH 12.5, in presence or absence of Fe^{2+}

Needle age (years)	Ethylene	Ethane	Propane
1	7.5	6.0	2.7
2	5.0	3.0	4.0
3	4.1	3.6	2.0
4	4.5	3.0	3.3

Table 7. The effect of 1.6 mM Cu^{2+} on ethylene release from pine needle extract at different pH. Hydrolysis was performed at 65 °C for 1 h. Data are means from five samples for each pH value ±SE

pH	Ethylene (nmol mL^{-1})	Increase (times)
3.7	20.0 ± 1.0	760
6.0	5.0 ± 0.5	60
13.4	1.0 ± 0.1	8

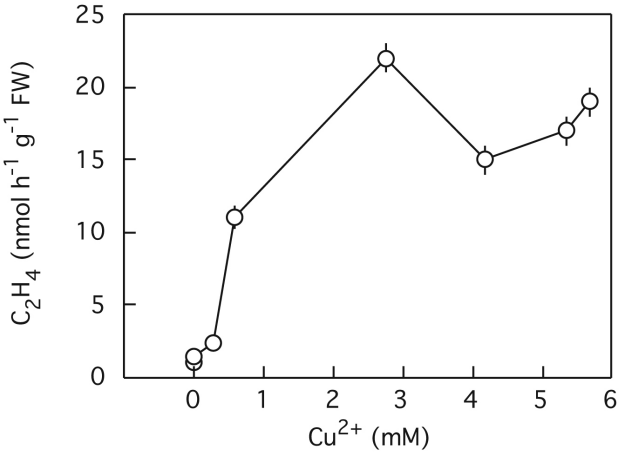

Fig. 3. Effect of different concentrations of Cu ions in the medium on ethylene release from pine needle homogenate during acidic hydrolysis. Hydrolysis were performed at 65 °C, pH 4.5, for 4 h. Data are means from four samples for each concentration ±SE.

needle water extract during hydrolysis at different pH in the incubation medium (Table 7). At the physiological pH for needle homogenate (pH 3.7), the activation effect of Cu^{2+} was most pronounced (760-fold) while the effect decreased with alkalinity of incubation medium (60-fold and eight-fold, for pH 6.0 and pH 13.2, respectively). The stimulatory effect of Cu^{2+} on ethylene release during hydrolysis was clearly dose dependent and saturation occurred at 2.76 mM Cu^{2+} in the incubation medium (Fig. 3). Cu^{2+} activated ethylene formation during hydrolysis of all fractions after DEAE cellulose chromatography (data not shown).

When intact detached pine needles were infiltrated with 1.6 mM solution of $CuSO_4$ and incubated for 2 h at 30 °C, ethylene production rose by about three-fold in comparison with control needles held in the same conditions (Table 8).

Table 8. The effect of 1.6 mM $CuSO_4$ on ethylene evolution (nmol h^{-1} g^{-1} FM) from detached intact pine needles. Needles were infiltrated and either immediately placed in closed bottles to accumulate ethylene for the next 2 h at 30 °C or were held for 72 h and then placed in bottes to accumulate ethylene. Data are means from three samples ±SE

	Without infiltration	Infiltrated with H_2O	Infiltrated with $CuSO_4$
Immediately after detachment	0.057 ± 0.004	0.064 ± 0.003	0.190 ± 0.030
After 72 h	n.d.	0.560 ± 0.030	4.250 ± 0.100

Table 9. The effect of cycloheximide on ethylene production from intact pine needles and ethylene release during alkaline hydrolysis from needle tissue. Detached needles were preincubated in water, 0.1 mM cycloheximide, 2 mM aminooxyacetic acid (AOA) or 10 mM Co^{2+} for 1 h, then incubated in water or 1 M H_2O_2 in continuous light for 16 h and 24 h, for analysis of ethylene production and ethylene release during hydrolysis, respectively

Treatment	Ethylene production (nmol h^{-1} g^{-1} FM)	Ethylene release during hydrolysis (nmol g^{-1} FM)
H_2O	0.26 ± 0.01	0.30 ± 0.07
H_2O + cycloheximide	0.43 ± 0.08	0.34 ± 0.03
H_2O + AOA	n.d.	0.28 ± 0.03
H_2O + Co^{2+}	n.d.	0.23 ± 0.04
H_2O_2	4.62 ± 0.05	1.40 ± 0.06
H_2O_2 + cycloheximide	0.42 ± 0.01	0.37 ± 0.04
H_2O_2 + AOA	n.d.	0.80 ± 0.08
H_2O_2 + Co^{2+}	n.d.	1.08 ± 0.07

Within 72 h after treatment, ethylene production in $CuSO_4$-treated needles reached an intensity eight times higher than the control level.

