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Ascorbate System in Plant Development

Oreste Arrigoni[1]

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By using lycorine, a specific inhibitor of ascorbate biosynthesis, it was possible to demonstrate that plant cells consume a high quantity of ascorbate (AA). The *in vivo* metabolic reactions utilizing ascorbate are the elimination of H_2O_2 by ascorbate peroxidase and the hydroxylation of proline residues present in the polypeptide chains by means of peptidyl-proline hydroxylase.

Ascorbate acts in the cell metabolism as an electron donor, and consequently ascorbate free radical (AFR) is continuously produced. AFR can be reconverted to AA by means of AFR reductase or can undergo spontaneous disproportion, thus generating dehydroascorbic acid (DHA).

During cell division and cell expansion ascorbate consumption is more or less the same; however, the AA/DHA ratio is 6–10 during cell division and 1–3 during cell expansion. This ratio depends essentially on the different AFR reductase activity in these cells. In meristematic cells AFR reductase is very high, and consequently a large amount of AFR is reduced to AA and a small amount of AFR undergoes disproportionation; in expanding cells the AFR reductase activity is lower, and therefore AFR is massively disproportionated, thus generating a large quantity of DHA. Since the transition from cell division to cell expansion is marked by a large drop of AFR reductase activity in the ER, it is suggested here that AFR formed in this compartment may be involved in the enlargement of the ER membranes and provacuole acidification.

DHA is a toxic compound for the cell metabolism and as such the cell has various strategies to counteract its effects: (i) meristematic cells, having an elevated AFR reductase, prevent large DHA production, limiting the quantity of AFR undergoing disproportionation. (ii) Expanding cells, which contain a lower AFR reductase, are, however, provided with a developed vacuolar system and segregate the toxic DHA in the vacuole. (iii) Chloroplast strategy against DHA toxicity is efficient DHA reduction to AA using GSH as electron donor. This strategy is usually poorly utilized by the surrounding cytoplasm.

DHA reduction does play an important role at one point in the life of the plant, that is, during the early stage of seed germination. The dry seed does not store ascorbate, but contains DHA, and several DHA-reducing proteins are detectable. In this condition, DHA reduction is necessary to form a limited AA pool in the seed for the metabolic requirements of the beginning of germination. After 30–40 h ascorbate *ex novo* synthesis starts, DHA reduction declines until a single isoform remains, as is typical in the roots, stem, and leaves of seedlings. Finally, DHA recycling also appears to be important under adverse environmental conditions and ascorbate deficiency.

KEY WORDS: Lycorine; AA-peroxidase; Prolyl-hydroxylase; AFR-reductase; DHA recycling.

INTRODUCTION

Many years ago it was reported that lycorine, an alkaloid extracted from *Amaryllidaceae*, at 10 mM

[1] Istituto di Botanica, Via E. Orabona, 4, University of Bari, 70126 Bari, Italy.

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concentration inhibited germination and growth in *Vicia faba* and induced chromosomal aberrations (Yamaguchi and Suda, 1952). Starting from these data we began to study the effects of lycorine on various plants in order to get additional information on its action. Thus, by using much lower concentrations, it was found that lycorine is a powerful growth inhibitor in higher plants and algae (Amico *et al.*, 1972; Liso and Calabrese, 1975). Lycorine also strongly inhibits cell division in rat fibroblasts, but does not affect division of guinea pig fibroblasts (unpublished data). The effect of lycorine (at micromolar concentration) on yeasts is more complex: most of the strains analyzed showed no sensitivity to the alkaloid. However, in some of them, *Schizosaccharomyces pombe* (I MAT-V Pbx) and *Aureobasidium pullulans* (DBV A77), the growth was significantly inhibited by lycorine, while, on the contrary, in some others, *Saccharomycopsis fibulifera* (DBV 3812) and *Cryptococcus terreus* (CBS 1895), it was clearly stimulated by the alkaloid (Garuccio and Arrigoni, 1989).

To better understand lycorine action we analyzed some metabolic responses to the alkaloid. Initial data obtained have shown that the alkaloid does not affect oxidative phosphorylation or translocation of several metabolites in both mitochondria and chloroplasts. Lycorine inhibits both ^{3}H-uridine and ^{14}C-leucine incorporation into RNA and protein, respectively; however, the time courses of these incorporations show that the drop in ^{14}C-leucine incorporated into protein appears prior to any inhibitory effect of ^{3}H-uridine incorporation into RNA (De Leo *et al.*, 1972). Later we reported that lycorine induces a drop in the ascorbate (AA)/dehydroascorbate (DHA) ratio in several tissues (Arrigoni *et al.*, 1975).

Micromolar lycorine concentrations induce a 50–90% decrease in ascorbate content in potato tuber slices. Potato slices, which are widely utilized to study the effects of lycorine, develop cyanide-resistant respiration while synthesizing ascorbate; lycorine inhibits both ascorbate biosynthesis and the elicitation of cyanide-resistant respiration. Actinomycin, puromycin, and cycloheximide also inhibit the development of cyanide-resistant respiration occurring during the aging of the slices (Arrigoni *et al.*, 1976). Since the ascorbate administration only prevents the lycorine effect and not those of puromycin, actinomycin, and cycloheximide action, we concluded that ascorbate is a factor required to develop cyanide-resistant respiration and that lycorine inhibition of the development of such respiration may be due to the primary action of lycorine on ascorbate biosynthesis (Arrigoni *et al.*, 1977a).

To obtain information on lycorine structure–activity relationships, we tested 23 lycorine derivatives for their ability to inhibit ascorbate biosynthesis. Data obtained have shown that: (a) cleavage of the acetate bonds on the dioxole ring has no effect on activity; (b) derivatives with a methoxy group on C-8 (A ring) are inactive; (c) oxidation of NCH_2-7 to an amide group (B ring) causes loss of activity; (d) stereoselective hydrogenation of the double bond of the C ring induces a considerable increase of the alkaloid activity (Evidente *et al.*, 1983, 1986). These results indicate that the 1,2-diol system of the C ring is essential for the inhibitory activity of lycorine on ascorbate biosynthesis (Fig. 1).

ASCORBATE AND CELL DIVISION

Lycorine has proved to be an excellent tool for studying ascorbate-dependent metabolic reactions in ascorbate-synthesizing cells. The use of this alkaloid has allowed, for the first time, the modulation of ascorbate content inside the cell, thus rendering possible the study of metabolic responses to ascorbate changes (Liso *et al.*, 1984).

