Environmental and Experimental Botany 109 (2015) 151–160

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Iron in complex with the alleged phytosiderophore 8-hydroxyquinoline induces functional iron deficiency and non-autolytic programmed cell death in rapeseed plants

Rajesh Kumar Tewari[a,*], Gert Bachmann[b], Franz Hadacek[c]

[a] Terrestrial Ecosystem Research (TER), Faculty of Life Sciences, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria
[b] Molecular System Biology (MOSYS), Faculty of Life Sciences, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria
[c] Albrecht-von-Haller Institut, Plant Biochemistry, Georg-August-Universität Göttingen, Justus-von-Liebig-Weg 11, 37077 Göttingen, Germany

ARTICLE INFO

Article history:
Received 2 February 2014
Received in revised form 23 July 2014
Accepted 25 July 2014
Available online 4 August 2014

Keywords:
Brassica napus
Iron
8-Hydroxyquinoline
Programmed cell death
Photosynthesis
Reactive oxygen species

ABSTRACT

The alkaloid 8-hydroxyquinoline (HQ) was pointed out as potential phytosiderophore that is taken up unspecifically by iron acquisition strategy I and II plants. HQ in root exudate of the knapweed *Centaurea diffusa* even was considered as a factor contributing to the invasive success of this species. This study compares the iron supply efficiencies of the Fe–HQ complex and Fe–EDTA in hydroponic cultures of rapeseed (*Brassica napus*) as a plant model system to explore the proposed function. Iron ($FeCl_3$) was supplied in 2 and 10 μM concentrations in a 1:1 ratio with the ligand (HQ). After 20 days, Fe content, lipid peroxidation, superoxide anion, hydrogen peroxide, and ascorbic acid concentrations, various enzyme activities associated with antioxidant defences and programmed cell death (PCD), and nuclear condensation were determined. Iron supply in the form of a Fe–HQ complex clearly was less efficient. These plants developed chlorosis and showed symptoms of non-autolytic PCD similar to those that had been subjected to Fe deprivation. Reactive oxygen species (ROS) concentration levels and enzyme activities (superoxide dismutase, catalase, alkaline protease, caspase-3-like and deoxyribonuclease) resembled more those observed in plants which suffered from Fe deprivation, than those of Fe-sufficient Fe–EDTA supplied plants. The results do not support the putative phytosiderophore function alleged to HQ in the studied concentration range (2–10 μM) but instead corroborate the one hypothesis explaining HQ toxicity: Fe in complex with HQ is not released efficiently enough from the complex to be available for metalloenzymes that require it as a co-factor.

1. Introduction

Iron is the fourth most abundant element in the earth's crust and an essential element for all forms of life (Frey and Reed, 2012). Its limitation has a profound impact on the productivity of photosynthetic organisms (Jeong and Guerinot, 2009). Similarly as Cu, Mn and Zn, Fe is an important cofactor of many enzymes (Broadley et al., 2012). Its solubilisation and long-distance transport from roots to shoots, as well as its subcellular storage and remobilization, require redox changes to obtain the best solubility for transport processes in a buffered environment and involve formation of specific coordination complexes with low- molecular-weight metabolites and proteins (Briat et al., 2007). Although Fe is abundant in the Earth's crust, much of it exists in insoluble forms and is thus not freely available to plants. In dicotyl angiosperms, which represent the majority of present-day flowering plants, the uptake of Fe is supported by reduction of external Fe^{3+} to Fe^{2+} by the cell membrane-associated protein ferric chelate reductase and acidification of the rhizosphere (Kobayashi and Nishizawa, 2012). By contrast, grasses exude low-molecular-weight molecules, such as mugineic acid, as specific phytosiderophores (Broadley et al., 2012; Ma, 2005).

Processes to remove redundant or damaged cells in eukaryotic tissues are designated as programmed cell death (PCD); however, the same phenomenon also represents an important process of cell differentiation and organ development (Greenberg, 1996; Lam,

Abbreviations: AA, ascorbate; DCF, dichlorofluorescein; DHA, dehydroascorbate; EDDHA, ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid); EDTA, ethylenediaminetetraacetic acid disodium salt; H_2DCF-DA, 2′,7,-dichlorodihydrofluorescein diacetate; HQ, 8-hydroxyquinoline; NBT, *p*-nitroblue tetrazolium; PCD, programmed cell death; ROS, reactive oxygen species.

