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PHYTOTOXICITY OF PYRROLINE-5-CARBOXYLATE REDUCTASE INHIBITORS

PETROLLINO D.*, LIBONI M.*, GIBERTI S.*, BERLICKI L.**, KAFARSKI P.**, FORLANI G.*

*) Department of Biology & Evolution, University of Ferrara, Via L. Borsari 46, 44100 Ferrara (Italy)
**) Department of Bioorganic Chemistry, Wroclaw University of Technology, 50-370 Wroclaw (Poland)

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Accumulation of high intracellular levels of proline has long been reported in many plant species under a variety of abiotic stress conditions. Unexpectedly, exogenously-supplied proline was found to exert phytotoxic effects and trigger the activation of programmed cell death (PCD). An early induction of the gene for δ^1-pyrroline-5-carboxylate (P5C) dehydrogenase, the enzyme that catalyses the second and last step in proline catabolism, was shown in several crops infected by virulent fungal strains. Moreover, proline accumulation through *de novo* synthesis has been reported in *Arabidopsis thaliana* during incompatible plant-pathogen interactions. Therefore the possibility exists that proline metabolism is involved in the process leading to PCD during the hypersensitive defence reaction. However, it is still unclear which may be the active molecule, whether proline itself or P5C, the intermediate in both its synthesis from, and its oxidation to glutamate. Some authors recently postulated that P5C might trespass the mitochondrial membrane, and that a P5C/Pro cycle would occur as a consequence, leading to reactive oxygen species production (Miller *et al*., J. Biol. Chem. 284, 26482–26492, 2009). This cycle would be mediated by the contrasting activity of proline dehydrogenase in the mitochondrion and P5C reductase in the cytosol.

The elucidation of these aspects has been hampered to date by the unavailability of p5cr$^-$ mutants. Proline can be synthesized from either glutamate or ornithine, but the two pathways share the last reaction, just catalyzed by P5C reductase. Therefore, in the absence of a functional enzyme, cells cannot synthesize proline. As a consequence, null mutations are embryo lethal. This also means that specific inhibitors of P5C reductase are expected to exert phytotoxic effects, and might represent new active principles for weed control. If available, they would represent as well useful tools to study the involvement of proline metabolism in the plant defence response against fungal pathogens.

We previously screened several aminomethylenebisphosphonates for their ability to inhibit *in vitro* P5C reductase. Some phenyl derivatives were indeed found to interfere in the micromolar range with the activity of the enzyme from *A. thaliana* (Forlani *et al*., J. Agric. Food Chem. 55, 4340-4347, 2007). Growth inhibition and reversal experiments performed with cell suspension cultures supported the possibility that P5CR inhibition does occur also *in vivo* (Forlani *et al*., J. Agric. Food Chem. 56, 3193–3199, 2008).

Here we report a troughout characterization of their effects at the whole plant level. The inhibitory potential of the most effective compounds, and of some new active analogues designed

on the basis of a structure-activity relationship analysis, was assessed on *Brassica napus* seedlings grown under axenic conditions. Data were compared with the inhibition brought about *in vitro* by the same substances on rapeseed P5C reductase, and related to the resulting steady-state levels of free amino acids in seedling tissues. Results showed phytotoxic effects in the micromolar to millimolar range, and confirmed the occurrence of proline starvation *in vivo*. Interestingly, significant variations in the homeostasis of other, unrelated amino acids were found, suggesting the existence of multiple targets of phosphonate action in plant amino acid metabolism.