When explants of lettuce pith parenchyma are cultured on a growth medium containing auxin and cytokinin, DNA synthesis and/or cell division is quickly induced and high mitotic activity begins after a two-day lag. The calluses continue to grow while about 8% of the dividing cells differentiate into tracheids. Adding different concentrations of lycorine (from 1 to 100 μM) to the growth medium inhibits cell division by up to 97%. Furthermore,

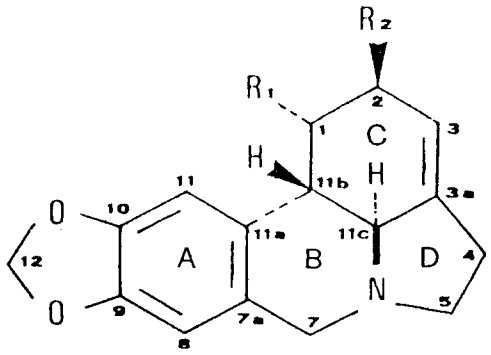


Fig. 1. The structure of lycorine.

lycorine-treated cells of the primary roots of *Vicia faba* show a rapid drop in the mitotic index while interphase cells increase proportionately (De Leo *et al.*, 1972).

Cytophotometric measurements relative to the DNA content in interphase nuclei indicate that lycorine-treated cells interrupt their cell cycle during the G1 and G2 phases. In the meristems of *Allium cepa* roots kept in water, cells with a 2c DNA content (namely, cells in the G1 phase) amounted to 37% of all the cells considered in the analysis; those with a 2c–4c content (cells in the S phase), 35%; and those with 4c (in G2), 28%. Conversely, in meristems treated with lycorine, the proportion of 2c cells increased to 70%, the remaining 30% being 4c cells, whereas no cells were present in the 2c–4c phase. These results clearly indicate that lycorine induces a disappearance of cells in the S phase. Further evidence for this assumption is given by results obtained with [methyl-^{3}H] TdR labeling, since the percentage of labeled interphase cells decreased progressively on increasing the incubation time of *Allium cepa* roots with lycorine. Under the same experimental conditions, the mitotic index (MI) dropped from 9.5 to 3.0 after 12 h. When the duration of lycorine treatment was extended to 21 h, the MI diminished to only 0.4 and 90% of the cells were accumulated in the G1 phase.

Treatment with lycorine drastically lowers the quantity of endogenous ascorbate in the root meristems. When *Vicia faba* roots were treated with lycorine for 12 h, the ascorbate content dropped from 360 μg/100 mg to 72 μg/mg fresh weight; in those of *Allium cepa* the corresponding decrease went from 1070 to 95 μg/100 mg fresh weight. The ascorbate decrease is due to the fact that it is the synthesis, not the utilization of ascorbic acid, which is inhibited in the presence of lycorine (Liso *et al.*, 1984). From these data one can deduce that large amounts of ascorbate are utilized by the meristematic cells.

The progression through the cell cycle does require ascorbate; in fact, the arrest of the cell cycle in the G1 phase produced by lycorine is prevented by exogenous ascorbic acid administration. Adding ascorbic acid not only prevents, but even reverses, the inhibitory effect of lycorine on cell division.

Quiescent-center cells, which are known to have their cycle extended mostly in G1 and to divide rarely, are stimulated to undergo DNA synthesis in root meristems of *Allium cepa* by treatment with ascorbate. The effect of 0.1 mM ascorbate is dramatic: 21% of all the cells in the quiescent center remain in the G1 phase while 79% enter the S phase. The volume of the quiescent center drops and, concomitantly, the volume of the proximal meristem zone increases (from $23230 \times 10^3\ \mu m^3$ in control roots to $30340 \times 10^3\ \mu m^3$ following ascorbate treatment). The resulting apices are wider with an increased number of cell files across the root (Liso *et al.*, 1988). This means that ascorbate stimulates not only the activity of the quiescent-center cells but also cell proliferation in the entire root meristem. Thus, ascorbate could well have a regulatory role in cell proliferation. Since the rate of cell progression through the cycle and cellular ascorbate content are correlated, it may be that higher ascorbate levels bring about a reduction in the duration of the cell cycle by shortening the G1 period. Considering that root meristems are made up of a heterogeneous population of proliferating and nonproliferating cells (Barlow and Macdonald, 1973), which also vary in the duration of their cell cycles (Thomas and Davidson, 1982), it is possible to envisage that the concentrations of ascorbate vary between meristemic cells: cells with low ascorbate could have a longer G1, as do those in the quiescent center, and those with higher ascorbate content could be fast cycling cells (Innocenti *et al.*, 1990). There is some evidence of differences in ascorbic acid levels between cells in the same tissue (Sreekumari and Shah, 1978; Hedge, 1985).

The effects of ascorbic acid on dividing cells in the meristem proper and on the quiescent center of *Allium cepa* root are long-lasting since they have been obtained in both short- and long-term experiments. It is noteworthy that, whatever the time of treatment with ascorbate and whatever the starting size of the quiescent center (in *Allium cepa* the Q.C. has 450 or 1000 cells), there is always a minimum number of quiescent center cells (90–100) which remain in the G1 phase. This supports the hypothesis that a correct root-growth strategy may be safeguarded by a minimum number of quiescent-center cells (Innocenti *et al.*, 1990; Bitonti *et al.*, 1992).

The effect of ascorbate is also detectable on the pericycle cells, which undergo differentiation at the 2c level, serving as an initial site for lateral root formation, and might be considered as a reservoir of quiescent meristematic cells (Clowes 1961; Corsi and Avanzi, 1970; Blakely and Evans, 1979). Ascorbate treatment affects the cellular division in the pericycle line during the development of the *Allium cepa* root. The treatment with ascorbate also extends the upper limits of mitosis

in pericycle cell lines. In other words, in control roots the pericycle stops dividing at a distance of 1000 μm from the tip, whereas in roots grown in ascorbate the capacity for division is maintained up to 1800 μm behind the tip. Furthermore, the labeling index is higher in the treated roots than in control roots, with a difference of more than 50% (Arrigoni *et al.*, 1989).

