South African Journal of Botany 99 (2015) 54–61

Contents lists available at ScienceDirect

South African Journal of Botany

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

Cytokinin dynamics in differently senescing laminae of *Phragmites australis* plants grown in different habitats

K. Conrad [a,*], V. Motyka [b], R. Bernhardt [a], C. Stein [a]

[a] Department of Botany, Ernst Moritz Arndt University, Grimmer Strasse 88, D-17487 Greifswald, Germany
[b] Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Rozvojová 263, CZ-16502 Praha 6, Czech Republic

ARTICLE INFO

Article history:
Received 10 July 2014
Received in revised form 27 February 2015
Accepted 1 March 2015
Available online 13 April 2015

Edited by K Dolezal

Keywords:
Chloride
Chlorophylls
Common reed
Cytokinins
Leaf senescence
Phosphate

ABSTRACT

Common reed is a cosmopolitan species, which occurs also in South Africa. At a bay of the Baltic Sea stands of tall, large-leafed reed (TR) and stands of short, small-leafed reed (SR) were found. While TR grew where deep soil with much humus and phosphate was available, SR was found growing on shallow soil, which was often of relatively high salinity. As changes in coloration and chlorophyll analyses showed, SR laminae senesced earlier than did TR laminae. The differences in growth and senescence were eliminated by transplanting TR and SR into garden earth. As literature data from other species show, soil components like phosphate and NaCl can influence levels of the antisenescence hormones cytokinins. Our aim was to find correlations between cytokinin levels and the vitality in reed laminae from natural sites. We used ELISAs for cytokinin analysis and completed the investigations by LC-MS determination of 17 cytokinins and cytokinin conjugates in laminae of HR and SR grown near Prague. From July to autumn the content of *trans*-zeatin, dihydrozeatin and their 9-ribosides decreased in third laminae under panicles at TR on an average to 1.3 nmol *trans*-zeatin-9-riboside equivalents kg^{-1} fresh mass and at SR to 0.7 (8 sample pairs). In autumn SR laminae had also lower levels of N^6-(Δ^2-isopentenyl)adenine and its 9-riboside and probably also of *trans*-zeatin-O-glucoside and *cis*-zeatin-9-riboside-O-glucoside compared to TR laminae. The putatively inactive or weakly active *cis*-zeatin compound increased during the summer to very high quantities. After isolation of laminae, the content of cytokinins declined and cytokinin application delayed yellowing. Contrary to some other examples, our results are in concordance with the rule that endogenous cytokinins retard leaf senescence and help thus to explain the senescence differences in TR and SR.

1. Introduction

Phragmites australis (Cav.) Trin. ex Steudel is one of the most distributed species and occurs also in South, East and North Africa (Rodewald-Rudescu, 1974). We investigated common reed at a Bay of the German coast of the Baltic Sea called Greifswalder Bodden. In addition to stands of tall reed with large leaves (TR) there were found those of short, small-leafed reed (SR). The TR and SR stands also differed in their green and yellow colors in autumn. To find reasons for the differences between the reed stands, especially for the senescence differences, we looked for differences between the soils on which they grew. Substrates of reed stands in many countries have been well investigated (e.g. review by Rodewald-Rudescu, 1974; Voigtland, 1983; Lippert et al., 1999) as has the influence of some minerals on reed growth (e.g. Ulrich and Burton, 1985; Matoh et al., 1988; Krumscheid-Plankert, 1992). Because even soil analyses of TR and SR stands at the Greifswalder Bodden were already available (Krisch, 1978), we restricted our analyses to determinations of utilizable phosphorus and nitrogen and of chloride and water. In addition, transplanting TR and SR into uniform soil was used to help clarify the influence of the natural soils on shoot growth and autumnal color changes of leaves.

We hypothesized that differences in the soil characteristics influence the metabolism of reed plants in such a way that the level of phytohormones of the cytokinin class in autumn is higher in TR laminae than in SR laminae. A higher cytokinin level would contribute to later senescing. Within this probable causal chain we concentrated our effort on differences in the cytokinin levels, while for the influence of single soil factors on leaf cytokinins only literature citations concerning other species are given in the discussion. The cytokinin contents of sample pairs of TR and SR laminae were determined by enzyme-linked immunosorbent assays (ELISAs). Analogous investigations were done by liquid chromatography-mass spectrometry (LC-MS) with laminae collected near Prague.

