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The ascorbate system in two bryophytes: *Brachythecium velutinum* and *Marchantia polymorpha*

C. PACIOLLA and F. TOMMASI*

Dipartimento di Biologia e Patologia Vegetale, Università di Bari, Via E. Orabona 4, I-70125 Bari, Italia

Abstract

The ascorbate system, one of the major antioxidant systems, has been studied in two bryophytes: a moss, *Brachythecium velutinum* (Hedw.) B., S. & G., and a liverwort, *Marchantia polymorpha* L. The moss and liverwort gametophytes contain ascorbate both in the reduced and oxidized form; utilize ascorbate in removing hydrogen peroxide by means of ascorbate peroxidase and reconvert to ascorbate its oxidation products by means of dehydroascorbate reductase and monodehydroascorbate reductase. Ascorbate oxidase activity was measured in the cytosolic fraction suggesting a localization of the enzyme different from more evolved organisms. The ascorbate content was maintained in the moss after drought stress while it declines in the liverwort, which seems more sensitive to water stress. Since ascorbate recycling is more efficient in the moss than in the liverwort, this seems to suggest a correlation between efficiency of ascorbate recycling and water stress tolerance.

Additional key words: ascorbate free radical, ascorbate peroxidase, dehydroascorbate, dehydroascorbate reductase, liverwort, moss.

Introduction

Environmental factors, including air pollution, heavy metals, drought, heat, cold, UV radiation, and chemical compounds, cause oxidative stress. During oxidative stress reactive oxygen species (ROS) such as singlet oxygen, hydrogen peroxide and radicals are formed and plant cells are equipped with enzymatic and non-enzymatic antioxidant systems to overcome oxidative damage. One of the major antioxidant species is ascorbate (ASC) which is actively synthesized in plant cells and metabolized mainly by two enzymes with different cell localization: ascorbate peroxidase and ascorbate oxidase. ASC acts as an electron donor and generates a hemi-quinone free radical, ascorbate free radical (AFR), also called monodehydroascorbate, that is spontaneously disproportionated to ascorbate and dehydroascorbate (DHA), the last product of ascorbate oxidation. AFR and DHA can be reconverted to ascorbate by means of two enzymes, AFR reductase and DHA reductase. The first utilizes NAD(P)H as electron donor, the second utilizes reduced glutathione. The network of reactions involving ascorbate is well known as the ascorbate-glutathione cycle and prevents the accumulation of toxic concentrations of the H_2O_2 and other ROS in many cell compartments (Horemans *et al.* 2000). The ascorbate system has been widely studied in many living organisms and a vast literature about ascorbate is available (for review see Noctor and Foyer 1998, Loewus 1999, Smirnoff and Wheleer 2000). The literature concerns mainly spermatophytes and less information regards the bryophytes. However, recently mosses and liverworts have been utilized as bio-indicators in an increasing number of programmes of environmental monitoring. The bryophytes, in fact, are a taxonomic group consisting of species more or less tolerant to abiotic stresses widespread in different environmental conditions. They are considered as "pioneer" organisms and can survive for a long time in unfavourable conditions. Very often the responses of the bryophytes to the external conditions depend on their water content and their capability to counteract drought stress. An extensive literature on desiccation tolerance of the bryophytes exists. Much of the earlier work sought simply to establish how long plants would survive in the dry state, while in more recent decades emphasis has moved to the basis for mechanisms

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Abbreviations: ASC - ascorbate; AFR - ascorbate free radical; DHA - dehydroascorbate; DIECA - diethyldithiocarbamic acid; EDTA - ethylenediaminetetraacetic acid; PAGE - polyacrylamide gel electrophoresis.

* Corresponding author; fax: (+390)805442155, e-mail: tommasi@botanica.uniba.it

of tolerance (Dhindsa and Matowe 1981, Dhindsa 1987, Seel *et al.* 1992a,b, Smirnoff 1992, 1993, Proctor and Smirnoff 2000). Studies aimed at understanding the molecular basis of desiccation tolerance have focused on the antioxidant systems in few species of different taxonomic groups. Nevertheless, although some data concerning antioxidant systems in *Tortula ruraliformis, Dicranella palustris* (Seel *et al.* 1992b), *Fontinalis antipyretica* (Roy *et al.* 1996), and a few other species of mosses are available, still little information about ascorbate metabolism of mosses exists and even less on liverworts. The aim of this work is to study one of the most important antioxidant systems in plant cells, the ascorbate system, and its metabolism in two cosmopolitan species: *Marchantia polymorpha*, a liverwort, and *Brachythecium velutinum*, a moss.