These data clearly show that the quiescence of pericycle cells in roots is reversed or delayed by ascorbic acid treatment; the extent and the frequency of cell division as well as the number of labeled cells along the pericycle cell line increases following the treatment. In *Lupinus albus* ascorbate also stimulates pericycle cell activity; in fact, a more abundant production of lateral primordia was observed in roots grown in ascorbic acid solution than in roots maintained in water alone. The histochemical localization of ascorbate, as revealed by the reduction of silver nitrate into metallic silver, gives a wide stain intensity in the pericycle nuclei in a zone ranging between 2000 and 25000 μm from the root tip, the zone of the root which corresponds to the onset of metabolic activation for the production of lateral root primordia (Innocenti *et al.*, 1993).

Ascorbate stimulation of cell division is not restricted to the primary root meristem; secondary meristems are also strongly stimulated (paper in preparation).

Ascorbate control of cell division is found to be specific: We have shown that isoascorbic acid has no effect *in vivo* on cell division of *Vicia faba* and *Allium cepa* roots, whereas all ascorbate effects on the cell cycle can be obtained by the addition of 0.5–1 mM galactone-γ-lactone (GL), the precursor of ascorbate. GL is promptly converted to ascorbate in the cell by a very active galactone oxidase. The use of GL instead of AA is preferable in that it avoids the various interactions occurring as ascorbate crosses the apoplast. Furthermore, since GL is converted into AA inside the cell, the effects obtained can be ascribed to the ascorbate actually present within cells.

One unexpected and noteworthy result should be stressed: the roots absorb at pH 6.0 ascorbic acid from the external medium in different ways at different times during the year. When the endogenous ascorbate content and ascorbate peroxidase activity of the roots are elevated, the uptake of external ascorbic acid is also high. When endogenous ascorbate content and ascorbate peroxidase are low, the uptake of ascorbic acid is also low (Innocenti *et al.*, 1994). It is not easy to explain this rhythmic uptake of exogenous ascorbate at a molecular level; however, it is possible to suggest the involvement of a specific carrier for ascorbate which could undergo rhythmic regulation.

ASCORBATE UTILIZATION BY CELL METABOLISM

The first important data obtained by employing lycorine was the demonstration that plant cells are very high ascorbate consumers. The cell metabolism utilizes ascorbate essentially as an electron donor, so that ascorbate undergoes continuous oxidation. The known plant cell metabolic reactions that utilize ascorbate are the elimination of the hydrogen peroxide by means of AA peroxidase and the hydroxylation of the proline residues present in the polypeptide chains which is catalyzed by prolyl hydroxylase. Ascorbate is also oxidized by $O_2^{\cdot-}$, $OH\cdot$, and H_2O_2, Fe^{3+}, Cu^{2+} etc.; however, it is difficult to quantitatively evaluate their contributions to the consumption of ascorbate in the cell.

As yet the role of ascorbate oxidase remains to be defined.

Ascorbate peroxidase is undoubtedly an important hydrogen peroxide-scavenging enzyme for plant cells (De Gara *et al.*, 1993a). Ascorbate peroxidase in higher plants was firstly identified in spinach chloroplasts, organelles lacking catalase (Groden and Beck, 1979), and then in the cytosol of *Euglena gracilis*, an alga that lacks catalase and uses ascorbate peroxidase as its only H_2O_2 scavenging enzyme (Shigeoka *et al.*, 1980). Subsequently, however, we have observed the presence of the enzyme in a wide range of different plants, and found that cytosolic ascorbate peroxidase and catalase both actually cooperate in the removal of hydrogen peroxide (Tommasi *et al.*, 1987). The activity of the enzyme is high and remains constant throughout cell division and cell expansion, 450–500 nmol min^{-1} mg^{-1} protein (Arrigoni *et al.*, 1992). When ascorbate peroxidase activity drops, many metabolic drawbacks occur in the cells; it has been reported that, in *Dasypyrum villosum*, the decrease of AA peroxidase is specifically correlated with the onset of a biochemical pathway leading to morphological anomalies and to the loss of seed germination capacity (De Gara *et al.*, 1991a).

Quantitative analyses of cytosolic ascorbate peroxidase performed in several species of higher

plants, on the basis of their phylogenetic position, show the existence of a consistent rise in enzyme activity along the two phylogenic lines starting from Magnoliales and proceeding, respectively, to advanced dicotyledons and monocotyledons; this trend is, however, much more evident for the latter line.

In the phyletic line proceeding toward advanced dicotyledons, the general rule is the presence of a single cytosolic form of the enzyme. On the other hand, the presence of isoforms is a common feature in advanced monocotyledons; typically three isoforms are seen in *Triticum aestivum* and *Zea mays*, two plants with active oxidative metabolism and high productivity, and consequently producing high rates of toxic activated oxygen species as well as the obvious hydrogen peroxide.

The presence of a single enzymatic form in almost all of the dicotyledons studied accounts for a well-conserved character in a wide variety of plants, and the similar electrophoretic behavior observed among plants belonging to the same families would seem to be a possible starting point for investigating taxonomic relationships. It is worth noting that the improvement

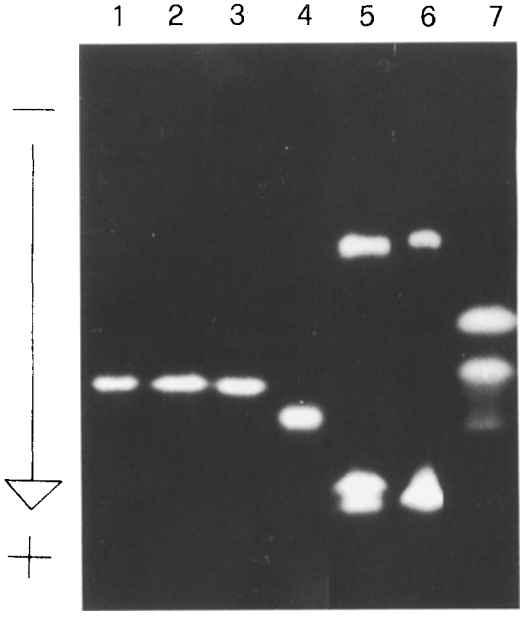


Fig. 2. Native-PAGE for ascorbate peroxidase activity in the dicotyledons *Pisum sativum* (1), *Phaseolus vulgaris* (2), *Vicia faba* (3), *Lupinus albus* (4) and in the monocotyledons *Triticum durum* (5), *Dasypyrum villosum* (6), and *Zea mays* (7). AA peroxidase was located as an achromatic band on a Prussian Blue background resulting from the reaction of ferrichloride and ferricyanide, the latter having been produced by the reduction of ferricyanide by unreacted AA (De Tullio et al., 1993).

in cytosolic ascorbate-dependent peroxidase activity occurring in advanced monocotyledons is not only a matter of quantitative enhancement, but also results from qualitative differences, i.e., the presence of isoforms of the enzyme (Fig. 2).

