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Enzymatic determination of ascorbic acid in leaf cell walls using acidic buffer during infiltration

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Abstract

A modification of the procedure of extraction of cell wall solution for enzymatic determination of ascorbic acid and its reduction level in the apoplast of leaf cells is proposed. The modification consists in infiltration of leaves with citric acid/sodium phosphate buffer, pH 3, instead of customarily used neutral solutions. In acidic media autooxidation of ascorbic acid is effectively suppressed, so that infiltration could be performed at laboratory temperatures. Using polyacrylamide gel electrophoresis and infiltration solutions of pH down to 1.5 it is shown, that at pH 3 the extracted fluid is not contaminated with intracellular substances if appropriate vacuum and centrifugation forces are used. The modification is shown to be more effective for leaves of *Phaseolus* than for those of *Spinacia*. In cell walls of mature leaves of these species the concentration of ascorbic acid was found to be around 1 mM, with reduction level up to 0.90. The role of ascorbic acid in cell walls as ozone scavenger is discussed.

Additional key words: ascorbate oxidase, ozone susceptibility, *Phaseolus vulgaris*, *Spinacia oleracea*.

Introduction

During last two decades measurement of ascorbic acid (AA) in leaf cell walls has obtained considerable attention, as this compound appears to be involved in detoxification of ozone, entering the leaf in increasing quantities from polluted atmosphere through the open stomata (Omasa *et al.* 1980). Mesophyll cell wall is the

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Abbreviations: AA - ascorbic acid; AAO - ascorbate oxidase; AM - acidic method; DHA - dehydroascorbate; DTT - dithiothreitol; EDTA - ethylenediaminetetraacetic acid; IWF - intercellular washing fluid; NM - neutral method; PAGE - polyacrylamide gel electrophoresis; PPFD - photosynthetic photon flux density; RL - reduction level of AA; SU - solute uptake by infiltration.

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single liquid phase defense line against penetration of ozone and its not less phytotoxic derivatives to the vulnerable plasmalemma, where various membrane components are attacked and impaired (Chevier and Sarhan 1992).

Ozone chemistry in the cell wall solution is very complex (Heath 1988). Among numerous others peroxidase-catalysed scavenging reaction of ozone-generated hydrogen peroxide with AA to form dehydroascorbate (DHA) and water has been frequently mentioned. Moreover, as pointed out by Chameides (1989), AA due to its carbon double bond has in aqueous solutions a high bimolecular rate constant with ozone itself, and in the case of sufficient concentration of AA in the cell walls, this direct reaction may play a decisive role in protecting the plasmalemma from ozone damage.

Among a variety of methods used to measure AA in whole leaf homogenate some were recently adapted to assess AA levels in the cell walls. The most attractive appears to be the enzymatic method where the vacuum-infiltrated leaves are subjected to "soft" centrifugation [from 160 *g* (Takahama and Oniki 1992) to 1500 *g* (Castillo and Greppin 1988)] depending on species. After dilution of the obtained intercellular washing fluid (IWF), its absorbance at AA UV absorption maximum (265 nm) is measured before and after oxidizing AA to nonselectively absorbing DHA by ascorbate oxidase (AAO). To get the reduction level of AA, in the second assay the original DHA is reduced to AA by adding dithiothreitol (DDT) (Okamura 1980).

As a rule, near-neutral solutes are used throughout the procedure to match a rather narrow maximum of AAO activity at pH 6.5 - 6.8 (Sereikaite *et al.* 1993) and reduction optimum of DTT at pH 6.5 - 8.0 (Okamura 1980). To suppress autooxidation of AA and DHA down the so called AA-cascade (Kimoto *et al.* 1993), infiltration and centrifugation steps are performed at +4 °C, but the final dilution solution [customarily sodium phosphate buffer, pH 6.8, (Luwe *et al.* 1993)] should bring the IWF to room temperatures to achieve sufficient oxidation and reduction activities. However, our preliminary experience with *Phaseolus vulgaris* leaves has shown that in spite of using low temperatures during extraction loss of AA may still reach tens of percents within 10 - 15 min. In addition, at low temperature infiltration tended to be poorer, especially when stomata were not fully open. The degree of infiltration could be increased by repeated under-pressurization/relaxing (Luwe 1994), but the procedure will be correspondingly longer.

