Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytochemistry 67 (2006) 1120–1126
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Enhanced oxidation of flavan-3-ols and proanthocyanidin accumulation in water-stressed tea plants

Iker Hernández, Leonor Alegre, Sergi Munné-Bosch *

Departament de Biologia Vegetal, Facultat de Biologia, Universitat de Barcelona, Avinguda Diagonal 645, E-08028 Barcelona, Spain

Received 18 November 2005; received in revised form 17 March 2006; accepted 3 April 2006
Available online 19 May 2006

Abstract

(−)-Epicatechin (EC) and (−)-epigallocatechin gallate (EGCG), two major tea flavan-3-ols, have received attention in food science and biomedicine because of their potent antioxidant properties. In plants, flavan-3-ols serve as proanthocyanidin (PA) building blocks, and although both monomeric flavan-3-ols and PAs show antioxidant activity in vitro, their antioxidant function in vivo remains unclear. In the present study, EC quinone (ECQ) and EGCG quinone (EGCGQ), the oxidation products of EC and EGCG, increased up to 100- and 30-fold, respectively, in tea plants exposed to 19 days of water deficit. Oxidation of EC and EGCG preceded PAs accumulation in leaves, which increased from 35 to 53 mg gDW^{-1} after 26 days of water deficit. Aside from the role monomeric flavan-3-ols may play in PAs biosynthesis, formation of ECQ and EGCGQ strongly negatively correlated with the extent of lipid peroxidation in leaves, thus supporting a protective role for these compounds in drought-stressed plants. Besides demonstrating flavonoid accumulation in drought-stressed tea plants, we show for the first time that EC and EGCG are oxidized to their respective quinones in plants in vivo.

Keywords: *Camellia sinensis*; Water deficit; Antioxidants; Flavonoids; Flavan-3-ols; Proanthocyanidins; Epicatechin; Epigallocatechin gallate; Quinones

1. Introduction

Flavonoids are phenolic compounds that show a basic C_6–C_3–C_6 skeleton: two benzene rings linked by a three-carbon chain. This structure is achieved by the condensation and cyclization of a 4-coumaroyl-CoA molecule with three malonyl-CoA molecules. Many different groups of flavonoids have been described according to their chemical composition, and over four thousand flavonoids have been identified and characterized in nature (reviewed by Shirley, 1996; Heim et al., 2002). Flavonoids are of great interest for human health due to their antiinflammatory (Subarnas and Wagner, 2000), antiplatelet (Lou et al., 1992), antiviral (Formica and Regelson, 1995) and antitumoral (Duthie et al., 2000) activities. In plants, flavonoids participate in a number of processes including protection from UV radiation (Ryan et al., 2002), flower and seed pigmentation (Weiss, 1991; Pourcel et al., 2005), pollen tube germination (Mo et al., 1992), and auxin transport (Jacobs and Rubery, 1988).

The potent antioxidant properties of tea (*Camellia sinensis*) extracts have been attributed to their major flavonoids, the monomeric flavan-3-ols, (−)-epicatechin (EC), (−)-epigallocatechin (EGC), and their gallate esters, such as (−)-epigallocatechin gallate (EGCG). Also, proanthocyanidins (PAs), which are also known as condensed tannins, are flavan-3-ol oligomers or polymers of great interest for human health, since they show anticancer (Ahmad et al., 2000), antimutagenic (Dauer et al., 2003), and immunomodulatory (Lin et al., 2002) activities, and promote cardiac recovery after ischemia (Pataki et al., 2002) and inhibit bacterial adhesion to the urinary tract (Foo et al., 2000), among other functions. PAs are therefore responsible for many of the health-promoting effects of green tea, wine, grape juice, grape seed extracts and cranberry juice, among many other natural sources of flavan-3-ols (Bagchi et al., 2000; Foo et al., 2000; reviewed by Dufresne and Farnworth, 2001).

* Corresponding author. Tel.: +34 934021463; fax: +34 934112842.
E-mail address: smunne@ub.edu (S. Munné-Bosch).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.04.002

Despite the in vitro antioxidant activity of monomeric flavan-3-ols has been estimated to be up to five times higher than that of α-tocopherol or ascorbic acid, their role as antioxidants in plants in vivo has not been demonstrated. Although flavan-3-ols may accumulate in plants exposed to a number of stresses (Hutzler et al., 1998; Tattini et al., 2000, 2004; Gould et al., 2002; Ryan et al., 2002; Winkel-Shirley, 2002), which supports a protective role for these compounds in plant responses to stress, there is no direct evidence that they are oxidized in vivo. Furthermore, flavan-3-ols can polymerize, possibly following an oxidative process, yielding PAs that are stored in the vacuole (Kondo et al., 2000; Tanaka et al., 2000). In such a way, PAs serve a significant protective role linked to biotic stress resistance such as defense against microbial pathogens, insect pests and larger herbivores (Kelsey et al., 1984; Li et al., 1996; González de Colmenares et al., 1998).

Taking advantage of the recent identification of several oxidation products of flavan-3-ols in vitro (Kondo et al., 1998; Sawai and Sakata, 1998; Valcic et al., 1999, 2000; Sawai and Moon, 2000; Mizooku et al., 2003; Krishnamachari et al., 2004), we examined whether flavan-3-ol quinones (Fig. 1), which are relatively stable oxidation products of flavan-3-ols derived from a phenoxyl radical intermediate (Tanaka et al., 2002), accumulate in stressed plants. With this aim, small leaf tea (*C. sinensis* cv. *sinensis*) plants, which have high flavan-3-ol contents, were exposed to water deficit in the field, and several indicators of stress, together with the levels of reduced and oxidized flavan-3-ols, were analyzed.