The presence of isoforms is also of great interest for its physiological implications, since isoforms could suggest differences in kinetic features, such as substrate affinity or differential regulation mechanisms of the isoforms. This means that advanced monocotyledons are seemingly supplied with a much more efficient and adaptable hydrogen peroxide scavenging system than other plants, a system potentially capable of coordinating plant responses to oxidative stresses by means of different expression of their cytosolic ascorbate peroxidase isoforms.

It is well known that prolyl hydroxylase is very active during cell division and cell expansion because a large amount of hydroxyproline proteins is synthesized (Lamport, 1965; Cleland 1968a,b; Chrispeels *et al.*, 1974). Stone and Meister (1962) reported that prolyl hydroxylase utilizes *in vitro* ascorbate as an electron donor; however, Rhoads and Udenfriend (1970) found that many other reducing compounds could replace ascorbate *in vitro*; on the basis of these data, they argued that ascorbate was not the physiological electron donor in the reaction catalysed by prolyl hydroxylase. However, by using lycorine, we obtained some data suggesting high ascorbate consumption could be related to the involvement of the ascorbate in the *in vivo* synthesis of hydroxyproline-containing proteins (Arrigoni *et al.*, 1977a,b).

From our subsequent studies on various plant materials (potato tuber slices, carrot root slices, pea embryos) treated with 3,4-dehydro-D,L-proline (DP), an analogue of proline which specifically inhibits prolyl hydroxylase and does not affect protein synthesis (Cooper and Warner, 1983), it has been possible to confirm that ascorbate is the physiological electron donor in the reaction (De Gara *et al.*, 1991b).

Potato slices incubated in DP have a much lower hydroxyproline proteins content than those kept in water for the same period of time. The process of hydroxylation of the proline residues in the presence of DP is inhibited by 80%. Under these conditions there is a large increase in the AA content; in fact, in the potato slices treated with DP there was 200 nmol/100 mg^{-1} dry weight more than in those kept in water. In carrot slices and pea embryos, the treatment with DP also strongly inhibits the biosynthesis of protein hydroxyproline and at the same time leads to an

increase in AA content with respect to the untreated sample.

The increase in AA found in the tissues treated with DP can be interpreted as a consequence of the reduced activity of the prolyl hydroxylase; in the presence of the inhibitor, the AA, which is used for hydroxylation of the proline in untreated tissues, remains unused and accumulates in the cell. It is therefore reasonable to suppose that such an increase indicates that the AA acts *in vivo* as an electron donor in the process of proline hydroxylation. The data obtained also indicate that there is a correlation between the increase in AA and the decrease in hydroxyproline proteins. The relationship between the percentage AA increase and the percentage hydroxyproline inhibition in all three materials used is of an order of approximately 2.

THE AA/DHA RATIO IN THE CELL

The ascorbate system in plants includes a complex net of AA and DHA-producing reactions (Fig. 3).

The AA/DHA ratio in the cell mainly depends on two events: ascorbate biosynthesis and AA oxidoreduction processes. Ascorbate biosynthesis occurs in all plant cells; however, there is an exception: embryos of *Vicia faba* are not capable of AA biosynthesis until 30 days after anthesis, i.e., during the period of intense growth. During this phase of embryogenesis, characterized by elevated cell division and cell expansion, embryo cells contain large amounts of ascorbate. Therefore it is possible to conclude that the AA present in the young seed is furnished entirely by the parent plant.

Ascorbate is utilized in the cell as an electron donor, and the first AA oxidation product, whatever the oxidative reaction occurring in the cell, is always a semiquinone-like free radical, i.e., AFR. The ascorbate free radical can either be reconverted to ascorbate by AFR reductase, an enzyme catalyzing the reduction of AFR by using NAD(P)H, or spontaneously undergo disproportionation (2 AFR $\rightleftharpoons$ AA + DHA), thus generating DHA (Foerster *et al.*, 1965).

As a third possibility, AFR could be directly oxidized to DHA by means of a specific metabolic reaction requiring AFR as electron donor, which we suggested some years ago, but at the present time there are no data on this subject.

The amount of AFR which undergoes disproportionation in the cell depends on: (a) the extent of the ascorbate oxidative processes, i.e., when ascorbate utilization is high, more AFR is formed; (b) the level of AFR reductase activity. In fact, if AFR reductase activity is high, a large quantity of AFR is reduced to ascorbate, and a small amount of AFR remains available for disproportionation, so that DHA formation is low; conversely, when AFR reductase is low, a smaller amount of AFR is reconverted to ascorbate, and most of the AFR undergoes disproportionation, generating DHA in large quantities.

DHA accumulation is generally considered as a negative event for cell metabolism. The changes in the AA/DHA ratio are constantly accompanied by a decrease in growth and respiratory activity (Laudi, 1955). Dehydroascorbate inhibits some dehydrogenase activity in both plant and animal

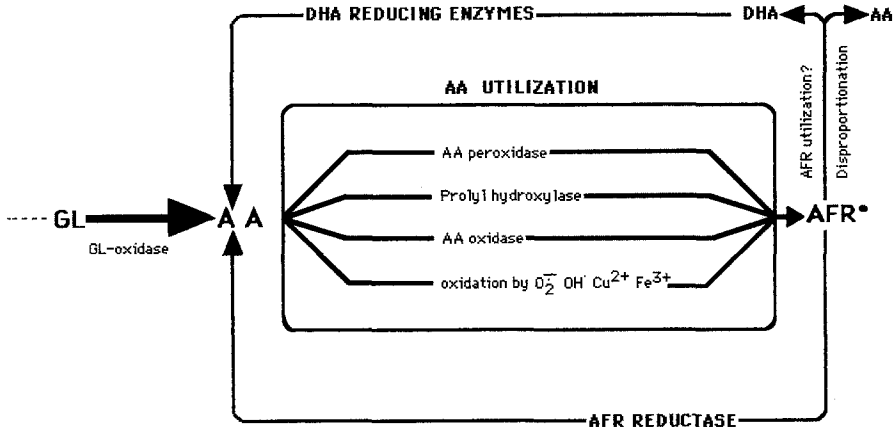


Fig. 3. General scheme of the ascorbate system in higher plants.

