Induced Accumulation and Potential Antioxidative Function of Rutin in Two Cultivars of *Nicotiana tabacum* L.

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The rutin (quercetin-3-rhamnosyl-glucoside) content of two tobacco cultivars (*Nicotiana tabacum* L.) which differ in their ozone-sensitivity was assayed after exposure to various rutin-inducing stimuli. In the growth-chamber, UV radiation in combination with white light led to the accumulation of similar amounts of rutin in both cultivars. Treatment with radical producing agents (*tert*-butylhydroperoxide and paraquat) also led to rutin accumulation. In this case, the rutin content was higher in the tolerant cultivar. The rutin content was also higher in the tolerant cultivar upon exposure of the plants on an out-door stand, even when the UV-part of the sun spectrum was excluded by cut-off filters.

The potential role of rutin as antioxidant was tested with an ion leakage assay. Plants with relatively high rutin content were less sensitive towards paraquat-induced ion leakage than plants without rutin. Thus, the higher rutin content of the ozone-tolerant cultivar Bel B may well contribute to its tolerance against oxidative stress.

Introduction

Various biological roles have been attributed to flavonoids, including coloring of flowers, fruits, and pollen and acting as protectants against UV radiation, herbivory and microbial attack [1, 2]. More recently, it has been suggested that flavonoids might serve as intracellular antioxidants in plants [3].

The tobacco cultivars Bel B and Bel W 3 (*Nicotiana tabacum* L. var. Bel B and *Nicotiana tabacum* L. var. Bel W 3) differ in their ozone-sensitivity. The Bel W 3 cultivar is highly sensitive to ozone, whereas the Bel B cultivar is relatively tolerant [4]. The differing sensitivity of the two cultivars was suggested to be related to a lower content of hydroxy-cinnamoyl conjugates of polyamines in the apoplast of the sensitive cultivar [5]. These conjugates have been shown to be effective antioxidants and radical scavengers [6].

Rutin (quercetin-3-rhamnosyl-glucoside) is one of the predominant flavonoids in most tobacco species and cultivars [7], and *in vitro* experiments have shown rutin to have antioxidative and radical scavenging properties [8, 9]. The question thus arises as to whether differences in the rutin content of the two cultivars, Bel B and Bel W 3, might also play a role in their differing sensitivity to ozone.

In this paper, experiments are described in which plants of the two cultivars were treated with various stimuli to induce rutin accumulation. *t*-BuOOH[1] or paraquat-induced membrane damage and subsequent ion leakage from leaf discs with different rutin contents was monitored to ascertain whether rutin might have a potential antioxidative or radical scavenging role in tobacco.

Materials and Methods

Chemicals

t-BuOOH was purchased from Fluka (Buchs, Switzerland); paraquat was obtained from Sigma (Deisenhofen, FRG) and rutin from Roth (Karlsruhe, FRG). HPLC solvents and citric acid were obtained from Merck (Darmstadt, FRG).

Plant material

Seeds of both cultivars (*Nicotiana tabacum* var. Bel B and *Nicotiana tabacum* var. Bel W 3) were germinated in a growth chamber (12:12 h light: dark; total energy fluence rate (400–800 nm): 48.8 W/m^2 from Xenon arcs; Osram XQO, temperature: 25 °C) on vermiculite and the seedlings transferred to pots filled with a mixture of garden soil, sand and vermiculite (1:1:1, v/v/v) as soon as they reached a height of about 2 cm. The plants were fertilized weekly with half strength Hoagland's

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nutrient solution [10]. Experiments were carried out with 5 to 7 week-old plants.

Light treatments

a) UV + white light (growth-chamber): One half of each of the leaves of the mid-stem region was covered with WG[1] 305 nm cut-off filters (Schott and Genossen, Mainz, F.R.G.) and the other half with PG[1] 370 nm cut-off filters (Röhm and Haas, Bellefonte, U.S.A.). The remainder of the plant was covered with black cloth. The plants were placed for four days in 12:12 h light/dark cycles under a field of UV + white light which consisted of two Osram L 40 W/73 fluorescent tubes, two Philips TL 40 W/18 tubes and one Philips TL 40 W/12, as described by Beggs and Wellmann [11]. Total energy fluence rate (400–800 nm) of the field was 11.02 W/m². The temperature was held between 22 and 25 °C.

b) Natural outdoor conditions: Plants were transferred from the growth chamber to the roof of the Botanical Institute during periods of clear sky in the month of August. One half of each of the leaves of the mid-stem region was covered with UV-transmitting quartz glass, and the other half with PG 370 nm cut-off filters. In a first experiment, the plants were exposed for 26 h, in a second for 96 h. At the time of transfer, the rutin content was below the detection limit in these plants.