Below, a modification of the procedure is proposed, where during infiltration an acidic buffer is used to suppress AA and DHA oxidation before spectrophotometric assaying. As a result, infiltration could be performed at laboratory temperatures.

Materials and methods

Plant material and growth conditions: *Spinacia oleracea* L. and *Phaseolus vulgaris* L. plants were grown in a growth chamber in stainless steel pots containing well fertilized commercial peat. Light/dark periods were 14/10 h (*Phaseolus*) and 10/14 h (*Spinacia*), PPFD was 260 - 360 μmol m^{-2} s^{-1} (100 - 140 W m^{-2}, mercury fluorescent

lamp). Air relative humidity was 30 - 40 %, light/dark temperatures were 27/22 °C (*Phaseolus*) and 25/16 °C (*Spinacia*). Substrate water content was held at 50 - 60 % of maximum capacity by adding tap water daily.

Cell wall and whole leaf solute extraction: 13 - 15 d (*Phaseolus*) and 30 - 40 d (*Spinacia*) after seedling emergence fully expanded leaf blades (1 to 2 g of fresh mass) were excised in the middle of the light period, weighed and vacuum infiltrated (-90 kPa, see below) for 2 to 3 min either with 0.1 M of cold (4 °C) potassium chloride solution (customary neutral method, NM) or at room temperature (18 - 20 °C) with citric acid/sodium phosphate buffer (pH 3.0) (proposed acidic method, AM). To suppress possible AA oxidation by catalytic action of metal ions present as occasional impurities, EDTA was added to both solutions in concentration 2.5 mM. It was shown that concentrations of EDTA up to 10 mM did not reduce AAO activity during the subsequent spectrophotometric assay. Osmotic potentials of the both solutions were similar (0.48 ± 0.02 MPa, measured with *Roebling* osmometer (*12/12 DR*, Berlin,Germany) and close to leaf water potential after excision (measured with a pressure chamber). Sometimes a second vacuum application/release was necessary to achieve full infiltration. The time interval between the leaf excision and the end of infiltration did not exceed 5 to 7 min. Infiltrated leaves were blotted, weighed, rolled carefully and inserted into a 20 × 80 mm polypropylene cylinder with perforated bottom. The cylinder was put over a centrifuge tube and centrifuged at 160 *g* (see below) for 5 to 6 min. at 4 °C (both methods). The amount of IWF obtained was 0.1 - 0.2 cm^3 (*Phaseolus*) or 0.2 - 0.3 cm^3 (*Spinacia*) per g leaf fresh mass.

To determine the AA and DHA contents in whole leaf homogenate for comparison, 1 g of uninfiltrated leaf material was ground in cooled mortar with 2.5 cm^3 of cold 2 % *m*-phosphoric acid. The homogenate was centrifuged at 16 500 *g* for 15 min at 4 °C and the supernatant was used for determination.