mitochondria (Marrè *et al.*, 1995; Marie and Laudi, 1955; Pece *et al.*, 1956) and inhibits oxidative phosphorylation (Marrè *et al.*, 1956; Arrigoni, 1957; Forti, 1958). It is necessary to mention that among the three chemically close related compounds, ascorbic acid, dehydroascorbic acid, and diketogulonic acid (DKG), only DHA inhibits *in vitro* phosphorylation and dehydrogenase activity. DHA inhibition of these metabolic reactions is detectable at pH 7, i.e., a pH at which, in pure solutions, DHA is quickly transformed into the ineffective diketogulonic acid. The explanation of this apparent contradiction is that in the presence of proteins, DHA escapes its spontaneous conversion into the inactive DKG. This is due to the formation of complexes between DHA and mitochondrial proteins, presumably at the SH level since linking between proteins and dehydroascorbate is prevented by *p*ClHgB, a typical SH reagent (Arrigoni, 1958).

Different strategies are employed by cells to counteract dehydroascorbic acid toxicity. Meristematic cells are endowed with an elevated AFR reductase activity (Arrigoni *et al.*, 1957). This enzyme is present in mitochondria, the ER, the cytosol, plasma membrane, and other cell compartments (Arrigoni *et al.*, 1981; Craig and Crane, 1982; Navas, 1991; Luster and Buckhout, 1988; De Gara *et al.*, 1993b); this means that the overall capability of recycling AA from AFR is much higher in meristematic cells. As a consequence, the formation of DHA is low and the AA/DHA ratio is very high, 6–8, in these cells (Laudi, 1955; Trezzi, 1956).

In differentiated cells, i.e., parenchymatic cells, total AFR reductase activity is less than 50% of that in meristematic cells. In these cells AFR is less efficiently recycled to AA than in meristematic cells, and the ascorbate free radical is massively disproportionated into DHA; since DHA recycling is also not very efficient because DHA reductase activity is low or lacking, the AA/DHA ratio in these cells goes down to much lower values, 1–3 (Trezzi, 1956; Tommasi *et al.*, 1990). Differentiated cells, which are provided with a developed acid vacuolar system, use this cellular compartment to store toxic dehydroascorbic acid. We have demonstrated that in parenchymatic cells in potato tuber slices more than 70% of the total DHA is segregated in the vacuole (Liso *et al.*, 1977); similarly, in pea internodes the DHA reducible by dehydroascorbate reductase is about 25% of the total (Laudi 1955; Liso and Calabrese, 1974).

The problematic presence of DHA is resolved differently in chloroplasts. It is well known that during photosynthesis chloroplasts produce superoxides $O_2^{\cdot-}$, H_2O_2, and also $OH\cdot$, as a consequence of the reaction between these compounds. The high level of ascorbate they contain is used to scavenge $O_2^{\cdot-}$ and $OH\cdot$, probably nonenzymatically, and H_2O_2 by a means of a specific ascorbate peroxidase, an enzyme which is highly sensitive to the presence of ascorbate (Groden and Beck, 1979). In all these reactions in which AA is oxidized, AFR is produced. Chloroplasts contain AFR reductase; however, the activity of the enzyme is low (Marrè and Arrigoni, 1958); consequently AFR largely disproportionates, forming a high amount of DHA, but dehydroascorbate does not accumulate in the chloroplast because it is continuously reduced to ascorbate by using reduced gluthatione as an electron donor (Foyer and Halliwell, 1976). Chloroplast DHA reduction is very efficient because a rise in pH occurs in chloroplast stroma during photophosphorylation caused by proton transport from the stroma into the thylakoid. This stroma alkalinization enables efficient GSH dependent dehydroascorbate reduction to ascorbate. It can therefore be assumed that the chloroplast strategy against toxic dehydroascorbate is efficient DHA recycling.

THE PROMINENT ROLE OF AFR REDUCTASE

A constant condition regarding both animal and plant cells is that high AFR reductase activity is always strictly correlated with intense cell division. AFR reductase activity in virus-transformed mouse fibroblasts, showing high cell proliferation, is two times that observed in normal fibroblasts (Arrigoni *et al.*, 1981). Further confirmation that high AFR reductase activity is closely associated with cell division comes from our recent data: when explant of lettuce pith cells restore *in vitro* cell division a prompt rise of AFR reductase occurs and the AA/DHA ratio in the cells rises (unpublished data).

Studies on the biochemical responses of the ascorbate system in plants under pathogen stress provide additional data. Pathogen attack induces a high biosynthesis of proteins containing hydroxyproline and at the same time an increase of AFR reductase activity in resistant cultivars of *Lycopersicum esculentum*; AFR reductase present in ER increases 2.5–4 fold, while no change in AFR reductase activity is detected in other cell compartments (Zacheo *et al.*, 1981).

It is well known that hydroxyproline proteins are synthesized as ordinary proline-containing polypeptide chains and that, subsequently, some of the proline residues present in the chain are hydroxylated by means of a peptidylproline hydroxylase. The hydroxylation process occurs in the endoplasmic reticulum; since ascorbate is specifically required as an electron donor (De Gara *et al.*, 1991), it follows that a high quantity of ascorbate is oxidized to AFR in this cell compartment. It can therefore be deduced that the large increase in AFR reductase observed in the endoplamic reticulum is correlated to the biosynthesis of hyproproteins. In susceptible plants no increase of AFR reductase nor increment of hyproproteins biosynthesis occur following nematode attack. If we consider that nematode attack occurs at the level of the root meristem, it appears quite clear that a high level of AFR reductase is necessary to maintain high cell division in the root apex.

We can ask ourselves why AFR reductase activity is so high in dividing cells. It is well known that meristematic cells, being large ascorbate consumers, are also high AFR producers. All the researchers who have been studying AFR reductase, this author included, always considered it an essential enzyme for maintaining the ascorbate system in the reduced state. It is self-evident that AFR reductase regenerates ascorbate, but I now believe that its major goal is to limit the amount of AFR undergoing disproportionation. To limit AFR disproportionation is of great relevance, since this is the reaction leading to DHA formation, and DHA, as already mentioned, is very dangerous for nonvacuolated meristematic cells. This explains how important it is to limit DHA formation by means of high AFR reductase activity in these cells.