Determination of ozone by surface active monitoring

An alkaline indicator solution containing starch, potassium carbonate and potassium iodide as described by Rumpel [12] was applied to glass-fiber filters (GF 92, 50 mm diameter, Schleicher & Schuell, Dassel, F.R.G.). Dried filters were exposed close to the plants over the period of the experiments. The filters were protected against direct UV-radiation by covering them with sheets of paper. Every day, two filters were collected and the iodine-starch complex present was eluted with potassium iodide solution. The absorption of the eluate was determined at 575 nm. The ozone-dosage over the time of exposition was calculated from the absorption data using the calibration curve given in Rumpel [12].

Treatment with radical producing compounds

The second and the third leaves with a midrib longer than 7 cm were painted on both sides three times with 100 μM paraquat solution or seven times with 100 mM *t*-BuOOH. Leaves of control plants were painted with water. The leaves were harvested 6 days after the first paintings. The rutin content of the painted leaves and of the first and the fourth leaves was determined.

Rutin determination

a) Extraction: Leaf discs were cut out of leaf tissue free of midribs and major lateral veins with a cork borer (10 mm diameter) immediately after the irradiation. Each sample consisted of five discs which were transferred to a tube and frozen in liquid nitrogen. After thawing, 200 μl of mixture of ethanol:water:acetic acid (70:29:1, v/v/v) were added. The leaf discs were crushed with a small plastic rod. The brei was incubated for 20 min at 80 °C and cell debris subsequently spun down. The supernatant was collected and centrifuged at 6200 × g for 10 min. The clear extract was subjected to HPLC.

b) Separation of the phenolic extract was carried out on a C-18 RP-column (μBondapak, 3.9 mm diameter, 300 mm length, Millipore, Eschborn, F.R.G.) with a mobile phase of solvent A: water + 0.4% (w/v) citric acid and solvent B: water (+ 0.4% (w/v) citric acid):acetonitrile:2-propanol (100:47:5, v/v/v). The elution started with 38% of solvent B and increased hyperbolically to 100% of solvent B in 13 min. The flow rate was 1.5 ml/min. The detection wave length was set at 350 nm. Rutin eluted at 9.2 min as verified by the addition of a rutin standard. Rutin was further identified by TLC and UV spectroscopy. The detection limit for rutin was at 0.25 ng per cm² leaf surface.

The rutin content is given as amount per leaf surface [μg/cm²], but the same relative values were obtained when related to fresh or dry weight.

Determination of ion leakage

Three sets of leaf discs were obtained from previously irradiated plants as described above. A set consisted of 12 leaf discs, which were incubated in 24 ml water. One set of leaf discs served as control and the radical producing compound (final concentration: 20 mM *t*-BuOOH or 5 μM paraquat) was added to a second set before the first measurement. A third set was used to determine total ion leakage after freezing and thawing discs in water, as recommended by Whitlow *et al.* [13]. Changes in conductivity were monitored with flow-through conduc-

tivity electrodes (PW 9513, Philips, Zürich, Switzerland) connected to a conductometer (K 320, Consort, Turnhout, Belgium). During the experiments the leaf discs were illuminated with four Osram L 20 W/25 fluorescent tubes. The difference in the electrical conductivity between samples treated with *t*-BuOOH or paraquat and the water control is expressed as percent of the total ion leakage as determined by the freeze-thaw treatments.

Results

Light treatments

a) UV (growth-chamber): After 37 h irradiation with UV + white light, leaf areas which were covered with WG 305 nm cut-off filters showed rutin accumulation, whereas, in areas covered with PG 370 nm cut-off filters, rutin was below the detection limit (Fig. 1). The amount of rutin in the leaves did not differ between the two cultivars. The ozone-dosage in the growth-chamber over the period of the experiment was below 0.5 mg/m^3.