* Corresponding author. Tel.: +43 1 4277 54262.
E-mail addresses: rajesh.tewari@univie.ac.at, rktewari_bot@yahoo.com (R.K. Tewari), gert.bachmann@univie.ac.at (G. Bachmann), franz.hadacek@biologie.uni-goettingen.de (F. Hadacek).

http://dx.doi.org/10.1016/j.envexpbot.2014.07.016
0098-8472/

2004). In plants, autolytic and non-autolytic PCD are differentiated on the basis of cell shrinking and cell swelling. Necrotic death is included into non-autolytic PCD, which also occurs during the hypersensitive response to pathogen infections (van Doorn, 2011; van Doorn, 2005). One of the best characterized forms of PCD in animals is apoptosis, which involves activation of a battery of highly specific proteolytic enzymes called caspases [cysteine-dependent aspartate-specific proteases (Chichkova et al., 2012)]. Despite the absence of caspase orthologs in plant genomes, cells undergoing PCD show caspase-like activity (Belenghi et al., 2004). In contrast to animal pro-caspases, plant enzymes with caspase-like activity are generally secreted from healthy cells into the apoplast and transported back into the cell during PCD execution (Chichkova et al., 2010). ROS generation in chloroplasts may be involved in non-autolytic PCD (Tewari et al., 2013). Chloroplasts contain 80% of the cellular Fe (Solti et al., 2012; Broadley et al., 2012). Consequently, Fe deficiency often severely affects chloroplast function. Iron deficiency also induces PCD in animal models by increasing cytosolic Ca^{2+} concentrations, energy depletion and annexin expression in mouse erythrocytes (Kempe et al., 2006) and caspase-3 activation in human Raji cell lines (Koc et al., 2006).

8-Hydroxyquinoline (HQ) is a simple aromatic alkaloid with antibacterial, antifungal, cyto- and phyto-toxic activities (Leanderson and Tagesson, 1996; Tharayil et al., 2009; Chobot et al., 2011). The general view is that HQ toxicity may depend on too efficient transition metal chelation that negatively affects the cofactor availability for metalloenzymes (Pierre et al., 2003; Chobot and Hadacek, 2010). Alternatively, strong free radical scavenging activities that quench ROS signalling properties have been suggested also as a plausible mode of action (Chobot et al., 2011).

Despite its documented toxicity in higher concentrations in a wide range of organisms, HQ was suggested to be utilized by the invasive knapweed species *Centaurea diffusa* for Fe uptake (Tharayil et al., 2009). No structural homology exists between HQ and the phytosiderophores (nicotianamine derivatives, mugineic acid) released by grasses. However, Fe–HQ complexes were shown to be able to supply the *yellow stripe1* mutant of *Zea mays* with sufficient Fe to recover from chlorosis. This maize mutant lacks the *YS1* gene that encodes a transporter protein for Fe-phytosiderophore uptake. Iron–EDDHA complexes, by contrast – EDDHA [ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid)] is a commonly used Fe chelator to treat Fe deficiency on calcareous soils (Rojas et al., 2008) – failed in reverting the chlorosis symptoms. In consequence, Tharayil et al. (2009) proposed an unspecific uptake mechanism for Fe–HQ complexes, which is independent of YS1 transporter proteins and could be utilized theoretically both by strategy I and II plants. The YS1 transporter protein is a specific characteristic of strategy II grasses that rely on Fe-phytosiderophore coordination complexes for Fe uptake; strategy I plants – more or less all other plants apart from grasses – rely on Fe mobilization by NAD(*P*)H-dependent ferric chelate reductase and root surface acidification by ATPase (Ma, 2005).