We have suggested that the drop in AFR reductase activity could mark the passage from the cell division to the cell expansion phase (Arrigoni *et al.*, 1992), and that the decrease in AFR reductase and the subsequent rise in ascorbate free radical are possibly part of the complex phenomenon of vacuolization. This seems to be likely, since some years ago Hidalgo *et al.* (1989) reported an important finding. They discovered that administration to the roots of solutions containing equimolar concentrations of ascorbate and dehydroascorbate, the mixture that generates AFR, induces cell vacuolization. Although this exogenous AFR-generating system utilized for *in vivo* studies has some limitations, we believe that the data of Hidalgo *et al.* make it possible to hypothesize the existence of a reaction specifically requiring AFR as an electron donor which could work at the endoplasmic reticulum level and could be closely related to provacuole formation. Since ascorbate utilization remains constant throughout cell division and cell expansion, while AFR reductase activity in the ER strongly decreases, we suggest that AFR formed in this compartment may be utilized as an electron donor to activate an allosteric proton pump involved in ER membrane enlargement and provacuole acidification. By means of this reaction AFR is oxidised and the DHA produced is vectorially transported into the forming vacuolar system together with the much more abundant DHA formed by AFR disproportionation in the surrounding cytosol (Fig. 4).

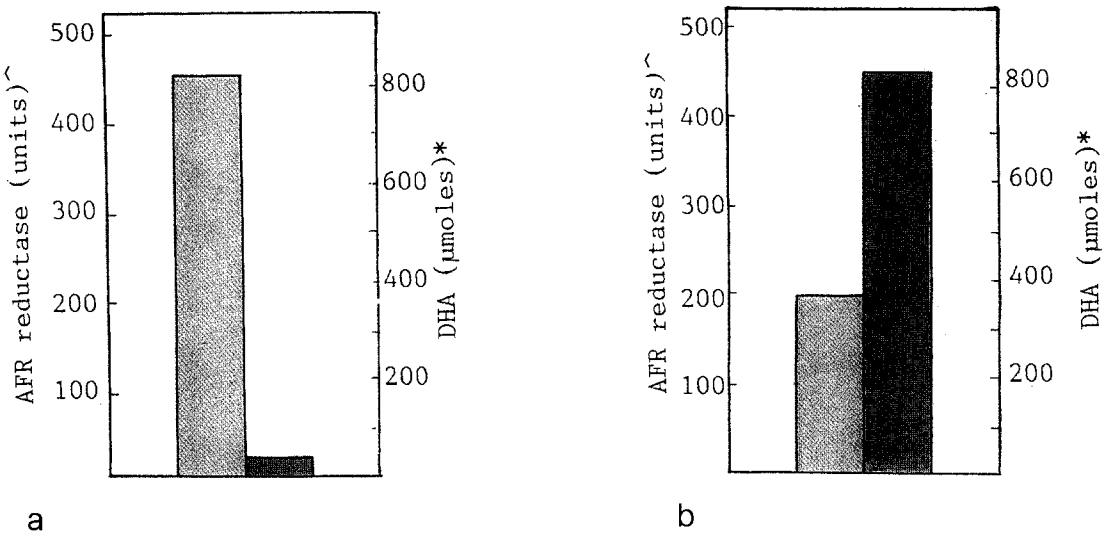
ASCORBATE DEFICIENCY AND DHA RECYCLING

Ascorbate recycling by DHA reduction is usually little used by plant cells since, as previously stated, DHA reductase is low or lacking. Our recent data show that DHA reductase plays a key role in the seed during the early stages of germination.

Dry seeds of *Vicia faba* are devoid of ascorbate and only contain a small amount of dehydroascorbate and DHA reductase (Arrigoni *et al.*, 1992); moreover, consistent AA synthesis starts only after several hours of germination (De Gara *et al.*, 1987). Under these conditions, DHA reductase activity is necessary to form a limited initial AA pool adequate for the metabolic requirements of early germination by reducing the DHA present in the seed. We have observed that DHA reductase is already detectable in the embryos of dry seeds and its activity declines to a lower steady-state level when AA *de novo* biosynthesis becomes functional.

Time courses of dehydroascorbate reductase in embryos of *Pinus pinea* during the early stages of seed germination clearly show the transient role of some DHA-reducing proteins (Fig. 5A). After 40 hours germination a single isoform remains, as is typical in the roots, stem and leaves of seedlings (Fig. 5B).

Our recent data demonstrate that ascorbate recycling through DHA reduction appears important in adverse environmental conditions. Caryopses obtained from plants of *Dasypyrum villosum* grown under water stress produce seedlings with a high capability to reduce DHA, and a new protein showing DHA reductase activity appears (Fig. 6).