Materials and methods

Plants: A population of *Brachythecium velutinum* (Hedw.) B., S. & G., in the botanical garden of the Bari University and two populations of *Marchantia polymorpha* L. (Bari, from a greenhouse stock growing on soil, and Selva di Ostuni, on soil) were observed periodically and used for the sample collection. Samples were collected from the field fully hydrated and equilibrated in a controlled enviroment chamber for 48 d at 20 °C in white light (14-h photoperiod, 20 μmol m^{-2} s^{-1}) and utilized. In order to check the effect of the variation of the water content, some samples were placed in uncovered sterile glass Petri dishes under the air flow of a laminar flow hood for 2 h and 4 h, respectively. Water content was estimated using a *Mettler* (Switzerland) infra-red drier LP 16-M and expressed on a fresh mass basis.

Enzyme assays: Batches of 0.3 g of thalli were homogenised in a mortar at 4 °C in 1:4 (m/v) ratio with a medium containing 0.3 M mannitol, 1 mM EDTA, 50 mM Tris-HCl, pH 7.8, 0.1 % (m/v) bovine serum albumin (BSA) and 0.05 % (m/v) cysteine. The cytosolic fraction was obtained according to the procedure reported by Arrigoni *et al.* (1997). The activities of cytosolic ASC peroxidase (EC 1.11.1.11), dehydroascorbate (DHA) reductase (EC 1.8.5.1) and ascorbate free radical (AFR) reductase (EC 1.6.5.4) were tested according to Tommasi *et al.* (1999); the activity of ASC oxidase (EC 1.10.3.3) was tested in accordance with Arrigoni *et al.* (1981). Since no ascorbic acid was added to the grinding medium only the so-called cytosolic component of ASC peroxidase was detected (Amako *et al.* 1994). Proteins were determined according to Bradford (1976) with BSA as standard.

Native-PAGE analysis of ASC peroxidase and DHA-reducing proteins: Tissues were ground as previously described, the supernatant at 20 000 *g* was used for electrophoretic analysis. Native-PAGE was performed on the cytosolic fraction using a stacking gel containing 4.3 % acrylamide and a separating gel containing 7.3 % acrylamide with a running buffer composed of 4 mM Tris-HCl, pH 8.3, and 38 mM glycine. After non-denaturing electrophoresis, the gels were incubated for 15 min at room temperature under agitation in 0.1 M Na-phosphate buffer, pH 6.2, containing appropriate substrates for the enzymatic reactions to occur: 4 mM ASC and 4 mM H_2O_2 for ASC peroxidase, 4 mM GSH and 2 mM DHA for DHA-reducing proteins. The gels were then washed with distilled water and stained with a solution of 0.125 M HCl containing 0.1 % (m/v) ferricyanide and 0.1 % (m/v) ferrichloride. ASC peroxidase was located as an achromatic band on a Prussian blue background, as a result of the reaction between ferrichloride and ferrocyanide, the latter having been produced by the reduction of ferricyanide with unreacted ASC. In contrast, DHA-reducing proteins were observed as dark blue bands on a light blue background.

Native-PAGE analysis of ASC oxidase: The supernatant obtained as described above was used for the test. An aliquot of purified ASC oxidase from pumpkin was loaded as a marker. After the electrophoretic run, the gel was incubated for 15 min in 0.1 M Na-phosphate buffer, pH 5.8 under mild agitation and for 15 additional min in the same buffer containing 1 mM of ASC. Then the gel was washed and stained as described above for ASC peroxidase.

Since ASC oxidase is described as a protein containing copper, some gels were preincubated in DIECA 1 mM (able to chelate copper) and transferred to the solution containing ASC 1 mM as described above in order to check whether an inhibition in the enzyme activity occurs.

Ascorbate and dehydroascorbate contents assay: Samples (0.2 - 0.4 g) were homogenised in 5 % metaphosphoric acid. The homogenate was centrifuged at 18 000 *g* and the supernatant was used. The ASC and DHA contents were determined according to Arrigoni *et al.* (1997).