2. Results and discussion

The relative leaf water content (RWC) decreased from ca. 85% to 50% after 19 days of water deficit and then remained constant until day 26, coinciding with the accumulation of small rainfalls during this period (Fig. 2). This

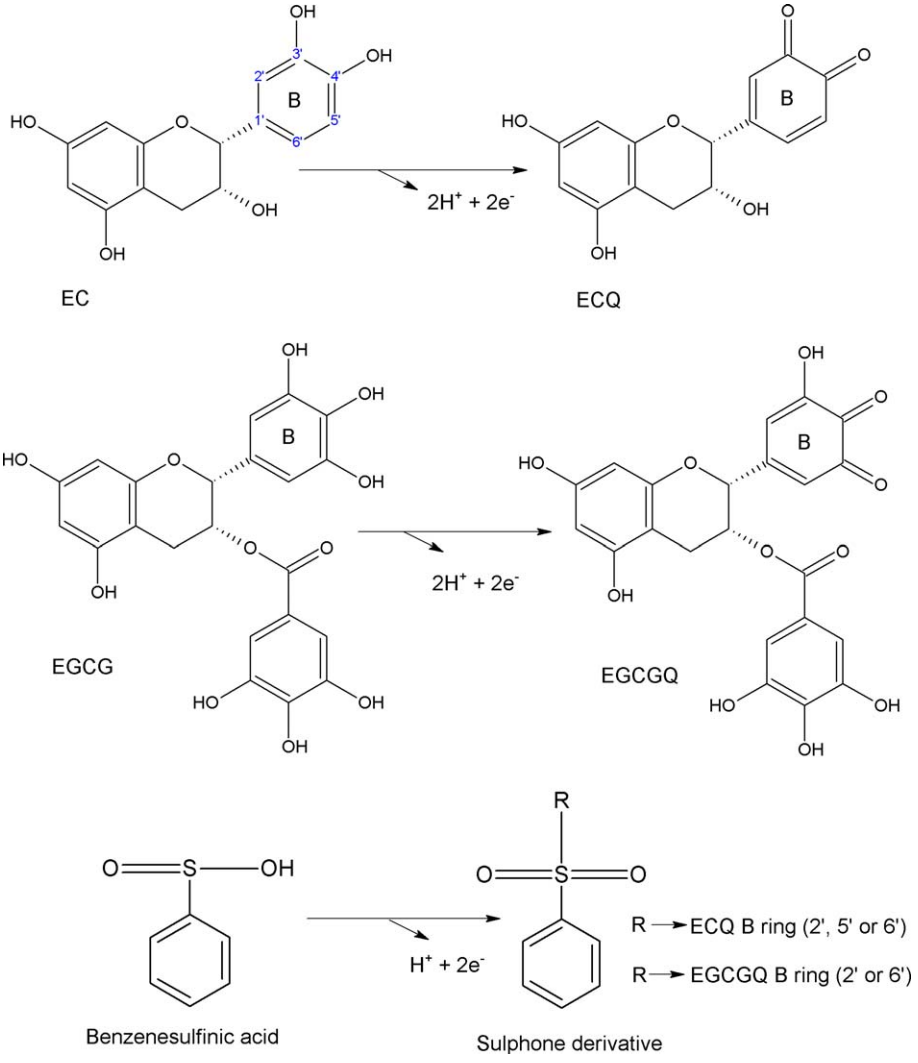

Fig. 1. Chemical structure of (−)-epicatechin (EC), (−)-epicatechin quinone (ECQ), (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin quinone (EGCGQ), benzenesulfinic acid and the sulphone derivatives. Note that although the formation of the quinonic form (and the sulphone derivative) has been indicated in the 4′,5′ OH position in the gallate, this may also potentially occur in the 3′,4′ OH position.