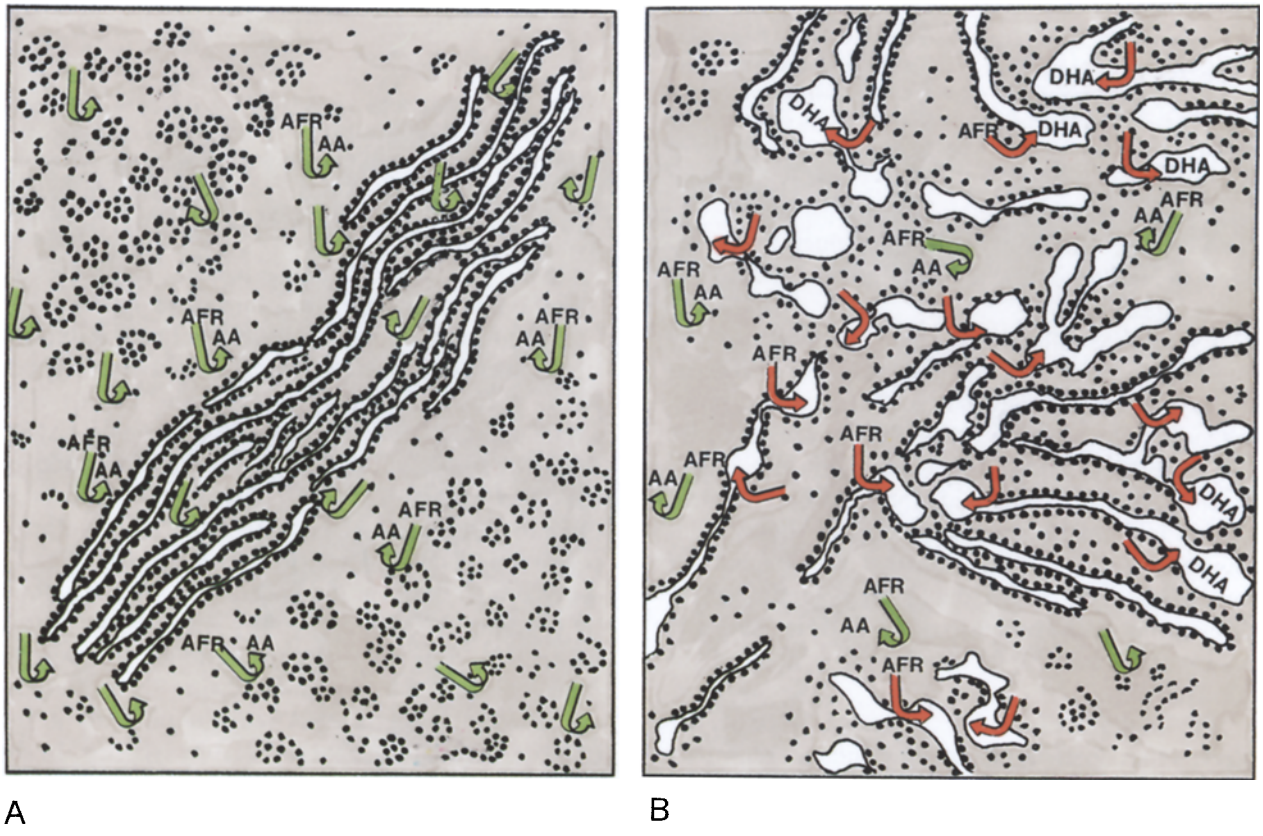


Fig. 4. A possible AFR reductase activity-regulated mechanism for provacuole formation. Above: AFR reductase activity (green bars) and DHA content (red bars) in dividing cells (a) and expanding cells (b) of *Vicia faba* developing embryos. (a) 12 days from anthesis; (b) 30 days from anthesis. ^1 unit = 1 nmol NADH oxidized/min/mg protein; *μmoles/embryo. Below: representation of the endoplasmic reticulum of a dividing cell (A) and an expanding cell (B) endowed with high and low AFR reductase activity (green arrows) respectively. In B, low AFR reductase allows disproportionation to DHA. Red arrows indicate a possible AFR-requiring reaction mediating enlargement of ER membranes.

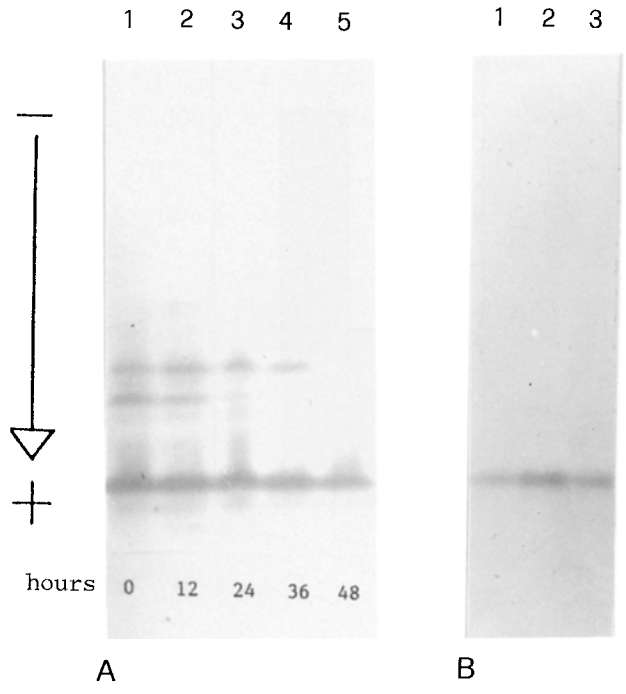


Fig. 5. Native-PAGE for DHA reductase activity in *Pinus pinea* (A) time-course of DHA reducing enzymes in germinating embryos. (B) DHA reducing enzymes in seedlings: (1) apical root meristem; (2) shoot apex; (3) cotyledon leaves. DHA reducing enzymes were detected as dark blue bands on a light blue background (De Tullio et al., 1993).

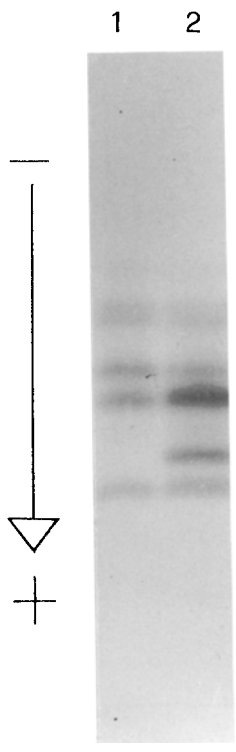


Fig. 6. Native-PAGE for DHA reductase activity in seedlings of *Dasypyrum villosum* showing the induction of a supplementary DHA reducing enzyme in water stressed plants. Lane 1, control; lane 2, stressed plant.

A strong increase of DHA reduction occurs under ascorbate deficiency conditions induced by lycorine treatment.

Lycorine inhibits ascorbate biosynthesis by inhibiting the activity of galactone lactone oxidase, the enzyme which catalyzes the convertion of galactone lactone to ascorbate (De Gara *et al.*, 1990; De Gara *et al.*, 1994). As ascorbate utilization in the cell is not affected by the alkaloid, the ascorbate content drops in lycorine treated tissues. Under these conditions cells undergo a situation that we designate as simulated scurvy, which could be potentially very troublesome both because of AA deficiency and because of the relative increase in the content of the toxic DHA.

In 20 years of studies involving lycorine, we observed that different plants show different sensitivity to the action of the alkaloid in terms of both AA content and cell viability. *Lupinus albus L.* is probably the most lycorine-sensitive plant every studied (1 μM lycorine clearly inhibits growth), but a great deal of other plants have also proved to be very sensitive. Advanced monocots are significantly less sensitive to the action of the alkaloid, especially *Zea mays L.* which is a fairly lycorine-resistant plant.