Plants from these experiments were used to compare radical induced ion leakage for leaves with different rutin content (see below).

b) Natural out-door conditions: Upon exposure to natural conditions, the rutin content of the leaves of both cultivars increased with exposure time and transmission of the used cut-off filters for shor-

Fig. 2. Rutin content in leaves of *N. tabacum* var. Bel B (■) and *N. tabacum* var. Bel W3 (□) upon exposure to natural outdoor conditions after 26 h (A) and 96 h (B). Leaves were covered by half with a PG 370 nm cut-off filter (>370 nm) and by the other half with UV-transmitting quartz-glass. Lower leaves were left uncovered (mean ± standard deviation, $n = 3$).

ter wave-lengths (Fig. 2). Generally the rutin content in the cultivar Bel B was higher than in Bel W 3. The highest rutin content was found for both cultivars in uncovered leaves. Covering the leaves with UV-transmitting quartz glass led to a decreased rutin content. Leaf areas covered with PG 370 nm cut-off filters showed the lowest rutin content for the cultivar Bel B; for the cultivar Bel W 3 no rutin accumulation was detected. The ozone dosage, *i.e.* the total amount of ozone the plants were exposed to, was 6.7 mg/m^3 for the first experiment and 16.5 mg/m^3 for the second as determined by the surface active monitoring method.

Treatment with radical producing compounds

Fig. 3 shows the effects of leaf treatments with *t*-BuOOH or paraquat on the accumulation of rutin. Leaves of the cultivar Bel B showed rutin accumulation upon treatment with *t*-BuOOH or paraquat. Leaves of the cultivar Bel W 3 showed significant rutin accumulation only after treatment with paraquat. This paraquat-induced rutin accumulation
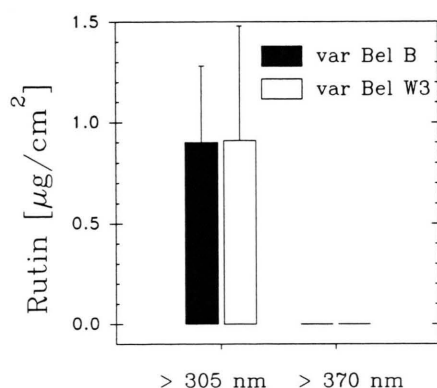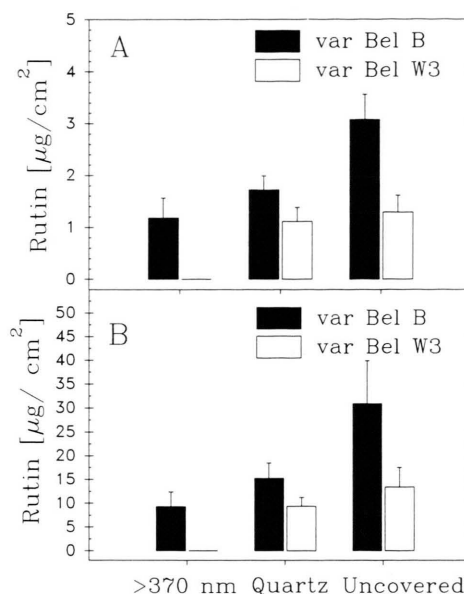

Fig. 1. Rutin content in leaves of *N. tabacum* var. Bel B (■) and *N. tabacum* var. Bel W3 (□) upon exposure to irradiation with UV + white light. Leaves were irradiated for four days with UV + white light (12:12 h light:dark cycle; total light duration: 37 h). One half of each of the leaves was covered with a WG 305 nm cut-off filter (>305 nm) the other half with a PG 370 nm cut-off filter (>370 nm) (mean ± standard deviation, $n = 6$).
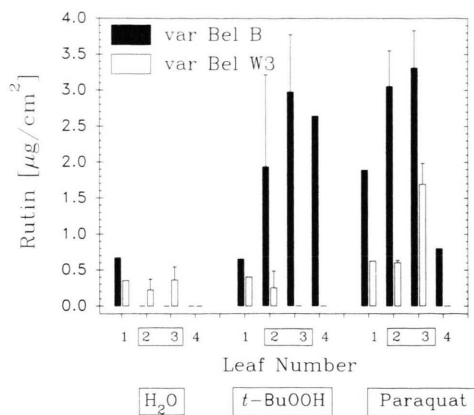
Fig. 3. Effect of paraquat or *t*-BuOOH-treatment on rutin accumulation in *N. tabacum* var. Bel B (■) and *N. tabacum* var. Bel W 3 (□). The second and the third leaf of a seven week old plant was painted three times with a paraquat solution (100 μM) or seven times with *t*-BuOOH (100 mM). Control plants were painted with water. Three samples were assayed from the painted leaves and a single sample was assayed from the non-painted leaves (mean ± standard deviation, *n* = 3).

was lower than in leaves of the cultivar Bel B. The fact that the first and the fourth non-treated leaves also showed rutin accumulation in the Bel B cultivar suggests that both agents have a systemic effect.