In contrast to ethylene release from tissue homogenate, where the highest increase due to Cu^{2+} treatment was found at pH 3.7, most pronounced effect of Cu^{2+} on breakdown of ACC was found at pH 6.5 (53-fold, data not shown).

Model experiments on the effect of stress on ethylene release during hydrolysis

Treatment of pine needles with a chemical stressor H_2O_2 (1 M) caused an increase in ethylene release from needle tissues during alkaline hydrolysis (Table 9). To test further the increase in ethylene release during alkaline hydrolysis of H_2O_2-incubated needles was due to an increase in the amount of particular precursors of ethylene release rather than chemical stimulation of breakdown during hydrolysis, control and H_2O_2-treated pine needle extracts were fractionated by gel filtration on Sephadex G-15. Chromatography of both control and treated needle extracts resolved four peaks of putative ethylene-releasing

substances. As was expected, in all peaks the release of ethylene during hydrolysis was higher for the H_2O_2-treated needle tissue (data not shown).

To study the possible role of protein synthesis and endogenous ethylene production in regulating H_2O_2-induced increase of ethylene release from disintegrated tissues, experiments with different inhibitors were carried out. The effect of cycloheximide, aminooxyacetic acid, and Co^{2+}, which are known to inhibit, respectively, protein synthesis, ACC synthesis, and ACC oxidation, was examined. Cycloheximide, a translation inhibitor, completely inhibited both the H_2O_2-induced increase in ethylene production from intact needles as well as ethylene release during hydrolysis (Table 9). Both aminooxyacetic acid and Co^{2+} partially inhibited the H_2O_2-induced increase in ethylene release during hydrolysis.

Discussion

Ethylene release from disintegrated plant tissues is likely a general characteristic of higher plants, as shown by the results obtained using wide array of plant tissues (Table 1). Brown needles of conifers released particularly high amounts of ethylene during hydrolysis. Our data are in accordance with earlier observations that showed organic peroxides to be precursors of ethylene formation in brown spruce needles (Chen et al. 1990). The fact that metal ions (Cu^{2+} and Fe^{2+}) significantly stimulated both ethylene production from intact pine needles (Table 8) as well as ethylene release from pine needle homogenates (Tables 6 and 8; Fig. 3) resembles the pine needle system in which metal ions were shown to be activators of ethylene formation (Chen, Wellburn 1991).

The rate of nonenzymatic ethylene formation was dependent on oxygen, temperature and pH. In general, these characteristics supported existence of a non-physiological system. Our experiments clearly showed that plant tissues contain several different substances (ACC being only of minor importance), which can serve as a substrate for nonenzymatic ethylene release. This is not surprising, as a large variety of organic substances can release ethylene *in vitro* (Abeles et al. 1992). An important question is: is it possible that similar reactions can occur in living plants, at least, locally, leading to release of physiologically important concentrations of ethylene, which may affect adjacent tissues? There is some evidence that this may be true. Firstly, at physiological pH of pine needles (around pH 4.0) the amount of ethylene released during alkaline hydrolysis (Fig. 2A) was comparable to that produced by intact pine needles in physiological conditions (Ievinsh, Tillberg 1995). Secondly, pollutant metals (e.a., Fe and Cu) can accumulate in pine needle tissue and may contribute to ethylene production both through the ACC-dependent ethylene biosynthesis pathway and by nonenzymatic ethylene release. It was shown previously that in *Spirodela* plants, Cu-induced ethylene production was associated with disintegration of intracellular membranes and organelles (Mattoo et al. 1986). In addition, earlier it was found that toxic concentrations of Cu ions generate strong lipid peroxidation concomitant with a burst of ethane and ethylene production (Sandmann, Boger 1980). On the other hand, the results of our investigation might explain, at least in part, the insensitivity in stressed pine needles of ethylene formation from to inhibitors of ACC-dependent ethylene biosynthesis (Ievinsh, Tillberg 1995). Conifers (and other gymnosperms) seem to be exceptional group of plants in respect to ethylene biosynthesis (Reynolds, John 2000).