Statistics: All the experiments were repeated at least six times; values reported are means of 6 experiments ± SD.

Results

The water content of *B. velutinum* and *M. polymorpha* varies between 75 - 93 %, the lowest value was observed in *B. velutinum,* the highest in *M. polymorpha* from the Ostuni stand. The protein content is remarkably higher in *M. polymorpha* where no significant differences are evident between the two different stands (Table 1).

The total ascorbate (ASC plus DHA) content was lower (67 %) in the moss than in the liverwort, while the ASC/DHA ratio was lower (40 %) in the liverwort than in the moss. No significant differences were found between the two stands of the *M. polymorpha* (Table 2).

Table 1. Water and protein contents of *B. velutinum* and *M. polymorpha* fully hydrated after the collection. Means ± SD, $n = 6$.

Sample	Water content [%]	Protein content [mg g^{-1}(d.m.)]
B. velutinum	75 ± 4	28 ± 2
M. polymorpha (Bari)	87 ± 5	60 ± 6
M. polymorpha (Ostuni)	93 ± 6	58 ± 4

Table 2. Ascorbate (ASC) and dehydroascorbate (DHA) contents, ASC plus DHA and ASC/DHA ratio in the gametophyte of *B. velutinum* and *M. polymorpha*. Means ± SD, $n = 6$.

Sample	ASC [nmol g^{-1}(d.m.)]	DHA [nmol g^{-1}(d.m.)]	ASC+DHA	ASC/DHA
B. velutinum	1290 ± 45	730 ± 28	2020	1.76
M. polymorpha (Bari)	3000 ± 167	3045 ± 199	6045	0.98
M. polymorpha (Ostuni)	3250 ± 183	2950 ± 210	6200	1.10

Table 3. Activities [nmol mg^{-1}(protein) min^{-1}] of oxido-reductive enzymes of ascorbate system in *B. velutinum* and *M. polymorpha* gametophytes. Means ± SD, $n = 6$.

Sample	ASC peroxidase	ASC oxidase	DHA reductase	AFR reductase
B. velutinum	505 ± 31	350 ± 26	250 ± 20	1516 ± 73
M. polymorpha (Bari)	477 ± 40	394 ± 26	300 ± 27	496 ± 25
M. polymorpha (Ostuni)	448 ± 32	352 ± 16	260 ± 18	510 ± 29

The activities of ASC peroxidase, ASC oxidase, DHA and AFR reductase were measured, both in the moss and in the liverwort, in the cytosolic fraction. The first three enzymes show activity similar in the two species considered, while the last one is considerably higher in the moss than in the liverwort (about 3-fold) (Table 3). Since the ASC oxidase activity was measured as oxidation of ascorbate, the extract was denaturated in order to exclude unenzymatic oxidation and the activity of the crude extract was also measured in the presence of an inhibitor of the proteins contanining copper (the DIECA). In both cases the activity was completely suppressed. No differences were evident between the two stands of *M. polymorpha.*

The activities of the ASC peroxidase, ASC oxidase and DHA reductase were also tested by native-PAGE techniques on the sample of *B. velutinum* and on the samples of *M. polymorpha.* One band of ASC peroxidase with different migration rates occurred in the two bryophytes analysed (Fig. 1*A*).

One protein oxidating ascorbate was present in *B. velutinum* and *M. polymorpha*; the migration pattern was similar to that shown by the enzyme purified from

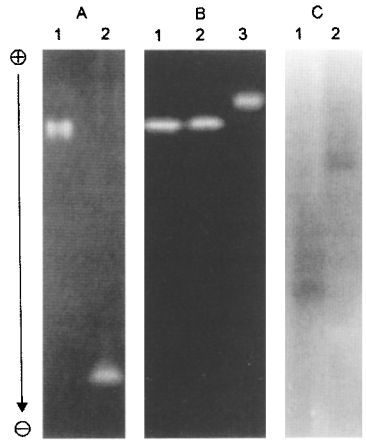

Fig. 1. Native-PAGE of cytosolic ascorbate peroxidase of *Marchantia polymorpha* (*lane 1*) and *Brachythecium velutinum* (*lane 2*) gametophytes (*A*), 250 μg of protein was loaded per lane; ascorbate oxidase of *Cucurbita* spp, 3U (*lane 1*), *Marchantia polymorpha* (*lane 2*) and *Brachythecium velutinum* (*lane 3*) gametophytes (*B*), 150 μg of protein was loaded per lane; DHA-reducing proteins of *Marchantia polymorpha* (*lane 1*) and *Brachythecium velutinum* (*lane 2*) gametophytes (*C*). 300 μg of protein was loaded per lane.