Ion leakage

Ion leakage upon incubation with radical producing agents was determined for leaf discs after irradiation of the plants with UV + white light. Discs from leaf areas covered by WG 305 nm cut-off filters (rutin accumulation) were compared to discs covered by PG 370 nm cut-off filters (no rutin accumulation). Controls without addition of radical producing compounds showed only negligible conductivity changes in the bathing solution. Upon incubation of the discs in 20 mM *t*-BuOOH, the electrical conductivity of the bathing solution increased to about 40 to 50% of the maximal conductivity observed after freeze-thaw treatments of the discs (Fig. 4). No difference was observed between the two cultivars and between discs with different rutin contents. However, upon incubation with 5 μM paraquat, discs from leaves kept under WG 305 nm cut-off filters (rutin accumulation) showed considerably lower ion leakage than discs from leaves kept under PG 370 nm cut-off filters (no rutin accumulation) (Fig. 5).
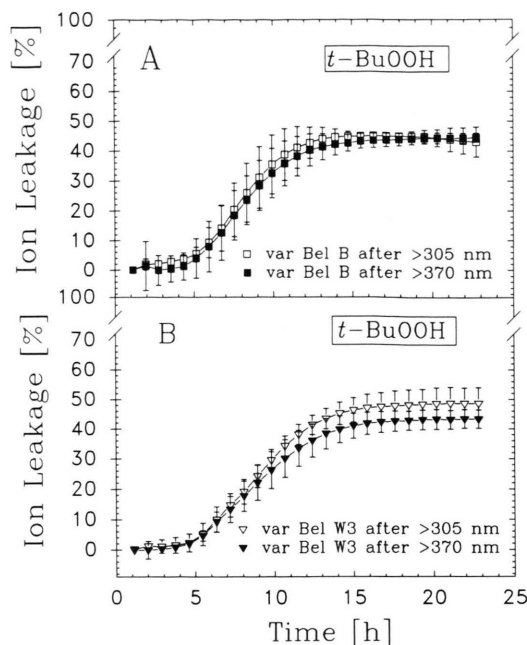

Fig. 4. Effect of light pretreatment on *t*-BuOOH-induced ion leakage of *N. tabacum* var. Bel B (A) and *N. tabacum* var. Bel W 3 (B). Discs were sampled from leaves kept under WG 305 nm (rutin-accumulation) (□, ▽) and PG 370 nm (no rutin accumulation) (■, ▼) cut-off filters as described in Fig. 1. The discs were incubated in water with 20 mM *t*-BuOOH. *t*-BuOOH was added at time zero (mean ± standard deviation, *n* = 3).

Discussion

Our findings that rutin accumulation in the two tobacco cultivars can be induced by UV radiation (Fig. 1 and 2) are in good agreement with observations for other flavonoids in various species [14]. In the growth-chamber experiments no differences in rutin content between the two cultivars could be detected when UV acted as a stimulus for rutin accumulation (Fig. 1). In contrast, upon outdoor exposure to natural sunlight, the rutin content was higher in the ozone-tolerant cultivar Bel B than in the ozone-sensitive cultivar Bel W 3 (Fig. 2). However, if the UV-part of the sunlight is excluded by PG 370 nm cut-off filters, rutin accumulation can be observed in Bel B, but not in Bel W 3. This indicates that another induction stimulus besides UV might be involved. A difference between the two locations is the dosage of ozone, which was found to be considerably higher out of doors than in the growth-
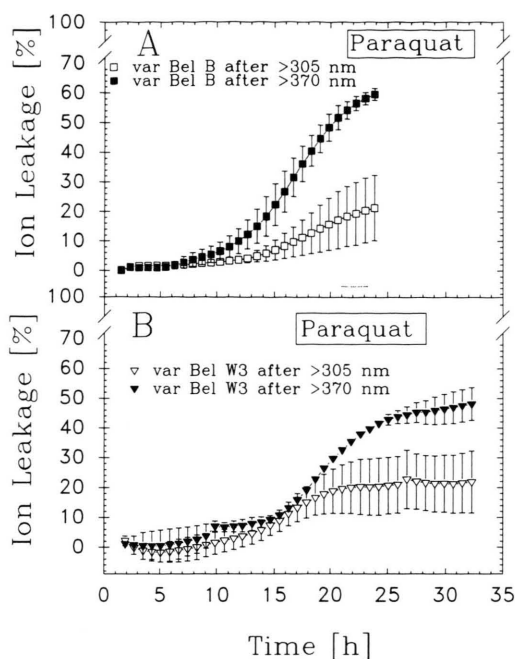
Fig. 5. Effect of light pretreatment on paraquat-induced ion leakage of *N. tabacum* var. Bel B (A) and *N. tabacum* var. Bel W 3 (B). Discs were sampled from leaves kept under WG 305 nm (rutin accumulation) ($\square$, $\triangledown$) and PG 370 nm (no rutin-accumulation) ($\blacksquare$, $\blacktriangledown$) cut-off filters as described in Fig. 1. The discs were incubated in water with 5 μM paraquat. Paraquat was added at time zero (mean ± standard deviation, $n = 3$).