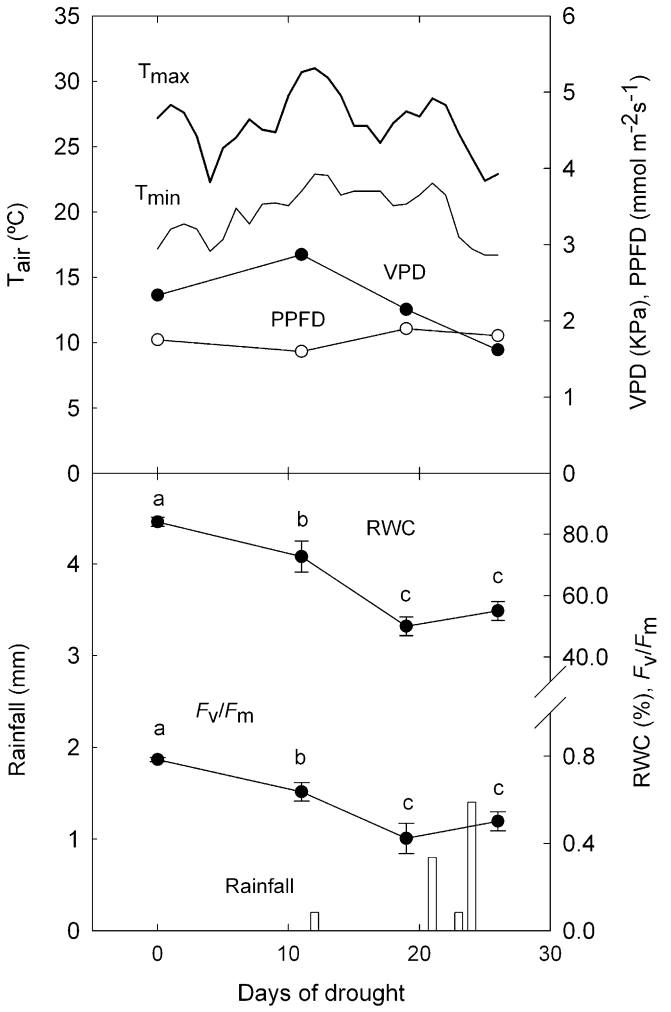

Fig. 2. Climatological conditions (maximum and minimum daily air temperatures [T_{max} and T_{min} respectively], maximum diurnal vapor pressure deficit [VPD], maximum diurnal photosynthetically-active photon flux density [PPFD], and rainfall), relative leaf water content (RWC) and maximum efficiency of photosystem II photochemistry (F_v/F_m) during the experiment. RWC and F_v/F_m data correspond to the mean ± SE of nine independent measurements. Letters indicate significant statistical difference ($p < 0.05$, ANOVA).

indicates that tea plants were not efficient in controlling water loss by stomatal closure, and thus, suffered a severe water deficit. The maximum efficiency of photosystem II photochemistry (F_v/F_m) changed in parallel with the RWC and decreased significantly from ca. 0.8 to 0.4 by day 19, remaining constant until day 26 (Fig. 2), indicating that this water deficit caused photoinhibition. Chlorophyll $a + b$, total carotenoids, and the chlorophyll a/b ratio remained constant throughout the experiment between 3.2 and 3.5 mg gDW^{-1}, 0.06 and 0.08 mg gDW^{-1}, and 1.9 and 2.3, respectively (data not shown).

The levels of reduced flavan-3-ols, EC and EGCG, remained unaltered throughout the experiment. Levels of EC were around 0.25 mg gDW^{-1}, while those of EGCG were 10-fold higher than EC, throughout the experiment (Fig. 3). Endogenous concentrations of EC and its gallate ester, EGCG, have been shown to increase (Jeyaramraja et al., 2003; Kirakosyan et al., 2003; Hernández et al., 2004) but also to remain unchanged (Arts et al., 2000) in previous studies on tea and other plant species exposed to water deficit. Together, these findings suggest that the effect of water deficit on EC and EGCG accumulation strongly depends on the species and conditions of the study.

In the present study, we show for the first time the oxidation of EC and EGCG to their respective quinones in plants in vivo. The levels of oxidized flavan-3-ols, ECQ and EGCGQ increased sharply under water deficit (Fig. 3). The strongest increases were observed after 19 days of stress, when levels of ECQ and EGCGQ rose from 0.08 to 8 mg gDW^{-1} and from undetectable levels to 1.4 mg gDW^{-1}, respectively. ECQ and EGCGQ decreased slightly at the end of the experiment, concomitantly with a small, but non-significant, increase of RWC due to small rainfalls. The redox state of EC and EGCG, estimated as ECQ/(EC + ECQ) and EGCGQ/(EGCG + EGCGQ), respectively increased from 0.23 to 0.98, and from 0 to 0.36 after 19 days of water deficit, when RWC values were at their lowest. Since levels of EC and EGCG remained unaltered throughout the experiment, this sharp increase suggests that the flavan-3-ols synthesized de novo in water-stressed tea plants are rapidly oxidized to their respective quinones.

The extent of lipid peroxidation, estimated as malondialdehyde (MDA) formation, decreased significantly from ca. 75 to 40 nmol gDW^{-1} after 19 days of stress, and then remained constant until the end of the experiment (Fig. 4). The MDA levels indicate that although plants were subject to severe water deficit and photoinhibition, they did not show enhanced lipid peroxidation in leaves. The accumulation of ECQ and EGCGQ correlated with a decrease in MDA levels in leaves ($r^2 = 0.975$ and 0.798, respectively), which suggests that the oxidation of EC and its gallate ester, EGCG, to their respective quinones may prevent lipids from oxidation. This is consistent with studies showing that these flavan-3-ols, especially EGCG, can integrate themselves deeply into lipid bilayers, acting as membrane antioxidants (Kumazawa et al., 2004; Saffari and Sadrazeh, 2004). Quinonic forms of flavonoids may be formed by non-enzymatic oxidation, or as a result of the activity of peroxidases (in the presence of H_2O_2) and other polyphenol oxidases such as tyrosinase and laccase (in the presence of oxygen) (Mayer and Harel, 1979; Guyot et al., 1996). The accumulation of ECQ and EGCGQ in water-stressed tea plants may therefore happen as intermediate products of PA biosynthesis, but also as a result of H_2O_2 scavenging by peroxidases.