Table 4. Water [%] and protein [mg g^{-1} (d.m.)] contents of *B. velutinum* and *M. polymorpha* maintained under the air flow of a laminar flow hood for 2 and 4 h, respectively. Means ± SD, $n = 6$.

Treatment	*B. velutinum*		*M. polymorpha*	
	water	protein	water	protein
Control	75 ± 4	28 ± 2	90 ± 5	60 ± 2
2 h	70 ± 3	27 ± 2	85 ± 3	50 ± 2
4 h	65 ± 2	28 ± 1	80 ± 2	25 ± 1

pumpkin (Fig. 1*B*). As far as the DHA reducing proteins are concerned, three bands were present in *B. velutinum* and only one in *M. polymorpha* (Fig. 1*C*). No differences in the electrophoretic patterns were found between the two stands of *M. polymorpha* (data not shown).

When samples of *B. velutinum* and *M. polymorpha* were maintained under the air flow of a laminar flow hood for 2 and 4 h, respectively, their water content was lower (Table 4). A remarkable decrease in the protein content occurred only in the liverwort, while in the moss this parameter did not shows significant variations.

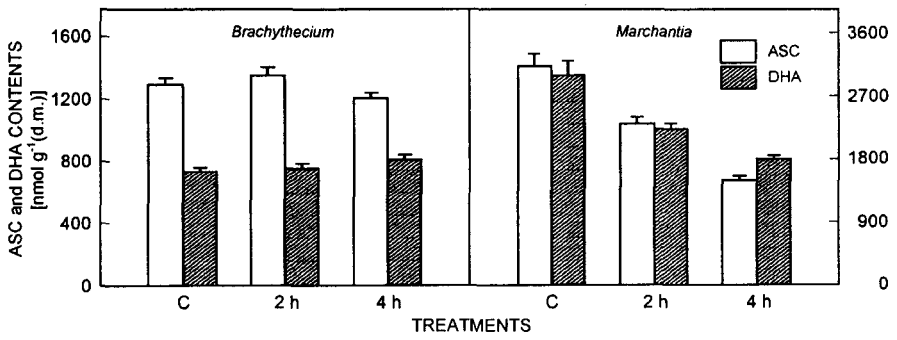

Fig. 2. Ascorbate (ASC) and dehydroascorbate (DHA) contents of *Brachythecium velutinum* and *Marchantia polymorpha* gametophytes fully hydrated (C) and maintained under the air flow of a laminar flow hood for 2 h and 4 h, respectively. Means ± SD, $n = 6$.

The moss did not have changes either in the total content (ASC + DHA) or in the redox state (ASC/DHA), while the liverwort showed a decrease in the ASC and DHA content (Fig. 2).

The ASC peroxidase activity decreased both in the moss and in the liverwort: in the first organisms the final value was only the 20 % lower than the initial while in *M. polymorpha* the enzyme activity dropped to the 75 %.

The activities of AFR reductase showed a moderate decrease (15 %) in the moss and an evident diminution (32 %) in the liverwort. The DHA reductase activity increased (36 %) in the moss while it decreased (35 %) in the liverwort. The ASC oxidase remains unchanged in the moss, while it decreased (20 %) in the liverwort after 4-h treatment (Fig. 3).