AA and DHA determination: From the IWF obtained a 0.05 - 0.1 cm^3 sample was pipetted into a 1-cm quartz cuvette, diluted with 1.5 cm^3 of either 0.1 M sodium phosphate buffer, pH 6.8, 20 °C (NM) or citric acid/sodium phosphate buffer, pH 6.8, 20 °C (AM). Absorbance of the assay was measured within 2 min at 265 nm in a double-beam spectrophotometer *Specord* (*UV VIS, Carl Zeiss*, Jena, Germany) with appropriate buffer in the reference position. Then 5 mm^3 (1 unit) of AAO (EC 1.10.3.3 from *Cucurbita* sp.) was added to the assay. After complete oxidation of AA to DHA (within no more than 3 min) absorbance of the assay was measured again. Into a second assay 0.02 cm^3 of 0.1 M DTT was added to reduce original DHA to AA. AA and DHA concentrations in the cell wall solution were calculated from changes in absorbances, using extinction coefficient for AA at 265 nm 18.0 $mmol^{-1}$ cm^{-1} (Takahama and Oniki 1992). It was assumed that the aqueous space of the cell wall was 10 % of the leaf fresh mass (Speer and Kaiser 1991). AA and DHA concentrations in the whole leaf extracts were measured in a similar way, the reaction mixture containing 1.5 cm^3 of 0.1 M sodium phosphate buffer (pH 6.4), 0.02 cm^3 of leaf extract and 5 mm^3 (1 unit) AAO or 0.02 cm^3 of 0.1 M DTT.

The reduction level (RL) of AA was calculated as RL = [AA]/([AA]+[DHA]). Each measurement was repeated 4 to 5 times using separate leaf samples.

Control of cell integrity: The maximum possible infiltration and centrifugation forces for IWF extraction (90 kPa and 160 *g*, respectively) were chosen in separate experiments as values where intracellular marker enzymes started to appear in trace amounts in the extracted fluid (exceptions see Results), being indicative of broken membranes and leakage of intracellular AA. To assess cell integrity after infiltration separately from the total effect of infiltration + centrifugation, and also the possible influence of acidic pH on membrane integrity during infiltration, leaves with 1 to 2 cm petioles, infiltrated at -70 to -95 kPa, as described, were transferred into a cold room (+4 °C), inserted into a pressure chamber (Turner 1976), the cut end of the petiole remaining outside. After pressurization the chamber with argon (up to 0.85 MPa) the sap expelled through the petiole (except the first drop) was collected directly into a vial (chamber upside down) with saccharose and *Sephadex G100* as stabilizers in the bottom. Similar samples were taken from the fluid extracted from infiltrated leaves by centrifugation at forces up to 4000 *g*. Intracellular marker enzymes acid phosphatase, superoxide dismutase, malate dehydrogenase and aspartate amino-transferase were determined in these samples using onedimensional polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the gels were stained for isoenzymes by applying standard histochemical methods (Wendel and Weeden 1989) with modifications described in Jaaska and Jaaska (1989). Presence of the enzymes was estimated visually by using centrifuged extract from the whole leaf homogenate as reference. As aspartate aminotransferase and malate dehydrogenase tended to precipitate at pH 3, an alternative test of the lack of destroying influence of low pH on membrane integrity was done by lowering pH of the infiltration solution down to pH 1.5. There was no increase in AA content of the IWF down to pH 2.5, but at still lower pH AA content sharply increased, indicating contamination with intracellular AA.

Results

Solution uptake by infiltration (SU) varied little among individual measurements and represented in average 28 % of the initial fresh mass in *Phaseolus* and 30 - 33 % in *Spinacia* (Table 1). This suggests slightly higher volume of air spaces in *Spinacia*. The differences in SU between the methods was insignificant in the both species. At the same time standard deviations (S.D.) for [AA] and [DHA] were large, indicating differences between individual measurements. In spite of this in *Phaseolus* the difference in the average values of [AA] between the methods was highly significant, the AM giving 5 times higher values than NM. At the same time [DHA] values were 34 % lower using AM. As a result, the sum [AA]+[DHA] was 2.75 times, and the reduction level (RL) 1.77 times higher using AM instead of NM. Both differences were significant.