Although flavonoids have been generally considered to be exclusively synthesized in the cytoplasm of plant cells, it has been recently shown that some key enzymes of the pathway such as chalcone synthase co-localize in the nuclei of several *Arabidopsis* cell types (Saslowsky et al., 2005). Although EC may be synthesized from cyanidin by the action of the enzyme anthocyanidin reductase, which is

localized in the cytoplasmic face of the endoplasmic reticulum (reviewed by Marles et al., 2003), further research is needed to clarify the subcellular localization of flavan-3-ols in plants.

An enhanced accumulation of PAs was observed in our study after the drought treatment, their levels increasing from 35 to 53 mg gDW^{-1} (Fig. 5). ECQ and EGCGQ appearance and increment preceded PAs accumulation after 26 days of water deficit. Flavan-3-ols such as EC and EGCG may finally polymerize and accumulate in vacuoles as PAs. The PA polymerization mechanism and the origin of the initiation and elongation building blocks remain to be fully understood (reviewed by Dixon et al., 2005), but there is general agreement that an oxidative

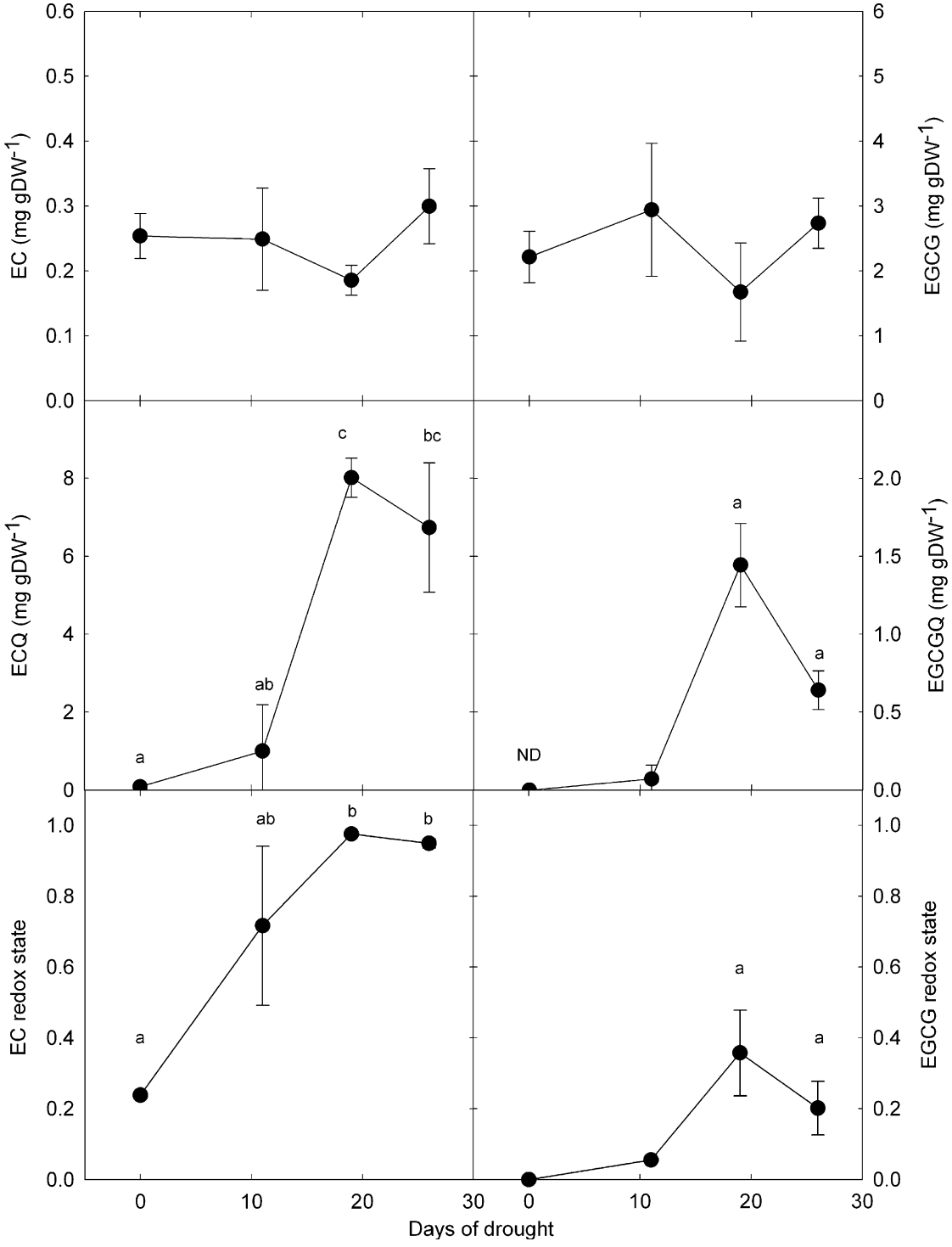

Fig. 3. Changes in epicatechin (EC), epigallocatechin gallate (EGCG), epicatechin quinone (ECQ), epigallocatechin gallate quinone (EGCGQ), and the redox state of EC and EGCG, estimated as ECQ/(ECQ + EC) and EGCGQ/(EGCGQ + EGCG), respectively, in *C. sinensis* leaves during the 26 days of water deficit. Data correspond to the mean ± SE of three independent measurements. Letters indicate significant statistical difference ($p < 0.05$, ANOVA).

