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Research article

Toxicity of canavanine in tomato (*Solanum lycopersicum* L.) roots is due to alterations in RNS, ROS and auxin levels

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ABSTRACT

Canavanine (CAN) is non-proteinogenic aminoacid and a structural analog of arginine (Arg). Naturally, CAN occurs in legumes e.g. jack bean and is considered as a strong allelochemical. As a selective inhibitor of inducible nitric oxide synthase in mammalians, it could act as a modifier of nitric oxide (NO) concentration in plants. Modifications in the content of endogenous reactive nitrogen species (RNS) and reactive oxygen species (ROS) influence root structure and architecture, being also under hormonal control. The aim of the work was to investigate regulation of root growth in tomato (*Solanum lycopersicum* L. cv. Malinowy Ożarowski) seedling by application of CAN at concentration (10 and 50 μM) leading to 50% or 100% restriction of root elongation. CAN at higher concentration led to slight DNA fragmentation, increased total RNA and protein level. Decline in total respiration rate after CAN supplementation was not associated with enhanced membrane permeability. Malformations in root morphology (shorter and thicker roots, limited number of lateral roots) were accompanied by modification in NO and $ONOO^-$ localization; determined mainly in peridermal cells and some border cells. Although, CAN resulted in low RNS production, addition of exogenous NO by usage of NO donors did not reverse its negative effect, nor recovery effect was detected after roots imbibition in Arg. To build up a comprehensive view on mode of action of CAN as root growth inhibitor, it was shown an elevated level of auxin. To summarize, we demonstrated several secondary mode of action of CAN, indicating its toxicity in plants linked to restriction in RNS formation accompanied by simultaneous overaccumulation of ROS.

1. Introduction

Secondary metabolites play important role in plants as a weapon against herbivores, microbes and/or competing plants. They also function as signaling compounds to attract animals (Weston and Duke, 2003). In spite of the name, chemicals belonging to the group of secondary metabolites are critical for plant survival and reproductive fitness and have been subjected to natural selection during evolution, resulting in their wide distribution among plant families (Wink, 2003). Non-proteinogenic amino acids (NPAAs) are examples of bioactive secondary metabolites that can be considered as useful taxonomic markers e.g. in *Fabacea*. NPAAs are defined as simple amino acids not included in protein structure. In general, they serve two important roles in higher plants, as key defense compounds and as mobile nitrogen storage reservoirs for tissues requiring nitrogen sources e.g. seeds (Wink, 2003). There are many endogenous NPAAs: L-canavanine (CAN), mimosine, albizziine, lathyrine, *meta*-tyrosine, 5-hydroxytryptophan that are suspected to act as insect antifeedants, anti-microbials, or toxins responsible for plants' high

Abbreviations: ABA, abscisic acid; Arg, arginine; APF, 3′-(p-aminophenyl) fluorescein; BHT, butylated hydroxytoluene; CAN, canavanine; CTAB, cetyltrimethylammonium bromide; cPTIO, 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAB, 3,3'-diaminobenzidine; DAF-FM DA, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; GA, gibberellic acid; IAA, idolile-3-acetic acid; NBT, nitroblue tetrazolium; NO, nitric oxide; NOS, nitric oxide synthase; NPAA, non-proteinogenic amino acid; $O_2^{\bullet-}$, superoxide anion; $ONOO^-$, peroxynitrite; PBS, phosphate buffered saline; ROS, reactive oxygen species; RNS, reactive nitrogen species; SNAP, *S*-nitroso-N-acetylpenicylamine; SNP, sodium nitroprusside.

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allelopathic potential (Wink, 2003). CAN (2-amino-4-guanidinoxybutanoic acid) is found in legumes, such as jack bean (*Canavalia ensiformis* (L.) DC.) (reviewed by Vranova et al., 2011). Jack bean has been shown to inhibit growth of many other neighboring plants. Synthesis of CAN is connected with light, photosynthetically active tissues are its production site, as was reported for green callus of *Canavalia* spp. (Hwang et al., 1996). CAN is structurally similar to arginine (Arg), and is considered as the guanidinooxy structural analog of Arg. Therefore, CAN is likely to exert some effects on Arg metabolism and other related amino acids. Rosenthal (1990) reported that in insects CAN was incorporated into proteins instead of Arg, as this amino acid is a substrate for arginyl-tRNA synthetase. Dysfunction of proteins with CAN incorporated instead of Arg is connected with decrease of basicity (Rosenthal and Harper, 1996). In insects, production of proteins containing CAN affected developmental processes and contributed significantly to expression of CAN's potent antimetabolic properties. These properties were also indicated in viruses, bacteria and fungi, and corresponded to disruption in DNA and RNA synthesis (Ekanayake et al., 2007).

Growth of roots and its architecture is under control of phytohormones, mainly auxins. Indole-3-acetic acid (IAA) distribution in plants is connected with its polar (cell-to-cell) transport. Root architecture and IAA polar transport are also governed by reactive oxygen (ROS) and reactive nitrogen species (RNS) (Yu et al., 2014). Cross-talk of RNS/ROS and auxins, occurs in an model functional root system and is involved in regulation of development of adventitious roots, formation of lateral roots, growth of root hairs, and gravitropic response (review by Corpas and Barroso, 2015; Krasuska and Gniazdowska, 2015; Yu et al., 2014). It was observed for certain plants that phytotoxins inhibiting root growth may also induce oxidative stress manifested as overproduction of ROS and alterations in antioxidant enzymatic and non-enzymatic cellular system (for review see Gniazdowska et al., 2015).