In contrast, in *Spinacia* there was no significant difference in [AA] values between the methods, although 13 % higher values were obtained using AM. However, values for [DHA] obtained by AM were significantly lower, constituting only 27 % of [DHA] values obtained by NM. As a result, the sum [AA]+[DHA] was even 12 %

lower in AM, but this difference was insignificant. The difference in RL remained significant, apparently due to great difference in [DHA]. Occasionally clearly enhanced values of [AA] were obtained with both methods, in spite of using centrifugation forces no more than 160 *g*. Close inspection of these leaves after centrifugation revealed some broken veins and sometimes 1 - 2 mm holes, slashes and/or bruises in interveinal areas. PAGE analyses indicated enhanced quantities of intracellular markers in these cases. Apparently significant amounts of AA were derived from the symplastic compartment through the membrane woundings.

Table 1. Ascorbate in cell wall extracts, measured by the two methods: NM - neutral solution used during infiltration, AM - acidic solution (pH 3.0) used during infiltration. SU - solute uptake by infiltration, [g(solution) g^{-1}(f.m.)], [AA] - concentration of ascorbic acid [mM], [DHA] - concentration of dehydroascorbic acid [mM], RL - reduction level of ascorbic acid [mM(AA) mM^{-1}(AA+DHA)]. Values are means ± S.D. of 4 to 5 measurements using different leaf samples. The same superscript letters denote significant difference at the 5 % confidence level.

Method	SU	[AA]	[DHA]	[AA]+[DHA]	RL
Phaseolus					
NM	0.28 ± 0.03	0.24 ± 0.05^a	0.26 ± 0.11	0.50 ± 0.11^b	0.49 ± 0.13^c
AM	0.28 ± 0.01	1.20 ± 0.30^a	0.17 ± 0.06	1.37 ± 0.30^b	0.87 ± 0.05^c
AM/NM	1.00	5.01	0.66	2.75	1.77
Spinacia					
NM	0.33 ± 0.02	0.83 ± 0.41	0.32 ± 0.07^d	1.16 ± 0.44	0.69 ± 0.10^e
AM	0.30 ± 0.01	0.93 ± 0.30	0.09 ± 0.01^d	1.02 ± 0.31	0.91 ± 0.02^e
AM/NM	0.91	1.13	0.27	0.88	1.32

A representative average of these data is presented in Table 2, along with data for whole leaf extracts. The levels of AA and DHA were intermediate between corresponding values from unwounded leaves (Table 1) and those from whole leaf extracts (Table 2, last row). However, AM again resulted in several times higher values for [AA] than NM, whereas values for DHA were slightly lower.

Table 2. Ascorbate in wounded *Phaseolus* leaf cell walls extracts measured by the two methods, and in the whole leaf homogenate. Notations as in Table 1.

Method	SU	[AA]	[DHA]	[AA]+[DHA]	RL
wounded cell wall					
NM	0.30 ± 0.03	0.54 ± 0.20^a	0.60 ± 0.19	1.15 ± 0.36^b	0.47 ± 0.05^c
AM	0.30 ± 0.20	3.32 ± 0.99^a	0.54 ± 0.25	3.86 ± 1.11^b	0.86 ± 0.05^c
AM/NM	1.00	6.13	0.89	3.37	1.83
leaf homogenate		9.76 ± 0.43	0.96 ± 0.11	10.72 ± 0.44	0.91 ± 0.01

Discussion

The above comparative data definitely showed that in enzymatic detection of AA in cell walls of *Phaseolus* leaves use of acidic buffer solution of pH 3.0 instead of routinely used neutral solutions yielded markedly higher AA concentrations, even if the infiltration step was accomplished at laboratory temperatures. At the same time the content of DHA was slightly lower in acidic conditions. This indicated that in the acidic media AA oxidation was efficiently suppressed, the suppression being greater than the counteractive enhancement of AA oxidation due to higher temperature. In contrast, in *Spinacia* the effect of high acidity during infiltration on AA content was relatively low, suggesting nearly balancing sensitivities of AA oxidation to acidity and temperature. The measured depression of DHA content by high acidity was even greater than in *Phaseolus*.

