Food Research International 65 (2014) 207–214

Contents lists available at ScienceDirect

Food Research International

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

Enhancement of zeaxanthin in two-steps by environmental stress induction in rocket and spinach

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ARTICLE INFO

Article history:
Received 29 January 2014
Accepted 29 May 2014
Available online 6 June 2014

Keywords:
Health
Nutritional quality
Rocket
Spinach
Tocopherols
Xanthophylls

ABSTRACT

Humans are continuously exposed to oxidative damage risk and in order to counteract it, the consumption of antioxidants and carotenoids of plant origin is recommended. Numerous studies show the need to include substantial amounts of the carotenoid zeaxanthin (Z) in the diet, because its deficiency provokes the development of macular degeneration, which leads to irreversible loss of vision. However, Z is a less-abundant carotenoid in plants, because most of its pool is rapidly converted to the carotenoid violaxanthin (V) via antheraxanthin (A), due to its involvement in the operation of the xanthophyll (V + A + Z) cycle. The aim of this paper, therefore, was to develop a protocol to enhance the Z content in spinach and rocket through two strategies: firstly, by applying stress (chilling, high light and drought) in order to enhance the total pool of V + A + Z and, secondly, by applying post-harvest treatments before consumption in order to enhance Z formation. The results showed that high light was the most beneficial stress, increasing the fresh weight production in rocket and showing the highest accumulation of V + A + Z and carotenoids in both species. An enhancement of α-tocopherol in rocket was, as well, accomplished by the environmental stress induction. Besides, with the second strategy (post-harvest treatments before consumption, such as boiling and vinegar dressing), both species showed Z enhancement. By combining both approaches in two-steps, the Z content can be enhanced up to 15-fold in spinach and 28-fold in rocket, increasing, as a result, the nutritional value of food.

1. Introduction

There is growing evidence that specific dietary components, the so-called "nutraceuticals", often found in plant-based food, as carotenoids and tocopherols (Namitha & Negi, 2010), may prevent diseases and disorders (Davies, 2007). Indeed, α-tocopherol (vitamin E; DellaPenna & Pogson, 2006), found in green parts of plants, has an important role in protecting membrane lipids from oxidative damage and it has been suggested that it is effective in avoiding obesity (Lira et al., 2011). Among carotenoids, lycopene and β-carotene have been shown to be inversely related to the risk of cardiovascular diseases and certain cancers (Sharoni et al., 2012), while lutein (L) and zeaxanthin (Z) are believed to function as protective antioxidants both in plants and in human nutrition (Demmig-Adams & Adams, 2002), preventing eye diseases (Rao & Rao, 2007), coronary heart diseases and stroke (Chrong, Wong, Kreis, Simpson, & Guymer, 2007). In fact, it has been demonstrated that macular pigments (L and Z) limit retinal oxidative damage by absorbing blue light and quenching reactive oxygen intermediates (Beatty, Koh, Henson, & Boulton, 2000). Zeaxanthin cannot be replaced by any other carotenoid in the retina and its levels in blood plasma have been negatively correlated to the development of age-related macular degeneration (Gale, Hakk, Philips, & Martyn, 2003), which is the cause of blindness in the elderly (Bressler, 2004). The underlying mechanisms are not well understood, but the significance of carotenoids in human diet is considered to be attributable to their antioxidant properties (Asensi-Fabado & Munné-Bosch, 2010; Linnewiel et al., 2009).

An improvement in Z content would thus be a desirable trait to incorporate into crops in order to improve their nutrient intake. Progress in this direction has been made by biofortification (Bouis & Welch, 2010), either through conventional plant breeding (Stommel, 2001) or through

Abbreviations: A, antheraxanthin; α-toc, α-tocopherol; Ch, chilling; C, control; DW, dry weight; Dt, drought; HL, high light; L, lutein; FW, fresh weight; Fo, basal fluorescence; Fm, maximum fluorescence; Fv/Fm, maximum quantum yield of PSII; ML, medium light; PPF, photosynthetic photon flux; PSII, photosystem II; RWC, relative water content; TW, turgid weight; V, violaxanthin; V + A + Z, the xanthophyll cycle involving the carotenoids violaxanthin, antheraxanthin and zeaxanthin; VDE, violaxanthin deepoxidase; Z, zeaxanthin.