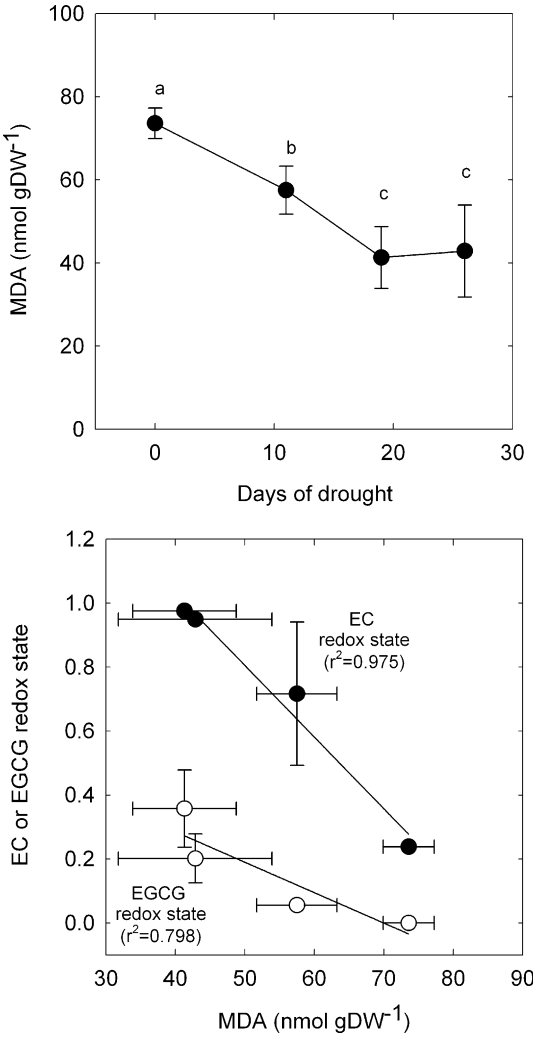

Fig. 4. Changes in malondialdehyde (MDA) levels, a marker of lipid peroxidation, in drought-stressed *C. sinensis* leaves, and correlation between MDA and the redox states of EQ and EGCGQ. Data corresponds to the mean ± SE of nine independent measurements for MDA and of three independent measurements for flavan-3-ol redox states. Letters indicate a significant statistical difference ($p < 0.05$, ANOVA).

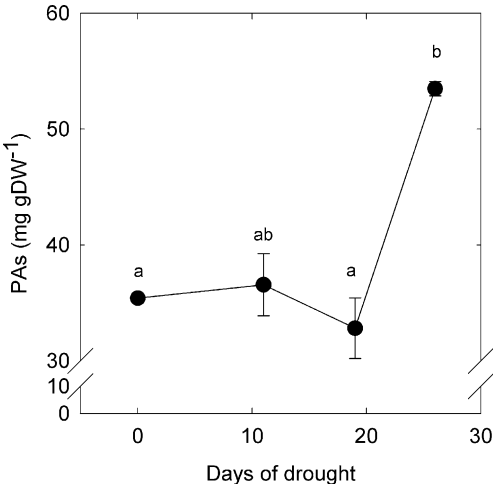

Fig. 5. Changes in proanthocyanidins (PAs) levels in *C. sinensis* leaves during the 26 days of water deficit. Letters indicate a significant statistical difference ($p < 0.05$, ANOVA).

metabolism may be required for PAs biosynthesis, at least during seed browning (Pourcel et al., 2005). Though still to be proven, it is likely that the oxidation of EC and EGCG to their respective quinones observed in water-stressed tea leaves may therefore be a part of an oxidative process linked to the biosynthesis of PAs.

3. Conclusions

We provide evidence for the first time of the in vivo oxidation of EC and EGCG to their respective quinones in plants. Moreover, we show that oxidation of flavan-3-ols precedes accumulation of PAs in water-stressed tea plants. Further research is however needed to better understand the mechanism by which flavan-3-ols are oxidized to their respective quinones in vivo, to what extent flavan-3-ols can protect membranes from lipid peroxidation, and by which mechanisms quinones contribute to the polymerization reaction yielding PAs.

4. Experimental

4.1. Plant material and water deficit treatment

Two-year-old *Camellia sinensis* cv. small leaf tea plants, obtained from cuttings in a nursery (Camforest, NC, USA), were placed in 1-L pots and watered twice a week with Hoagland's solution (Hoagland and Arnon, 1950) in a glasshouse at the University of Barcelona from January to May 2005. Plants were transferred to the soil of the Experimental Fields of the University on May 25, where they were watered twice a week with tap water until the experiment started on June 15. During the experiment (from June 15 to July 12), plants were water-stressed by withholding water so they received water exclusively from rainfall. The accumulated rainfall during the 26 days of the experiment was 2.6 mm only (Fig. 2). Environmental conditions throughout the experiment were monitored with a weather station. Maximum and minimum daily air temperatures ranged from 22.2 to 31 °C and from 16.4 to 22.2 °C, respectively, throughout the experiment. Maximum diurnal vapor pressure deficit ranged between 1.62 and 2.15 kPa, and maximum diurnal photosynthetically-active photon flux density ranged between 1752 and 1898 μmol m^{-2} s^{-1} (Fig. 1). Measurements of relative leaf water content, chlorophyll fluorescence, photosynthetic pigments, malondialdehyde, monomeric flavan-3-ols and PAs were performed at the beginning of the experiment (irrigated plants) and after 10, 19 and 26 days of water deficit on young, fully expanded, sun-exposed leaves at midday (12 h solar time). Leaves used for measurements appeared by mid April, so they were approximately two-months-old when the experiment started on June 15 2005. For the estimation of photosynthetic pigments, MDA, monomeric flavan-3-ols and PAs levels, leaves were immediately frozen in

liquid N_2 and stored at −80 °C until analysis. Necrotic leaves, which appeared approximately after 10 days of stress, were discarded for all measurements.