Discussion

Data are available in the literature concerning the ascorbate content, the ASC peroxidase and DHA reductase activities in some species of mosses submitted to desiccation and/or irradiance or observed in different geographic areas (Seel *et al.* 1992b, Roy *et al.* 1996). No information is available about the presence and the amount of DHA, and the activity of AFR reductase. Data reported in this work provide a complete picture of the ascorbate redox state and ascorbate metabolism in a moss and a liverwort. Both organisms considered are endowed with ascorbate either in the reduced or oxidized form. The total (ASC+DHA) content is higher in *M. polymorpha*, than in *B. velutinum* while the ASC/DHA ratio is higher in the moss than in the liverwort. That could be explained on the basis of different values of the AFR reductase activity measured in the two organisms. The moss shows an efficient AFR reductase: its activity is three-fold higher than in the liverwort. The AFR reductase plays an important role in ascorbate recycling because it avoids the DHA formation (De Gara and Tommasi 1999, Arrigoni and De Tullio 2002). When AFR reductase works very well, AFR is subtracted to disproportionation to ASC and DHA, the ASC/DHA ratio is maintained shifted towards the reduced form. On the contrary, low AFR reductase activity permits the accumulation of the DHA and the unbalance towards the oxidized form of the ASC/DHA ratio.

Since no data concerning ASC oxidase in bryophytes seems available, it is interesting to note that in both species studied the activity of this enzyme is measurable in the cytosolic fraction. ASC oxidase has been studied

and well characterised in higher plants. The gene and protein sequence, its three-dimensional structure and kinetic features (Messerschmidt *et al.* 1989, Esaka *et al.* 1990, Gaspard *et al.* 1997), as well as the leader sequence driving the apoplasmic enzymatic form into the cell wall (Ohkawa *et al.* 1989) have been published. Nevertheless the role in cell metabolisms at least in angiosperms, is still not clear and the enzyme has only been reported in the cell wall and in the apoplast. Although more studies are necessary to support these hypotheses, the presence of this enzyme in the cytosol could suggest the presence of a light link between cell wall and the enzyme or a new cell localization of the enzyme in bryophytes different from that of the higher plants.