The notion that in nature cytokinins delay leaf senescence was first suggested by the observation that cytokinin applications in many

Abbreviations: ABA, abscisic acid; Chl, chlorophyll; cZ, *cis*-zeatin; cZR, *cis*-zeatin-9-riboside; eq, equivalents; DHZ, dihydrozeatin; DHZR, dihydrozeatin-9-riboside; ELISA, enzyme-linked immunosorbent assay; FM, fresh mass; -7G, -7-glucoside; -9G, -9-glucoside; HPLC, high-performance liquid chromatography; iP, N^6-(Δ^2-isopentenyl)adenine; iPR, N^6-(Δ^2-isopentenyl)adenosine; Kin, kinetin; LC-MS, liquid chromatography-mass spectrometry; -OG, -O-glucoside; SR, short reed; TR, tall reed; tZ, *trans*-zeatin; tZR, *trans*-zeatin-9-riboside; *, significance of a difference.

* Corresponding author. Tel.: +49 3834 510021; fax: +49 3834 864114.
E-mail address: conrad@uni-greifswald.de (K. Conrad).

http://dx.doi.org/10.1016/j.sajb.2015.03.186
0254-6299/

species retard the leaf yellowing. However, the effect was usually only seen in experiments using isolated leaves (rev. by Van Staden et al., 1988). Stronger arguments have been provided by gene technologic experiments in which a transgene encoding isopentenyltransferase increased the cytokinin level and retarded the senescence in leaves (rev. by Gan and Amasino, 1996; Ghanem et al., 2011). A combination of the transgene with a senescence-specific promoter led e.g. in old wheat flag leaves to moderately higher levels of bioactive cytokinins and of chlorophyll (Chl) in comparison to the wild type (Sýkorová et al., 2008). In an analogous transgenic *Lactuca sativa,* however, higher cytokinin but lower Chl levels were found in the upper leaves than in the control (McCabe et al., 2001). The molecular mechanisms by which cytokinin may inhibit the leaf senescence were reviewed by Zwack and Rashotte (2013).

In general, a senescence delaying role of endogenous cytokinins in leaves is also suggested by the natural cytokinin dynamics before and during senescence. Bioassays showed a decline of cytokinin bases and ribosides in many but not all investigated species (rev. by Van Staden et al., 1988). Immunological assays and LC-MS also showed in most cases a negative correlation between the level of those and the leaf senescence (e.g. Yang et al., 2002; Ghanem et al., 2011). The opposite was found in certain attached rice leaves (Rubia et al., 2014) and in detached tobacco leaves (e.g. Faiss et al., 1997) and barley leaf sections (Zubo et al., 2008). We investigated in addition to slowly and fast senescing attached reed laminae also isolated ones.

By bioassays in several species an increase of cytokinin glucoside fractions in leaves was found but it was assumed that they were ineffective in delaying senescence because they might be compartmentalized (rev. by Van Staden et al., 1988). The LC-MS technique allowed us to detect O-, N9- and N7-glucosides. A review of the different metabolism of the three types of cytokinin conjugates is in Frébort et al. (2011). The reversible O-glucosides (-OG) are more active in a Chl retention assay than the N9-glucosides (-9G), while the N7-glucosides (-7G) are inactive (e.g. Žižková et al., 2009; Gajdošová et al., 2011). We determined also *cis*-zeatin (cZ) compounds, of which activity and function are currently under discussion (e.g. Gajdošová et al., 2011; Kudo et al., 2012). Our main aim, however, was to contribute to the clarification of the role of the strongly active cytokinins *trans*-zeatin (tZ), *trans*-zeatin-9-riboside (tZR), dihydrozeatin (DHZ) dihydrozeatin-9-riboside (DHZR), N^6-(Δ^2-isopentenyl)adenine (iP) and N^6-(Δ^2-isopentenyl)adenosine (iPR) in the senescence differences of reed plants grown under different soil conditions.

2. Materials and methods

2.1. Location of reed stands and transplantation

Most of the investigated stands of *Phragmites australis* were situated on the shore of the Greifswalder Bodden. While the stands of TR A and B were found at later closed outflows of old sewage ponds of the town of Greifswald into the bay (both about at 54°07′15″N, 13°26′23″E), the stands of SR K and L laid about 200 m further north. The stands of TR C and SR M were near the village of Kalkvitz and the stands of TR D and SR N near Greifswald-Eldena. While the extensive stands of TR A and SR K were the main subjects of investigation, the other ones, which were too small in area or were situated too far away, served for comparisons. For LC-MS analyses stands at the edges of fresh water ponds from the areas Tichá Šárka (50°05′56″N, 14°20′39″E) and Divoká Šárka near Prague were included. All stands grew above the median water range and received full sun light.