4.2. Plant water status and chlorophyll fluorescence

Plant water status was estimated by measuring the RWC as $100 \times$(fr. wt. − dry wt.)/(turg. wt. − dry wt.), where turg. wt. is the turgid weight after re-hydrating the leaves at 4 °C for 24 h in darkness, and dry wt. is the dry weight after oven-drying the leaves at 80 °C for 24 h. The F_v/F_m was estimated as $(F_m - F_0)/F_m$, where F_m and F_0 are the maximum and minimum fluorescence yields obtained from leaves adapted to darkness for 2 h with a portable fluorimeter mini-PAM (Walz, Effeltrich, Germany).

4.3. Photosynthetic pigments

Chlorophylls and carotenoids were extracted in 80% aqueous acetone and analyzed spectrophotometrically as described (Lichtenthaler and Wellburn, 1983).

4.4. Extent of lipid peroxidation

The extent of lipid peroxidation was estimated by measuring the amount of MDA in leaves as described by the method of Hodges et al. (1999), which takes into account the presence of interfering compounds in the thiobarbituric acid-reactive substances assay.

4.5. Flavan-3-ols and flavan-3-ol quinones

The monomeric flavan-3-ols, EC and EGCG, and their quinones, ECQ and EGCGQ, were extracted with EtOAc and derivatized with sodium benzenesulfinate as described (Vivas de Gaulejac et al., 2001). Monomeric flavan-3-ols and their quinones were analyzed by HPLC/MS. The HPLC separation was carried out with a Waters 2996 (Waters, Milford, MA) separation module and a Sunfire™ C_{18} column (3.5 μm particle size; 2.1 × 50 mm) with a pre-column of the same material (2.1 × 10 mm) (Waters). The gradient was applied at a 0.6 mL min^{-1} flow rate, as follows: initial conditions, 70% A and 30% B; min 1, 20% A and 80% B; min 4, 100% B; min 5, 100% B; min 6, 100% C; min 8, 100% C; min 10, 70% A and 30% B; and 3 min of column re-conditioning. Solvent A consisted of acidified water (0.05% HCO_2H); solvent B, 1:4 (v:v) MeCN:solvent A; solvent C, MeCN. The ESI/MS conditions were essentially the following. At the source: capillary, 3 kV; HV lens, 0 kV; Cone, −50 kV. At the MS: ion energy, 3.0 V; ion energy ramp, 6.0 V; LM and HM resolution 12.5; multiplier, 700 V. EC and EGCG were quantified by using the corresponding standards from Sigma (Steinheim, Germany). ECQ and EGCGQ were quantified by using these standards once they were oxidized and derivatized as described (Vivas de Gaulejac et al., 2001). The identity of the peaks corresponding to EC, EGCG, ECQ and EGCGQ (product ion scan of m/z 289, 457, 429 and 598, respectively) was confirmed further by their tandem mass spectra by using an HPLC (HP 1100 Series, Palo Alto, CA) coupled to an ESI-MS-MS system (API 3000, PE Sciex, Concord, Ont., Canada).

4.6. Determination of PAs

PAs were determined spectrophotometrically after acid hydrolysis as described (Dalzell and Kerven, 1998). For calibration, pure proanthocyanidin B2 standard was used (Sigma).

Acknowledgements

We are very grateful to Serveis Científic-Tècnics and Serveis de Camps Experimentals (University of Barcelona) for technical assistance. This study was supported by the Ministerio de Ciencia y Tecnología (Project MCYT BOS2003-01032). Iker Hernández holds a PhD grant by the Education, Research and Universities Department of the Basque Country Government.

References

Ahmad, N., Gupta, S., Mukhtar, H., 2000. Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor kB in cancer cells versus normal cells. Arch. Biochem. Biophys. 376, 338–346.

Arts, I.J., van de Putte, B., Hollman, P.C.H., 2000. Catechin contents of foods commonly consumed in The Netherlands. 2. Tea, wine, fruit juices, and chocolate milk. J. Agric. Food Chem. 48, 1752–1757.

Bagchi, D., Bagchi, M., Stohs, S.J., Das, D.K., Ray, S.D., Kuszynski, C.A., Joshi, S.S., Pruess, H.G., 2000. Free radicals and grape seeds proanthocyanidin extract: importance in human health and disease prevention. Toxicology 148, 187–197.

Dalzell, S.A., Kerven, G., 1998. A rapid method for the measurement of *Leucaena* spp. proanthocyanidins by the proanthocyanidin (butanol/HCl) assay. J. Sci. Food Agric. 78, 405–416.

Dauer, A., Hensel, A., Lhoste, E., Knasmueller, S., Mersch, S.V., 2003. Genotoxic and antigenotoxic effects of catechin and tannins from the bark of *Hamamelis virginiana* L. in metabolically competent, human hepatoma cells (Hep G2) using single cell gel electrophoresis. Phytochemistry 63, 199–207.