In *Phaseolus* the sum [AA]+[DHA] was also about 2.7 times higher in acidic buffer than in neutral solutions. This pointed to rapid further degradation of DHA, possibly to 2,3-diketo-L-gulonic acid (Kimoto *et al.* 1993), under neutral conditions and to effective inhibition of this degradation in acidic media. This is in agreement with data of Arrigoni *et al.* (1992), where oxidation of DHA in *Vicia faba* seed extracts was prevented by 5 % *m*-phosphoric acid.

In *Spinacia* the sum [AA]+[DHA] was hardly affected by the nature of the infiltration solution, suggesting either low dependence of DHA degradation on pH or different sensitivity of AA and/or DHA degradation to temperature in these two species. Higher temperature sensitivity of IWF for AA in *Vicia faba* if compared with sensitivity in *Spinacia* was observed (M. Luwe, personal communication).

Castillo *et al.* (1987) found [AA] values of 0.5 mM for intercellular fluid of spruce needles and Castillo and Greppin (1988) of 1.0 mM in *Sedum* leaves exposed to O_3. Polle *et al.* (1990) found [AA] up to 1 mM in intercellular fluid of nonozonated spruce needles. Data of Eckey-Kaltenbach *et al.* (1993) for parsley leaf cell walls covered the range 0.05 - 0.4 mM and Takahama (1993) gave for *Kalanhoe* [AA] = 0.7 mM. Takahama and Oniki (1992) and Luwe *et al.* (1993) estimated [AA] = 0.2 - 0.7 mM in the apoplast of spinach leaves. Our data for spinach approximated the upper limit of these published values. Also the average RL of 0.91, calculated from measurements in acidic buffer was close to upper values of the range (RL = 0.75 - 0.93) reported by Luwe *et al.* (1993), where neutral solutions were used. In our measurements with neutral solution the values of RL for spinach was around a rather low value (0.69, Table 1). The possible reason, already mentioned, was slower infiltration procedure where more AA may be oxidized.

The concentration of AA in young full-grown leaves of 2 to 2.5-week-old plants of *Phaseolus vulgaris* reached and slightly exceeded 1 mM, the upper limit, up to now reported for other species. The reduction level of 0.87 approached the range of 0.90 - 0.93, reported for apoplast in *Sedum album* (Castillo and Greppin 1988) and *Spinacia oleracea* (Luwe *et al.* 1993; this report). At the same time, our data indicated, that in comparison with *Spinacia*, in *Phaseolus* care had to be taken to avoid rapid autooxidation of AA when neutral solutions were attempted to use for infiltration.

Our data confirm the earlier findings that AA concentrations in mesophyll leaf cell

wall solution may reach 1 mM, the reduction level of AA being high. Considering also the high bimolecular reaction rate constant of AA with ozone (6×10^7 mol^{-1} s^{-1} at pH 7, Giamalva *et al.* 1985), AA in the cell wall may play an important role in protecting leaf interior from ozone injury. The model calculations of Chameides (1989) have shown that the ozone flux to the plasmalemma is very sensitive to AA in cell wall at physiological concentrations: two plants under the same ozone concentration in air and with similar stomatal conductance can experience orders of magnitude different influxes of ozone to the plasmalemma when concentrations of AA in the cell wall differ only by 2 - 3 times.

Finally, our data confirm the earlier warnings (Castillo and Greppin 1988, Takahama and Oniki 1992) that even small cell woundings can cause relatively high levels of AA in the extracted fluid (Table 2 *vs.* Table 1). This is not suprising, as AA concentration in whole leaf homogenate is approximately an order of magnitude higher (Luwe *et al.* 1993; this report, Table 2). Our experience, where the effect of infiltration was separated from the effect of centrifugation, indicated that the probability of breaks is greater during extraction where centrifugal forces tend to "flatten" the rolled leaves.

Factors determining the species specificity of AA and DHA oxidation in cell walls in response to pH and temperature, should be elucidated.

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