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Research article

A bifasic response to cadmium stress in carrot: Early acclimatory mechanisms give way to root collapse further to prolonged metal exposure

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ABSTRACT

Very few studies have provided information about the effects of cadmium (Cd) at histoanatomical and ultrastructural levels, along with potential localization of the metal *in planta*. In particular, from this standpoint, almost nothing is known in *Daucus carota* L. (carrot), a particularly important species for *in vitro* and *in vivo* functional investigations. In this work we hypothesized that 36 μM Cd, supplied for 1, 2, 3, 4, 7 and 14 days to 30-day-old *in vitro*-cultured plants, might induce an early acclimation, but a final collapse of roots and leaves. In fact, as a general feature, a biphasic root response to Cd stress actually took place: in the first phase (1–4 days of Cd exposure), the cytological and functional events observed – by light microscopy, TEM, epifluorescence, as well as by the time-course of thiol-peptide compounds – can be interpreted as acclimatory responses aimed at diminishing the movement of Cd across the root. The second phase (from 4 to 14 days of Cd exposure) was instead characterized by cell hypertrophy, cell-to-cell separation events, increase in α–β–γ–tocopherol levels and, not least, endocytogenic processes, coupled with a dramatic drop in the amount of thiol-peptide compounds. These events led to a progressive root collapse, even if they did not ingenerate macro/microscopic injury symptoms in leaf blades and petioles.

1. Introduction

Plants demonstrate a natural ability to extract minerals from soil, and to distribute them between the roots and the aerial organs, depending on the biological processes these elements are involved in [1]. Some heavy metals, such as zinc (Zn) and copper (Cu), in suitable concentrations, play vital roles in the metabolism of plants, and are indeed micronutrients, whereas metals such as cadmium (Cd), whilst actually toxic, are still extracted from the soil and introduced into the plant first and foremost at the root level, where they can cause damage to a greater or lesser extent [1,2].

The accumulation of Cd in roots and above-ground organs has been investigated in various species, mainly in terms of atomic absorption/emission spectrophotometric analysis, in order to gain quantitative information that might be of interest for basic and applied research on metal tolerance [3]. However, much less effort has been dedicated to investigating the spatial and temporal relationships of Cd effects on: i) cyto-histology, ii) ultrastructure, iii) parallel mounting of structural and functional responses addressed to circumscribe the metal toxicity and, above all, iv) determination of the metal localization sites *in planta*, by means of Cd-sensitive fluorochromes, use of which is still an unusual technique in plant tissues [4,5].

The objective of this work was to bridge these gaps in one species, *Daucus carota* L. (carrot), for which, in spite of its wide consumption as human and animal food, little is known from a cyto-histological, ultrastructural and functional standpoint when subjected to Cd stress. Carrot is a biennial dicot species, cultivated yearly as a crop for the production of its edible taproot. During the first year of vegetation, the plant forms some leaves showing deeply laciniated laminae and very long petioles, the latter fully resembling typical stems in their structure and functions [6]. Moreover, in the same year, a well developed root system is

Abbreviations: GSH, glutathione; LRs, lateral roots; PCs, phytochelatins; PR, primary root; TEM, trasmission electron microscopy.

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generated, composed of several thin lateral roots (LRs) emerging from a thick primary root (PR), the fleshy storage organ [6].

Previous functional studies [7,8] showed that *in vitro* cultured carrot plants and *Agrobacterium rhizogenes*-transformed carrot roots, exposed to 100–1000 μM Cd up to 4 days, actively produced glutathione (GSH), a γ-glutamyl-cysteinyl-glycine tripeptide antioxidant, as well as its oligomeric derivatives, phytochelatins (PCs), the main thiol-peptide compounds for Cd detoxification in plants [9]. Afterwards, studies from other authors [10] showed that in carrot roots Cd exposure affected the activities of some enzymes related to oxidative metabolism, such as catalase, peroxidase, polyphenol oxidase and superoxide dismutase, and influenced the levels of proline and malondialdehyde, the latter assumed to be the breakdown product of membrane lipid peroxidation [11]. However, in the above studies, no links were established over time between Cd localization *in planta*, possible root/leaf damage and response mechanisms addressed to circumscribe the metal stress. In general, the dose-response relationships set up in several plant systems evidenced that the response to different Cd concentrations and exposure-times is a very complex phenomenon, since the metal can evoke a number of parallel and/or consecutive events at a molecular, physiological and morphological level [2]. In this sense, it has been proposed that, above all in response to an acute Cd stress, various mechanisms can operate both in an additive and in a synergistic way, in terms of a multi-component model previously named "fan-shaped response" [2].

In this work we hypothesized that Cd might induce senescence in carrot plants, but that such a process could be effectively counteracted by an articulate "fan-shaped response" *sensu* Sanità di Toppi and Gabbrielli [2]. This hypothesis was verified in carrot primary root (PR), lateral roots (LRs) and leaves, in terms of the possible influence exerted by Cd on primary and secondary xylem development, second order root formation, schizogenous oil duct differentiation and chromoplast organization. Moreover, we also verified whether a main role in Cd detoxification was played by metal immobilization at cell wall and intercellular space levels, by thiol-peptide-mediated compartmentalization in the vacuole, as well as by α–β–γ–tocopherol variations consequent to the Cd-induced perturbation of the oxidative balance. An ordered spatial and temporal sequence of the above metal-generated events was set up.