chamber. It has been shown that ozone can stimulate the synthesis of flavonoids and other phenolic compounds [15]. A possible role for ozone in rutin induction is supported by the observation that treatment of leaves of the two cultivars with *t*-BuOOH or paraquat resulted in rutin accumulation and that this accumulation occurs with higher efficiency in the Bel B cultivar (Fig. 3). Paraquat acts in the chloroplast by the production of O_2^- radicals [16]. *t*-BuOOH decays under formation of a hydroxyl radical and a butoxyl radical [17]. Ozone also causes the production of reactive radicals when interacting with biological material, especially with constituents of membranes [18, 19]. Evaluating our data in the light of these findings we suggest that the higher ozone dosage during the outdoor exposure might be a stimulus for rutin induction in the two tobacco cultivars. Ozone fumigation of the plants under controlled conditions might further clarify this point.

Flavonoids have been discussed as radical scavengers and antioxidants in plants [6, 21] and their antioxidative potential has been demonstrated in *in vitro* assays [8, 9]. The higher rutin content of outdoor samples of the ozone-tolerant Bel B cultivar and its higher efficiency in rutin accumulation upon treatment with radical producing agents (Fig. 3) supports the view that rutin might play a role as antioxidant or radical scavenger in this tobacco cultivar. In this regard our findings parallel the results of Langebartels *et al.* [5], who reported a higher content of and a greater accumulation efficiency for hydroxycinnamoyl conjugates of polyamines for the Bel B cultivar. In addition to the antioxidative effect discussed for these conjugates in the apoplast, rutin may play a role as intracellular antioxidant. Although several authors postulate that almost all ozone which acts on plants decays in the apoplast [22–24], the light dependency of ozone damage in tobacco suggests that plastids may be an important target of ozone attack [4, 25]. Another indication for the light dependency of ozone damage is the fact that ozone flecks appear at first in the chloroplast-rich palisade-parenchyma and not in the spongy mesophyll [26], which is closer to the stomatal point of entry of ozone. Apparently, not only the reactions which take place in the apoplast are responsible for ozone damage, but also the light-dependent impairment of photosynthetic efficiency by ozone as proven by fluorescence measurements [27]. Thus, an intracellular antioxidant might contribute towards differing ozone sensitivity between the two tobacco cultivars. The cytoplasmic or plastidic localization of flavonoids has been postulated by several authors [28, 29].

An intracellular action of rutin is supported by our findings that a higher rutin content coincides with protection of leaf discs against paraquat damage (Fig. 5), but not against *t*-BuOOH derived radicals (Fig. 4). Paraquat causes an intracellular production of reactive radicals, whereas *t*-BuOOH-derived radicals mainly attack the cells from the extracellular space [17, 30]. Before the *t*-BuOOH-derived radicals could be detoxified by intracellular antioxidants, they would lead to oxidation of the plasmalemma or intrinsic plasma membrane proteins. In turn, this oxidation causes ion leakage from the damaged cell [20, 30]. In contrast, chloroplast-derived O_2^- faces the intracellular antioxidative system (including rutin), before it causes an oxidation

of the plasmalemma. Therefore, in the case of paraquat, damage of the plasmalemma causing ion leakage seems only to occur after exhaustion of the intracellular antioxidative system. We did not detect other phenolic compounds in the HPLC chromatograms, which showed similar accumulation patterns to rutin. However, with the method used, hydroxycinnamoyl conjugates of polyamines were not extracted.

In conclusion, the observed increase in rutin content after treatment with radical producing agents and exposure to ozone, particularly in the tolerant cultivar Bel B, as well as the correlation of rutin content with protection against paraquat-induced damage support the theory that rutin has an antioxidative function in tobacco.

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