We used H_2O_2 treatment for the induction of the stress responses in the pine needle model experiments. *De novo* protein synthesis at the translational level was essential for the observed H_2O_2-induced rise in ethylene release during alkaline hydrolysis (Table 9). This makes it very unlikely that the observed induction of ethylene release from disintegrated plant tissues was due simply to the chemical acceleration of breakdown of putative precursors during alkaline hydrolysis *in vitro*.

Our results tend to support the hypothesis that induction of formation of precursors for ethylene release is a part of the general stress response in pine needle tissues under the effect of H_2O_2. As inhibitory experiments showed a partial dependence of hydrolysis-related ethylene induction on ethylene biosynthesis in H_2O_2-treated needles (Table 9), it seems likely that in stressed plant tissues the precursors of ethylene release during hydrolysis can contribute to the ethylene mediated responses.

It can be concluded that, in severely stressed plant tissues with a certain degree of tissue disintegration, especially, in conifers, in presence of metal contaminants, substances releasing ethylene nonenzymatically can contribute to ethylene-mediated stress reactions.

Acknowledgements

The paper is dedicated to the memory of Dr. biol. Olgerts Kreicbergs (1928–2009), who significantly contributed to the field of ethylene research. He performed part of the experiments described here.

References

Abeles F.B., Morgan P.W., Saltveit M.E.Jr. 1992. *Ethylene in Plant Biology*. 2nd ed. Academic Press, San Diego. 414 p.

Castillo F.J., Miller P.R., Greppin H. 1987. Extracellular biochemical markers of photochemical oxidant air pollution damage to Norway spruce. *Experientia* 43: 111–115.

Chen Y.-M., Tabner B.J., Wellburn A.R. 1990. ACC-independent ethylene formation in brown Norway spruce needles involves organic peroxides rather hydroperoxides as possible precursors. *Physiol. Mol. Plant Pathol.* 37: 323–337.

Chen Y.-M., Wellburn A.R. 1991. Ethylene release from green spruce needles involves a combination of ACC-dependent and independent pathways. *Plant Growth Reg.* 10: 153-162.

Elstner E.F., Konze J.R. 1976. Effect of point freezing on ethylene

and ethane production by sugar beet leaf disks. *Nature* 263: 351–352.

Ievinsh G., Tillberg E. 1995. Stress-induced ethylene biosynthesis in pine needles: a search for the putative 1-aminocyclopropane-1-carboxylic acid-independent pathway. *J. Plant Physiol.* 145: 308-–314.

Kreitsbergs O., Vutsina I., Iljin V.V. 1989. Ethylene production in the process of chemical treatment of the plant material. II. Quantitative relations of ethylene produced by alkalic hydrolysis of different plant parts and organic compounds. *LPSR ZA Vēstis* 12: 71–75. (In Russian)

Mattoo A.K., Baker J.E., Moline H.E. 1986. Induction by copper of ethylene production in *Spirodela oligorrhiza*: evidence for a pathway independent of 1-aminocyclopropane-1-carboxylic acid. *J. Plant Physiol.* 123: 193–202.

Reynolds E.A., John P. 2000. ACC oxidase is found in seedlings of two (Coniferales, Gnetales) of the four gymnosperm orders. *Physiol. Plant.* 110: 38–41.

Sandmann G., Böger P. 1980. Copper-mediated lipid peroxidation processes in photosynthetic membranes. *Plant Physiol.* 66: 797–800.

Saxe H. 1991. Photosynthesis and stomatal responses to polluted air, and the use of physiological and biochemical responses for early detection and diagnostic tools. In: Callow J.A. (ed) *Advances in Botanical Research*. Vol.18, pp. 1–128. Academic Press, London.

Stegink S.J., Siedow J.N. 1986. Ethylene production from 1-aminocyclopropane-1-carboxylic acid *in vitro*: A mechanism for explaining ethylene production by a cell-free preparation from pea epicotyls. *Physiol. Plant.* 66: 625–631.

Tingey D.T., Standley C., Field F.W. 1976. Stress ethylene evolution: a measure of ozone effects on plants. *Atm. Env.* 10: 969–974.

Wolfenden J., Robinson D.C., Cape J.N., Paterson I.S., Francis B.J., Mehlhorn H., Wellburn A.R. 1988. Use of carotenoid ratios, ethylene emissions and buffer capacities for the early diagnosis of forest decline. *New Phytol.* 109: 85–89.

Yang S.F., Hoffman N.E. 1984. Ethylene biosynthesis and its regulation in higher plants. *Annu. Rev. Plant Physiol.* 35: 155–189.

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