We have utilized hydroponically grown *Brassica napus* as a model plant system that allows to explore Fe uptake mechanisms when it is present either as ion or in coordination complexes with natural and synthetic ligands. In this system, Fe deprivation triggers non-autolytic cell death (Tewari et al., 2013). A sufficient Fe supply was achieved by supplying Fe in the form of a Fe–EDTA complex, a commonly used Fe-source in hydroponic studies. To explore the effect of Fe–HQ complex as a potential supply source to enhance Fe availability in plants (Tharayil et al., 2009), we compared the effects of an Fe–EDTA supply to that of the Fe–HQ complex in identical concentration ranges (2 and 10 μM; higher concentrations were not possible due to phytotoxic effects of HQ at higher concentrations). The ratio of the potential central atom and the ligand was always 1:1. If the Fe–HQ complex represents a universal Fe source for plants, as suggested by Tharayil et al. (2009), no non-autolytic PCD symptoms should develop in Fe–HQ supplied plants. Similar to a previous study (Tewari et al., 2013), apart from the actual tissue Fe concentration, effects on chloroplast pigment biosynthesis (chlorophyll and carotenoids) and photosystem II efficiency were determined to obtain information about electron transport system-specific effects; oxidative stress was assessed from the relative levels of superoxide anion ($O_2^{\cdot -}$), H_2O_2 and lipid peroxidation (•OH, hydroxyl radical); antioxidant defences were characterized by the ascorbic acid concentration and superoxide dismutase, catalase and peroxidase-like activities. Development of non-autolytic PCD was monitored by nuclear condensation and caspase-3-like, alkaline protease and DNase activities.

2. Material and methods

2.1. Chemicals

All non-specifically annotated chemicals were analytical grade and obtained from Sigma-Aldrich (Schnelldorf, Germany). Water was Milli-Q quality.

2.2. Iron complexes

Stock solution of ethylenediaminetetraacetic acid disodium salt (EDTA, 10 mM) and ferric chloride (10 mM) were prepared in Milli-Q water. A stock solution of 8-hydroxyquinoline (8-HQ, 10 mM) was prepared in 50% (v/v) ethanol. Fe–EDTA and Fe–HQ complexes were prepared by mixing equal volumes of ferric chloride and solutions of EDTA and 8-HQ, respectively. Fe–HQ was prepared freshly and stored in a dark bottle before supply. The blue-coloured Fe–HQ complex and red coloured Fe–EDTA complex were supplied to the plants in the nutrient solution.

2.3. Hydroponic cultures and iron supply variations

B. napus plants were grown in aerated hydroponic culture in the glasshouse (night and day temperature 20 and 25 °C, respectively; relative humidity 50% and light intensity at noon 400 μmol m^{-2} s^{-1}). Initially, 15-day-old seedlings were grown in full nutrient solution for one week (Hewitt, 1966): 2.0 mM KNO_3, 2.0 mM $Ca(NO_3)_2$, 1.0 mM $MgSO_4$, 0.67 mM NaH_2PO_4, 0.05 mM NaCl, 0.05 mM Fe–EDTA, 5.0 μM $MnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM $ZnSO_4$, 16.5 μM H_3BO_3, 0.1 μM Na_2MoO_4, 0.05 μM $CoSO_4$ and 0.05 μM $NiSO_4$. The pH of the nutrient solution was adjusted to 6.7 ± 0.2 at the time of supply. The pH (6.7) of nutrient solution was based on previous studies (Hewitt, 1966) and was adequate for luxurious availability of Fe and other mineral ions in hydroponic culture of rapeseed (Tewari et al., 2013). After a week of transplantation, pots were divided into five groups. Roots of plants rinsed with water before initiation of treatments. Whereas plants in group 1 were not supplied with Fe (Fe-deprived, a positive control for cell death), those in group 2 and 3 were supplied with 2 and 10 μM Fe–EDTA and group 4 and 5 supplied with 2 and 10 μM Fe–HQ. Since we dissolved 8-HQ in 50% (v/v) ethanol, a similar concentration of ethanol (2.2 or 10.8 μM) was supplied to the plants treated with Fe–EDTA. The volume of nutrient solution was adjusted to the same volume with Milli-Q water daily, and the nutrient solution changed every third day. Plant leaves were sampled after 20 days of differential Fe supply for the different analyses.

2.4. Ferric chelate reductase activity of roots

The activity of root ferric-chelate reductase was quantified using the ferrozine assay as described elsewhere (Schmidt et al., 2000). The assay solution consisted of 0.5 mM Fe–EDTA, 0.5 mM $CaSO_4$