From TR A and SR K rhizome clogs were transferred into uniform garden earth in concrete containers with an area of 0.75 m^2 and a depth of 1.4 m. The cultures were situated in the open-air garden and were watered in dry periods.

2.2. Soil analyses

Soil samples were cored according to Pürckhauer from 0 to 1.0 m depth and divided in 10-cm parts. After discarding some rough material, utilizable phosphate, ammonium, nitrate and chloride were extracted with 0.5 M $NaHCO_3$, 1 M KCl, 12 mM $CaCl_2$ and water, respectively. Photometrical analyses were carried out after reaction of orthophosphate with ammonium molybdate and of NH_4^+ with phenol (Marr et al., 1988) and in the case of NO_3^- directly (Hoffmann, 1991). Chloride was titrated with $AgNO_3$ (Marr et al., 1988). Ground water samples, taken from vertically installed tubes 5 cm wide with openings 50 cm under the soil surface, were filtered and analyzed in the same way as the soil extracts. All determinations in each extract or water sample were done at least twice.

2.3. Preparation of lamina samples for analyses

For cytokinin, abscisic acid (ABA) and pigment analyses, near Greifswald leaves were taken from the reed plants between 8 am and 9 am by cutting immediately above the adjacent nodes. The enclosed internode pieces were left in the sheats. The leaves were placed in a closed metal vessel, in which their sheats were immersed to a depth of 5 cm in water. They were then quickly transferred to the laboratory where the laminae were cut, weighed and stored at −75 °C until analyzed. Cytokinin analyses of such material gave the same results as cytokinin analyses of comparable samples which were frozen in liquid nitrogen immediately at the reed stands. For cytokinin analyses in Prague, the middle parts of laminae were taken, weighed and frozen in liquid nitrogen at the stands and stored at −75 °C.

2.4. Isolation of laminae and application of cytokinins

Laminae were kept on moist filter paper in closed glass boxes at room temperature under diffuse daylight for ELISA analyses. To demonstrate an effect of cytokinins on senescence, the laminae were divided longitudinally before the incubation and one half was sprayed with a 10^{-4} M, 10^{-5} M or 10^{-6} M kinetin (Kin) solution or with a 10^{-4} M or 10^{-5} M tZR solution while the other half with water only. The spraying was repeated on each of the next 2 days. The colors of the half laminae were compared over 3 weeks. Analyses by LC-MS were carried out using laminae kept on moist filter paper in Petri dishes under a light/dark regime of 18 h white light of 130 μmol photons m^{-2} s^{-1} (lamps of Toshiba, Beijing, China) and 6 h darkness at 20 °C/18 °C.

2.5. Phytohormone and pigment analyses

In each case paired TR and SR samples were analyzed simultaneously. For cytokinin extraction in experiments carried out between 1991 and 1994 10–150 laminae [20–40 g fresh mass (FM)] were homogenized and incubated in methanol/chloroform/7 M formic acid (12:5:3, v/v/v). The cytokinins were partitioned between water and 1-butanol at pH 7.0. After purification on a phosphocellulose column, the "butanol-soluble" cytokinins were separated by chromatography on a column of Sephadex™ LH-20 (Amersham Pharmacia Biotech, Freiburg, Germany) with water as eluent. Thirty column fractions were assayed in triplicate for cytokinins by ELISAs using polyclonal antibodies directed primarily towards tZR and iPR, respectively (Conrad et al., 1992). In some cases aliquots of selected Sephadex eluate fractions were further purified by a Sep-Pak C18 cartridge (Waters, Eschborn, Germany) and re-chromatographed by high-performance liquid chromatography (HPLC) using a Merck Hitachi apparatus on a LiChrospher 100 RP-18, 5 μm column (Merck, Darmstadt, Germany). The column was developed isocratically with methanol/10 mM NH_4 formate buffer, pH 3.7 (23:77, v/v) as eluent. The flow rate amounted to 0.5 ml min^{-1} and the temperature to 25 °C. In further analyses a purification with a RP-18 column (Baker, Gross Gerau, Germany) and a tZR immunoaffinity column was