RNS include nitric oxide (NO) and peroxynitrite ($ONOO^-$) - the product of reaction of NO and superoxide anion ($O_2^{\bullet-}$). NO biosynthesis in plant cells occurs *via* several pathways which are classified into the oxidative and reductive ones. Reductive pathways depend on availability of nitrite (NO_2^-), while oxidative pathways require Arg as a substrate (for review see Gupta and Igamberdiev, 2015). It is also considered the existence of nitric oxide synthase like (NOS-like) enzyme, activity of which was detected mainly in peroxisomes, but also mitochondria and chloroplasts (Corpas et al., 2009). NOS activity depends on Arg and, as reported for mammalian, inducible isoforms of NOS (iNOS) is selectively inhibited by CAN (Abd El-Gawad and Khalifa, 2001). Thus, we suspect a close connection between CAN toxicity on root growth of tomato plants and modifications in NO tissue level. In the present study, the involvement of RNS and ROS was evaluated during CAN-induced restriction in growth of tomato roots. To investigate mode of action of CAN in plants we measured RNS (NO and $ONOO^-$) localization in roots, mainly in root tips. We have also determined concentration of NO_2^- to find correlation of CAN treatment to reductive pathway of NO biosynthesis. Moreover, we focused on superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) localization in roots of plants subjected to CAN at concentration leading to 50% inhibition of elongation growth or at concentration restricting root growth in 100%, as it is known that both ROS compounds influence root architecture in a different manner (Tsukagoshi et al., 2010). This RNS/ROS view was enriched by measurement of IAA content in roots. Moreover, to characterize harmful action of CAN during restriction of growth of tomato roots we investigated impact of tested NPAA on: total root respiration, membrane permeability, total protein and RNA level and DNA fragmentation. Data presented in the work broaden our knowledge on mode of action of potential allelopathic compounds; as so far, although ROS action in phytotoxicity of many allelochemicals is obvious (review by Gniazdowska et al., 2015), information on RNS involvement in their phytotoxicity are rare and very limited.

2. Material and methods

2.1. Plant material

Tomato (*Solanum lycopersicum* L. cv. Malinowy Ożarowski) seeds were germinated in water in darkness at 20 °C for 3 days. After radicle protrusion (day 0) seedlings of equal roots length (5 mm) were transferred to Petri dishes (Ø 15 cm) containing filter paper moistened with distilled water (control) or CAN (Sigma–Aldrich) aqueous solutions (10, 50, 250 μM) (Fig. 1A). Dishes were carried in a growth chamber with 23/20 °C, 12/12 h day/night regime for 3 days. Length of roots of seedlings was determined after 24 and 72 h of culture. The concentrations of CAN required for reduction of root length to 50% of the control, was accepted as IC_{50}. The concentration, which totally inhibited elongation growth of roots, was accepted as IC_{100}.

2.2. Germination test

Tomato seeds were placed (25 per dish) on Petri dishes (Ø 9 cm), filled with filter paper moistened with distilled water (control) or CAN aqueous solutions (50, 250, 500 μM). Culture was carried for 4 days in darkness in 20 °C. Seeds were considered germinated if the radicle had emerged through the seed coat. Experiments were repeated three times.

2.3. NO and cPTIO treatment of seedlings exposed to CAN

To investigate the consequence of exogenous NO application on physiological effect observed after CAN treatment two different experiments were performed (Fig. 1).

Single NO fumigation at the time point "0", before exposition to CAN: tomato seedlings (developed from seeds imbibed for 3 days in water), at the time point "0" were shortly (3 h) treated with NO donors: sodium nitroprusside (SNP, 0.25 and 0.50 mM) and *S*-nitroso-N-acetylpenicylamine (SNAP, 0.25 and 0.50 mM) at light. Then seedlings were washed in distilled water and transferred to Petri dishes and cultured in distilled water or CAN (10, 50 μM) solutions for 72 h (Fig. 1C). Root length of seedlings was measured after 72 h of culture.

In the other test (repetitive, double fumigation with SNP at the time of seedlings treatment with CAN), seedlings treated with CAN (10, 50 μM) or seedling placed in distilled water for 24 h were shortly (3 h) treated with SNP (0.25 or 0.50 mM) at light, and placed again in CAN solutions or water. SNP treatment was repeated after 24 h, and seedlings were placed again in CAN solutions or water (Fig. 1E). The length of roots of seedlings was measured twice, 24 h after first SNP application, and then 24 h after second SNP application, which corresponds to 48 h and 72 h of the trial, respectively.

Additionally, to check the influence of NO scavenging on the effect of CAN on growth of tomato roots 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, 800 μM) was used. Seedlings were treated simultaneously with CAN (10, 50 μM) and cPTIO for 72 h (Fig. 1D). Length of the roots of tomato plants was determined after 72 h of culture period. Experiments were repeated three times.

2.4. Test of recovery effect after CAN treatment

After 24 h of CAN (10, 50 μM) treatment seedlings were