2. Results

2.1. In roots, cadmium anticipates primary xylem development, stimulates lateral root formation, and causes cell-to-cell separation events also leading to schizogenous oil duct formation

From a cyto-histological point of view, the light microscopy showed an evident root damage in Cd-exposed plants up to 14 days (Figs. 1 and 2), whereas no alteration was observed at any time in the above-ground organs, namely leaf blades and petioles (Fig. S1). The total Cd concentrations measured at 4- and 7-days ranged, respectively, from 999 to 1015 μg g^{-1} DW in roots, 4–5 μg g^{-1} DW in leaf petioles and 17–21 μg g^{-1} DW in leaf blades. After 14 days, the only significant change was the increase in Cd concentration in the blades, which reached an average value of 49 μg g^{-1} DW.

As opposed to the LRs, in the PR no significant Cd-induced changes were observed by light microscopy up to 3 days of metal treatment, regardless of the region considered. As far as the LRs are concerned, although after 1–3 days of Cd exposure no anomalies were observed in their apical meristems, at the primary structure level they showed a xylem overproduction leading to an alteration of the diarch plate (Fig. 1A). In contrast, at the same distance from the root apex, the LRs of control plants exhibited a diarch plate showing a not fully differentiated metaxylem (Fig. 1B). Moreover, only the Cd-exposed LRs showed primordia of second order roots (Fig. 1C, arrow), and the most elongated ones exhibited a precocious secondary vascular differentiation.

As for 1–3 days, no anomalies were observed in the apical meristems of LRs also after 4 days of Cd exposure (Fig. 1D). Significant alterations were however evidenced in the elongation zone and in the primary body region, where events of cell-to-cell separation occurred, respectively, in the differentiating and mature cortical parenchyma, leading as a consequence to the formation of wide lacunae (Fig. 1D, E). At the same time, both the differentiation of schizogenous oil ducts (Fig. 1E, circles and inset) and the formation of second order roots (Fig. 1F) were evident in Cd exposed LRs, but not in the control ones (Fig. 1G). Finally, in the secondary body region, vascular differentiation precociously increased only in the Cd-treated LRs (Fig. 1H).

After 4 days of Cd exposure, alterations were observed not only in the LRs but above all in the PR, except for the apical meristem that was apparently not affected by the treatment (likewise the LRs, as shown in Fig. 1D). Indeed, in the PR, at a distance from the apex where the rhizodermis and the cortex were well defined, and the metaxylem not yet completely differentiated (Fig. 2A), the presence of Cd: 1) accelerated and increased the differentiation of the primary xylem (Fig. 2B); 2) caused hypertrophy in the cortical and endodermal cells (Fig. 2B); and 3) generated cell-to-cell separation events in the cortex (Fig. 2B). Moreover, the pericycle showed localized occurrence of cell proliferation, in particular near the phloem (Fig. 2B, arrow), leading to meristematic clumps, likely initiation sites of LR (Fig. 2C, circle) and of oil duct formation (Fig. 2C, arrows and inset). At the transition region between hypocotyl and PR, more abundant and earlier protrusion of LR primordia were observed (Fig. 2D). Moreover, at the hypocotyl base of untreated PR, the secondary growth started through the development of secondary, centrifugally developing xylem elements (Fig. 2E), whereas in the Cd-treated PR the hypocotyl showed a much more developed secondary structure, as well as a higher frequency of cell-to-cell separation events (Fig. 2F).

After 7 days of Cd treatment, the PR appeared to be much more affected by the metal than the LRs. In the elongation zone the procambium appeared collapsed and necrotic, and the primary body, other than cell hypertrophy, revealed cell-to-cell separation events in the cortex (Fig. 2H, in comparison with the control, Fig. 2G), as well as an anticipated formation of schizogenous oil ducts (Fig. 2H, circle and inset). Furthermore, the xylem seemed crushed by, or embedded into, a central core of irregular and necrotic cells (Fig. 2I). Even if strongly altered in the intrastelar region, the Cd-exposed PR maintained the capability of LR formation (Fig. 2I, arrow, and J), in contrast with control PR, which did not form LRs within the primary body (Fig. 2K). In the PR, at a distance from the apex where the early transition to the secondary growth occurred (Fig. 2L), as well as the very first schizogenous events leading to oil duct formation were observed (Fig. 2L, arrows), the 7-day-Cd exposure caused intrastelar endocytogenesis [12], owing to the proliferation of parenchyma cells surrounding the central xylem (Fig. 2M, N). Within the proliferated cells, schizogenous events leading to oil duct formation occurred (Fig. 2N, arrows), even near the xylem and the pericycle (Fig. 2M, arrowhead and arrows, respectively), and episodic necrosis was detected in the pericycle, too (Fig. 2M). At the same time, alteration in shape and crushing of the endodermal cells was observed (Fig. 2O). At the transition region from the PR to the hypocotyl, the oil ducts in Cd-exposed plants had completed the development (Fig. 2P, arrows), whereas such formation was still at the beginning in the untreated PR (Fig. 2Q, arrows).