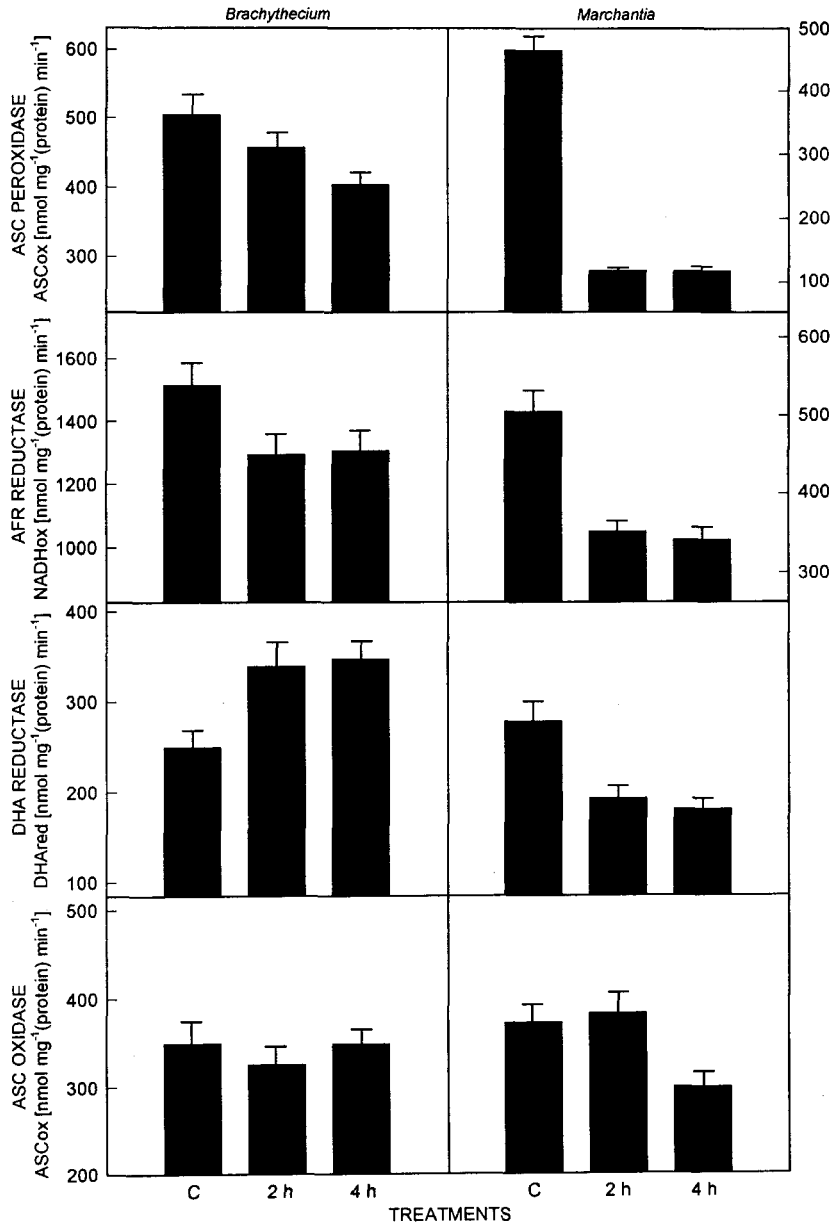

Fig. 3. Activities of oxido-reductive enzymes of ascorbate system in *Brachythecium velutinum* and *Marchantia polymorpha* gametophytes fully hydrated (C) and maintained under the air flow of a laminar flow hood for 2 h and 4 h, respectively. Means ± SD, $n = 6$.

As far as ASC peroxidase was concerned, it is well known that this enzyme plays an active role in the plant to survive in environments polluted by many organic chemical compounds (Roy *et al.* 1996). The values of the specific activity of ASC peroxidase found in *B. velutinum* and *M. polymorpha* are comparable to those reported in *Dicranella palustris*, *Tortula ruraliformis* (Seel *et al.* 1992b) and *Fontinalis antipyretica* (Roy *et al.* 1996). The enzyme is present in both organisms tested with a single protein, as reported in the majority of angiosperms and

gymnosperms previously examined (Paciolla *et al.* 1996, Tommasi and Paciolla 2000). A very different migration rate was observed in the ASC peroxidase of *B. velutinum* and *M. polymorpha*, as observed in organisms belonging to different taxonomic groups (Tommasi and Paciolla 2000). The DHA reductase shows comparable values of activities in the two organisms, higher than those observed by Seel *et al.* (1992b). In addition, native-PAGE shows that many different proteins with "*in vitro*" capability for reducing DHA are present in the moss. This could indicate the existence of proteins showing ability to reduce DHA "*in vitro*" with different roles in cell metabolism, as reported in literature (Tommasi *et al.* 1995, Trumper *et al.* 1994) but also could indicate the presence of different DHA reductase isozymes with different kinetic properties. In the last case, numerous isozymes could provide a better capability to ensure ASC in the reduced form in the cell.

As far as the water content variations are concerned, the liverwort seems to be sensitive to dehydration as indicated by the decrease in the protein content. This is in agreement with data reported by Pence (1998). *B. velutinum* shows a greater resistance to water content variation, confirmed by the stability of protein content. The enzymes of the ascorbate metabolism shows a different behaviour after desiccation: a decrease of the ASC peroxidase and AFR reductase activities occur in both organisms, while the ASC oxidase seems not affected by the water content variations. DHA reductase shows a different behaviour in the two organisms: an increase in the moss after desiccation, a decrease in the liverwort.

The ascorbate pool also does not change in *B. velutinum*, but declines rapidly in *M. polymorpha*. The failure to maintain sufficient ascorbate levels in *Marchantia* could be correlated to oxidative damage, as reported widely in the literature. The greater resistance to drought stress of the studied moss could be due to a higher ability to preserve the ASC pool.

In fact the increase in DHA reductase activity in the moss after desiccation could support the occurrence of an efficient capability to maintain the ASC pool in the reduced form by means of a mechanism controlling the DHA reductase activity. Moreover the following data are reported in the literature: *1)* in *Tortula ruraliformis* a decrease of ASC content occurs together with a low DHA reductase activity in response to desiccation and irradiance stress (Seel *et al.* 1992b); *2)* the desiccation of the sensitive species *Dicranella palustris* results in a slight increase in the enzyme activity in light, while a decrease in the dark was reported (Seel *et al.* 1992b).

Since different behaviours were observed in the ASC metabolism in different species and/or environmental conditions, further studies are necessary to extend knowledge of other antioxidant systems, biochemical and molecular aspects in these two species.

These data seem to indicate that these two species, very common in Italy, could be useful to increase knowledge about the physiology of these organisms and even for their utilization as environmental markers.

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