Lycorine treatment of maize embryos induces a strong increase in DHA reduction activity, whereas AFR reductase activity seems unchanged. The electrophoretic assay, performed using our native-PAGE method, allowed further characterization of the effect of lycorine on DHA reductase activity: a dramatic increase in the activity of the isoforms performing DHA reduction was observed (Fig. 7). As lycorine treatment diminishes the AA/DHA ratio by lowering AA content, embryos were also treated with DHA, thus affecting the ratio by following a different approach. Treatment with DHA induces a marked increase of DHA reduction activity in the cells, quite similar to that induced by lycorine. In the presence of both 0.5 mM DHA and 0.1 mM lycorine, the effect on DHA reduction appears clearly higher.

Interestingly, lycorine seems to have no effect on DHA reduction in lycorine-sensitive plants. Spectrophotometric analysis and the electrophoretic profile of DHA reductase from *Lupinus albus* L. embryos show

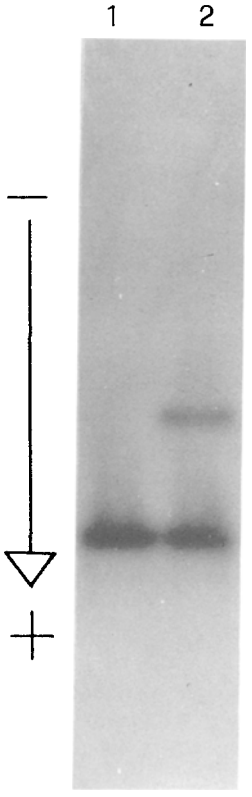


Fig. 7. Native-PAGE for dehydroascorbate reductase activity in *Zea mays* embryos incubated for 12 h in water (1) and 50 μM lycorine (2).

that lycorine treatment induces neither activity nor appearance of isoforms. Similarly, no response was observed in other lycorine-sensitive plants, such as *Pisum sativum* L. embryos and *Vicia faba* L. root meristems. All these data suggest that lycorine-resistant plants, in addition to the higher levels of DHA reductase observed in physiological conditions, are also endowed with a molecular mechanism allowing them to minimize the negative effect of lycorine on AA biosynthesis by eliciting AA recycling be means of DHA reductase activity.

In spite of the large number of early reports of the presence of DHA reductase in plant and animal tissue (Szent Gyorgyi, 1928; Borsook *et al.*, 1937; Yamaguchi and Joslyn, 1951), little is known as yet about the physiological role of this enzyme that catalyzes the reduction of DHA to AA by using glutathione (GSH) as a reducing substrate. Recent findings about the DHA reduction activity of thioredoxin, glutaredoxin, and protein disulfide isomerase (Wells *et al.*, 1990), as well as the presence of the latter as a subunit of the AA-dependent enzyme prolyl-hydroxylase (Pihlajaniemi *et al.*, 1987), have given new elements to the discussion on the physiological role of enzymatic DHA reduction in plant cell.

The large number of isoforms observed using our technique (De Tullio *et al.*, 1993), which highlights activity as opposed to specific proteins, is likely to indicate that enzymatic DHA reduction is performed by different proteins, in accordance with the above-mentioned papers. At the present time we do not have precise information concerning the molecular mechanism leading to DHA reduction activation. It has been reported that a decreased level of AA could act as a metabolic signal leading to increased GSH synthesis in both animals (Meister, 1992) and plants (May and Leaver, 1993). Although we lack direct

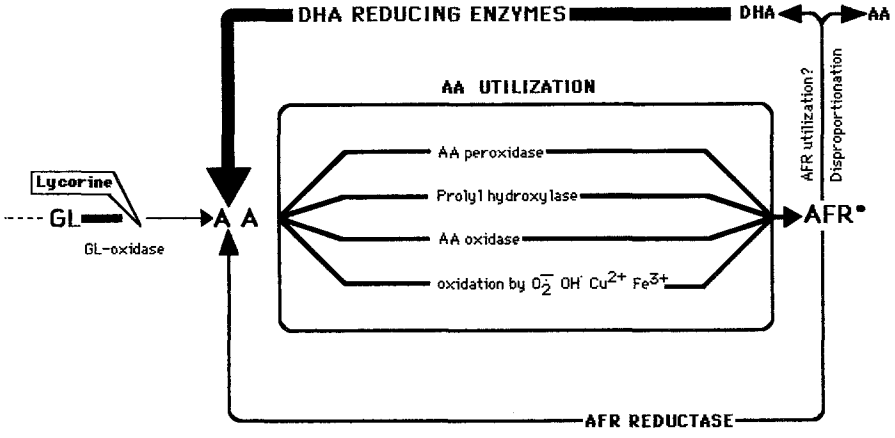


Fig. 8. Scheme of ascorbate system showing the enhancement of DHA-reducing enzymes occurring in lycorine-treated maize embryos. When ascorbate biosynthesis decreases, a prompt rise of DHA-reducing enzymes occurs.

information on GSH content in maize embryos, the possible activation of GSH synthesis and/or enhancement of GSH reductase activity could also be involved in the activation we observed in DHA reduction.

Our data seem to indicate that the AA/DHA ratio could play a critical role in starting a possible pathway that leads to DHA reduction activation. We hypothesize that, in cases of both lycorine and DHA treatment, the key molecule that induces the response is DHA, as lycorine administration results in a relative rise in DHA content.

The observations that lycorine treatment is ineffective in enhancing AFR reductase activity while on the contrary, it does enhance DHA reduction allow us to hypothesize that AFR reductase, DHA reductase, and, more generally, DHA reduction have different roles in plants.

A major role for AFR reductase in AA recycling, under physiological conditions, is widely accepted. AFR reductase activity is a key enzyme for dividing cells. High AFR reductase present in these cells is essential in order to prevent excessive DHA formation by AFR disproportionation; dehydroascorbate accumulation is very dangerous for nonvacuolated cells.

DHA reductase has an important physiological role in the early stages of seed germination before the beginning of ascorbate *ex novo* biosynthesis, but its activity is clearly less relevant in the subsequent stages of the plant development. DHA reduction becomes a determinant for plant growth when marked conditions of ascorbate deficiency or imbalanced AA/DHA ratios occur (Fig. 8). However, there are remarkable differences between the capability of various plants to tolerate low ascorbate conditions. The plants best equipped to survive prolonged periods of ascorbate deficiency are those capable of enhancing DHA reduction whereas those least resistant are unable to increase DHA reduction. In this context it appears quite interesting that, among the plants capable of efficiently counteracting ascorbate deficiency, the advanced monocotyledons are the most successful.

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