Dixon, R.A., Xie, D., Sharma, S.B., 2005. Proanthocyanidins – a final frontier in flavonoid research? New Phytol. 165, 9–28.

Dufresne, C.J., Farnworth, E.R., 2001. A review of the latest research findings on the health promotion properties of tea. J. Nutr. Biochem. 12, 404–421.

Duthie, G.G., Duthie, S.J., Kyle, J.A.M., 2000. Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants. Nutr. Res. Rev. 13, 79–106.

Foo, L.Y., Lu, Y., Howell, A.B., Vorsa, N., 2000. The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic P-fimbriated *Escherichia coli* in vitro. Phytochemistry 54, 173–181.

Formica, J.V., Regelson, W., 1995. Review of the biology of quercetin and related bioflavonoids. Food Chem. Toxicol. 33, 1061–1080.

González de Colmenares, N., Ramírez-Martínez, J.R., Aldana-Méndez, B., 1998. Isolation, characterization and determination of biological activity of coffee proanthocyanidins. J. Sci. Food Agric. 77, 368–372.

Gould, K.S., Mickelvie, J., Markham, K.R., 2002. Do anthocyanins function as antioxidants in leaves? Imaging of H_2O_2 in red and green leaves after mechanical injury. Plant Cell Environ. 25, 1261–1269.

Guyot, S., Vercautern, J., Cheynier, V., 1996. Structural determination of colourless and yellow dimers resulting from (+)-coupling catalysed by grape polyphenol oxidase. Phytochemistry 42, 1279–1288.

Heim, K.E., Tagliaferro, A.R., Bobilya, D.J., 2002. Flavonoid antioxidants: chemistry, metabolism and structure–activity relationships. J. Nutr. Biochem. 13, 572–584.

Hernández, I., Alegre, L., Munné-Bosch, S., 2004. Drought-induced changes in flavonoids and other low molecular weight antioxidants in *Cistus clusii* grown under Mediterranean field conditions. Tree Physiol. 24, 1303–1311.

Hoagland, D.R., Arnon, D.I., 1950. The water culture method for growing plants without soil. Calif. Agric. Exp. Sta. Circular, 347.

Hodges, D.M., DeLong, J.M., Forney, C.F., Prange, K., 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanidin and other interfering compounds. Planta 207, 604–611.

Hutzler, P., Fischbach, R., Heller, W., Jungblut, T.P., Reuber, S., Schmitz, R., Veit, M., Weissenböck, G., Schnitzler, J., 1998. Tissue localization of phenolic compounds in plants by confocal laser scanning microscopy. J. Exp. Bot. 49, 953–965.

Jacobs, M., Rubery, P.H., 1988. Naturally occurring auxin transport regulators. Science 241, 346–349.

Jeyaramraja, P.R., Pius, P.K., Kumar, R.R., Jayakumar, D., 2003. Soil moisture stress-induced alterations in bioconstituents determining tea quality. J. Sci. Food Agric. 83, 1187–1191.

Kelsey, R.G., Reynolds, G.W., Rodriguez, E., 1984. The chemistry of biologically active constituents secreted and stored in plant glandular trichomes. In: Rodriguez, E., Healey, P.L., Mehta, I. (Eds.), Biology and Chemistry of Plant Trichomes. Plenum Press, New York, NY, USA, pp. 187–241.

Kirakosyan, A., Seymour, E., Kaufman, P.B., Warbe, S., Bolling, S., Chang, S.C., 2003. Antioxidant capacity of phenolic extracts from leaves of *Crataegus laevigata* and *Crataegus monogyna* (Hawthorn) subjected to drought and cold stress. J. Agric. Food Chem. 51, 3973–3976.

Kondo, K., Kurihara, M., Miyata, N., Suzuki, T., Toyoda, M., 1998. Mechanistic studies of catechins as antioxidants against radical oxidation. Arch. Biochem. Biophys. 362, 79–86.

Kondo, K., Kurihawa, M., Fukuhara, K., Tanaka, T., Suzuki, T., Miyata, N., Toyoda, M., 2000. Conversion of procyanidin B-type (catechin dimer) to A-type: evidence for abstraction of C-2 hydrogen in catechin during radical oxidation. Tetrahedron Lett. 41, 485–488.

Krishnamachari, V., Levine, L.H., Zhou, C., Paré, P.W., 2004. In vitro flavon-3-ol oxidation mediated by a B ring hydroxylation pattern. Chem. Res. Toxicol. 17, 795–804.

Kumazawa, S., Kajiya, K., Naito, A., Saitô, H., Tuzi, S., Tanio, M., Suzuki, M., Nanjo, F., Suzuki, E., Nakayama, T., 2004. Direct evidence of interaction of a green tea polyphenol, epigallocatechin gallate, with lipid bilayers by solid-state nuclear magnetic resonance. Biosci. Biotech. Biochem. 68, 1743–1747.

Li, Y.G., Tanner, G., Larkin, P., 1996. The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. J. Sci. Food Agric. 70, 89–101.

Lichtenthaler, H.K., Wellburn, A.R., 1983. Determination of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem. Soc. Trans. 11, 591–592.