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http://dx.doi.org/10.1016/j.foodres.2014.05.044
0963-9969/

biotechnology, engineering carotenoid biosynthesis (Sandmann, Romer, & Fraser, 2006). However, as pointed out by Mou (2009), breeding efforts for the nutrition and commercialization of transgenic crops would depend on progress in transgene expression, public acceptance, economic and marketing challenges, intellectual property issues and risk assessment. Alternatively, the biofortification of crops, understood as a broad term, including agronomic practices and post-harvest modifications, may be effective in increasing the micronutrient concentration of food crops to avoid dietary deficiencies. This strategy of tackling micronutrient malnutrition is considered to be among the best investments, as this will generate a high return in the form of socio-economic benefits (The Copenhagen Consensus, 2004).

Several factors affect the content of these compounds in food: variety, genotype, environmental growing conditions and/or post-harvest treatments (Maiani et al., 2009). In the case of L and Z, plants typically have high levels of L, however, Z is usually found in low amounts, due to the dynamic interconversion of Z to the carotenoid V (violaxanthin) via antheraxanthin (A) within minutes to hours of darkening by the enzyme zeaxanthin epoxidase. When plants are illuminated, V is again converted to Z by the enzyme violaxanthin deepoxidase (VDE), completing the xanthophyll (V + A + Z) cycle (Yamamoto, Nakayama, & Chichestser, 1962). The balance between V and Z is then controlled by periods of darkness and light. Apart from this light-induced interconversions on time scale of hours to days, this cycle can also be regulated over a longer time scale (days to weeks) in response to sustained environmental changes, such as low temperature, water deprivation (Demmig-Adams & Adams, 2006) or increasing light (Niinemets, Kollist, García-Plazaola, Hernández, & Becerril, 2003). Thus, inducing moderate environmental stress during the crop growing period may be a suitable approach to increase some phytochemicals and enhance the nutritional quality of crops, without having an important effect on crop yield.

Another main point to consider in the change of the green vegetable profile of phytochemicals is what occurs at the different phases after the growing period, scilicet, in the post-harvest handing, in the vegetable storage and with the consumers' habits before consumption. Actually, the V + A + Z cycle is active in green vegetables and as a consequence of dark storage prior to consumption, the Z content dramatically decreases due to the operation of the cycle upon darkness in a short-term (Yamamoto, 1979). In addition, at the consumption time, vegetables are traditionally consumed as salad or cooked (generally boiling), which probably may affect phytochemical stability and carotenoid isomerization (Yang, He, & Zhao, 2013). Thus, studying how to improve the Z content by the post-harvest approach before consumption will complement the strategies for growing vegetables.

Taking into account that guaranteeing the presence and accumulation of these phytochemicals may help human nutrition and health, the goals of the present work were: (i) to activate the long-term up-regulation of V + A + Z cycle components (enhancing the total pool of V + A + Z cycle pigments) in green vegetables during the growth period in the presence of mild (stress that does not induce senescence) environmental stress (chilling, high light and drought), (ii) to activate the conversion of the V + A + Z cycle (from V to Z) and (iii) to stabilize the Z content after the usual post-harvest treatments for consumption. The experiments were carried out with spinach (*Spinacia oleracea* L.), a leafy vegetable traditionally considered to have high nutritional quality and large amounts of antioxidants (Bergquist, Gertsonn, & Osllon, 2006), and rocket (*Eruca sativa* Mill.), which has attracted considerable interest recently as a culinary vegetable for salads because of its strong flavor and content of putative health-promoting compounds (Pasini, Verardo, Cerretani, Caboni, & D'Antuono, 2011). Both vegetables are widely accepted by consumers because they are easy to prepare for eating. This paper will attempt to set out a number of simple recommendations in order to achieve the aforesaid goals by manipulating the environmental conditions during the growth period and/or during post-harvest treatments.