Lin, L.C., Kuo, Y.C., Chou, C.J., 2002. Immunomodulatory proanthocyanidins from *Ecdysanthera utilis*. J. Nat. Prod. 65, 505–508.

Lou, F.Q., Zhang, M.F., Zhang, X.G., Liu, J.M., Yuan, W.L., 1992. A study on tea pigment in the prevention of atherosclerosis. Prev. Med. 21, 333.

Marles, M.A.S., Ray, H., Gruber, M.Y., 2003. New perspectives on proanthocyanidin biochemistry and molecular regulation. Phytochemistry 64, 367–383.

Mayer, A.M., Harel, E., 1979. Polyphenol oxidases in plants. Phytochemistry 18, 193–215.

Mizooku, Y., Yoshikawa, M., Tsuneyoshi, T., Arakawa, R., 2003. Analysis of oxidized epigallocatechin gallate by liquid chromatography/mass spectrometry. Rapid Commun. Mass Spectrom. 17, 1915–1918.

Mo, Y., Nagel, C., Taylor, L.P., 1992. Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. Proc. Natl. Acad. Sci. USA 89, 7213–7217.

Pataki, T., Bak, I., Kovacs, P., Bagchi, D., Das, D.K., Toskai, A., 2002. Grape seed proanthocyanidins improved cardiac recovery during reperfusion after ischemia in isolated rat hearts. Am. J. Clin. Nutr. 75, 894–899.

Pourcel, L., Routaboul, J.M., Kerhoas, L., Caboche, M., Lepiniec, L., Debeaujon, I., 2005. *TRANSPARENT TESTA10* encodes a laccase-like enzyme involved in oxidative polimerization of flavonoids in Arabidopsis seed coat. Plant Cell 17, 2966–2980.

Ryan, K.G., Swinny, E.E., Markham, K.R., Winefield, C., 2002. Flavonoid gene expression and UV photoprotection in transgenic and mutant *Petunia* leaves. Phytochemistry 59, 23–32.

Saffari, Y., Sadrazeh, S.M.H., 2004. Green tea metabolite EGCG protects membranes against oxidative damage in vitro. Life Sci. 74, 1513–1518.

Saslowsky, D.E., Warek, U., Winkel, B.S.J., 2005. Nuclear localization of flavonoid enzymes in *Arabidopsis*. J. Biol. Chem. 280, 23735–23740.

Sawai, Y., Sakata, K., 1998. NMR analytical approach to clarify the antioxidative molecular mechanism of catechins using 1,1-diphenyl-2-picrylhydrazyl. J. Agric. Food Chem. 46, 111–114.

Sawai, Y., Moon, J., 2000. NMR analytical approach to clarify the molecular mechanisms of the antioxidative and radical-scavenging activities of antioxidants in tea using 1,1-diphenyl-2-picrylhydrazyl. J. Agric. Food Chem. 48, 6247–6253.

Shirley, B.W., 1996. Flavonoid biosynthesis: 'new' functions for and 'old' pathway. Trends Plant Sci. 1, 377–382.

Subarnas, A., Wagner, H., 2000. Analgesic and anti-flammatory activity of the proanthocyanidin shellegueain A from *Polypodium feei* METT. Phytomedicine 7, 401–405.

Tanaka, T., Kondou, K., Kouno, I., 2000. Oxidation and epimerization of epigallocatechin in banana fruits. Phytochemistry 53, 311–316.

Tanaka, T., Mine, C., Inoue, K., Matsuda, M., Kouno, I., 2002. Synthesis of theaflavin from epicatechin and epigallocatechin by plant homogenates and role of epicatechin quinone in the synthesis and degradation of theaflavin. J. Agric. Food Chem. 50, 2142–2148.

Tattini, M., Gravano, E., Pinelli, P., Mulinacci, N., Romani, A., 2000. Flavonoids accumulate in leaves and glandular trichomes in *Phillyrea latifolia* exposed to solar irradiation. New Phytol. 148, 69–77.

Tattini, M., Galardi, C., Pinelli, P., Massai, R., Remorini, D., Agati, G., 2004. Differential accumulation of flavonoids and hydroxycinnamates in leaves of *Ligustrum vulgare* under excess light and drought stress. New Phytol. 163, 547–561.

Valcic, S., Muders, A., Jacobsen, N.E., Liebler, D.C., Timmermann, B.N., 1999. Antioxidant chemistry of green tea catechins. Identification of products of the reaction of (−)-epigallocatechin gallate with peroxyl radicals. Chem. Res. Toxicol. 12, 382–386.

Valcic, S., Burr, J.A., Timmermann, B.N., Liebler, D.C., 2000. Antioxidant chemistry of green tea catechins. New oxidation products of (−)-epigallocatechin galate and (−)-epigallocatechin from their reactions with peroxyl radicals. Chem. Res. Toxicol. 13, 801–810.

Vivas de Gaulejac, N., Vivas, N., Nonier, M., Absalon, C., Bourgeois, G., 2001. Study and quantification of monomeric flavan-3-ol and dimeric procyanidin quinonic forms by HPLC/ESI-MS. Application to red wine oxidation. J. Sci. Food Agric. 81, 1172–1179.

Weiss, M.R., 1991. Floral colour changes as cues for pollinators. Nature 354, 227–229.

Winkel-Shirley, B., 2002. Biosynthesis of flavonoids and effects of stress. Curr. Opin. Plant Biol. 5, 218–223.