2. Materials and methods

2.1. Pre-harvest experimental design: growth conditions and treatments

Seeds of spinach (*S. oleracea* L. var. Viroflay, Chenopodioideae) and rocket (*E. sativa* Mill. var. Golden line, Brassicaceae) were germinated at 26 °C in darkness for 96 h, in a mixture of peat and vermiculite (2:1, v:v) and moistened with deionized water, prior to placement in a growth chamber under controlled conditions (25/18 °C day/night, relative humidity of 60/70% day/night and a photosynthetic photon flux, PPF, of 300 μmol m^{-2} s^{-1}) Ten days after sowing, two seedlings of each species were transplanted to 1.5 l pots (two plants per pot and 6 pots per species and per treatment), filled with a mixture of peat and vermiculite (3:1, v:v). Prior to treatments, plants were allowed to grow for 14 days under the same controlled conditions as above. Irradiation from fluorescent lamps provided a PPF of 300 μmol m^{-2} s^{-1} during a 14 h photoperiod with a ramp for illumination at dawn and dark sunset of 30 min. These conditions were considered as medium light (ML) for light and drought treatments (see details in Sections 2.1.1 and 2.1.2) and control (C) for chilling treatment (see details in Section 2.1.3). The weekly watering regime consisted of watering the plants twice with 150–200 ml of distilled water and once with 150–200 ml of a Hoagland nutritive solution. After 15 days (day 24 of the experiment) under the conditions described above the following treatments were applied.

2.1.1. High light (HL) treatment

On day 24 of the experiment, one set of plants of both species (6 pots per species) was exposed to a HL regime (600 μmol m^{-2} s^{-1}), the other set continued with the ML regime (300 μmol m^{-2} s^{-1}) and we finally harvested the plants on day 42.

2.1.2. Drought (Dt) treatment

We tested the effects of drought stress and subsequent recovery on both species. To this end, in one set of ML plants (3 pots per species), we reduced watering with the Hoagland solution to only once a week, from day 36 to day 40 of the experiment, and from day 40 to the end, plants were watered as a regular regime until harvest. For comparison, one set of ML plants (3 pots per species) was watered with the regular regime indicated above. Finally, we harvested all the plants on day 42.

2.1.3. Chilling (Ch) treatment

On day 24 of the experiment, one set of plants of both species (6 pots per species) was exposed to chilling stress. First at 12/8 °C day/night, relative humidity of 91/100% day/night and a PPF of 300 μmol m^{-2} s^{-1} until day 35 of the experiment, and later the conditions were hardened to 9/4 °C day/night until harvest on day 38 of the experiment. The other set (6 pots per species) continued until harvest with control conditions (25/18 °C day/night).

Samples were collected, during the course of the experiment, every 3–4 days throughout the growth period and finally at harvest. For this purpose, 3–5 leaves from each species and treatments were randomly selected and analyzed for fluorescence. Then, leaf discs (3 mm diameter) were collected from leaves, where fluorescence was measured, frozen in liquid nitrogen and stored at −80 °C until the carotenoid, chlorophyll and tocopherol analyses were conducted. Sampling was performed after a period of dark incubation (12 h) in order to reduce the variability and to provide comparable conditions (Tausz, Wonisch, Grill, Morales, & Jiménez, 2003).

2.2. Post-harvest experimental design

On day 42, ML spinach and rocket shoots were exposed to post-harvest treatment in accordance with the way each species is normally consumed (boiling for spinach and vinegar dressing for rocket). Prior to treatments, spinach and rocket plants were dark adapted for 10 min and exposed to direct sunlight for 30 min (PPF of 1001 ±