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Chemical-induced resistance against post-harvest infection enhances tomato nutritional traits

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Abstract

Grey mould post-harvest infection caused by *Botrytis cinerea*, one of the main causes of decay in harvested tomato, was controlled by inducing resistance with the plant activator benzothiadiazole (BTH, Bion™). Ripened red tomatoes (cv. Ciliegino), of uniform size and colour, were sprayed every other day with 0.3 mM BTH, three times, then inoculated by injection of a conidial suspension (10^5 spores ml^{-1}) deep into the pericarp. In BTH-untreated controls, mycelium developed concentrically from the inoculation site at the 48th h after incubation at 20 °C, 99% relative humidity (RH), and disease markedly increased after the 4th day, with a maximum development at the 8th day, when most of the berry was infected. In BTH-treated tomatoes, the reduction of mycelium spreading was 71%, though in many cases fungal growth was completely inhibited. HPLC analysis showed an increase of lycopene content in treated vs. control tomatoes by 15.7%, while tocopherol and salicylic acid remained unchanged. The increased lycopene content was confirmed by an innovative technique, based on the microscopic analysis of lycopene crystalloids with epifluorescence and a dichroic mirror. These results showed that post-harvest induced resistance, besides being successfully employed in crop protection, could be a valid tool for improving the health benefits of plant foodstuffs.

Keywords: Lycopene; α-Tocopherol; Salicylic acid; *Botrytis*; Tomato; Benzothiadiazole

1. Introduction

Tomato is one of the most important food crops worldwide, and a main component of the traditional Mediterranean diet. According to several epidemiological reports, regular consumption of tomatoes and tomato products has been associated with a lower incidence of some major chronic diseases, preventing the development of some types of cancer and reducing the risks of cardiovascular diseases (Giovannucci, 1999). It has been postulated that the tomato health-promoting effects are due to its chemical composition. Tomato is a significant source of micronutrients, such as carotenoids, ascorbic acid, tocopherols, phenylpropanoids (e.g., monophenols and polyphenols) and folates, apart from constituting almost the sole available source of lycopene, the carotenoid responsible for their deep red colour (Beecher, 1998).

Carotenoids are highly unsaturated tetraterpenes with an extensive double-bond system (Della Penna & Pogson, 2006). In particular, lycopene ($C_{40}H_{56}$) is the most efficient antioxidant among the carotenoids, because of its molecular structure: a symmetrical open hydrocarbon chain, including 11 conjugated and 2 non-conjugated π bonds (Mortensen & Skibsted, 1997). Indeed, lycopene can be

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considered as the most bioactive compound of tomato, due to its anti-proliferative activity. In fact, it inhibits the proliferation of endometrial, mammary and lung human cancer cells more efficiently than do α- and β-carotene (Levy et al., 1995). In plant cells, lycopene occurs in chromoplasts, a class of plastids developed from chloroplasts. In tomato fruit, these cellular organelles differentiate into chromoplasts upon the initiation of ripening, when chlorophylls are degraded and carotenoids are deposited within the lumen of thylakoids (Ben-Shaul & Naftali, 1969).

Phytochemical composition of food crops depends on a variety of pre- and post-harvest factors: genetic traits, environmental conditions, agricultural techniques, plant disease control, ripening stage under harvest and storage conditions (Brandt, Pék, Barna, Lugasi, & Helyes, 2006; Iriti, Rossoni, Borgo, & Faoro, 2004; Iriti, Rossoni, Borgo, Ferrara, & Faoro, 2005; Raffo et al., 2002; Toor & Savage, 2006).

One of the main causes of decay, in harvested tomatoes, is grey mould, and the fungus *Botrytis cinerea* is the aetiological agent. The pathogen can penetrate the host still while in the field, thus causing latent infections that can develop after harvest, as a consequence of host physiological and biochemical changes and storage conditions (Coley-Smith, Verhoeff, & Jarvis, 1980). Furthermore, open wounds, created during harvesting, handling and packaging, are the major sites of invasion by *B. cinerea*.

Traditionally, synthetic fungicides are employed to control post-harvest diseases, and chemical treatments can be performed either in the field (pre-harvest treatments), or after the harvest (post-harvest treatments). In addition, a novel approach for controlling post-harvest diseases has been based on the activation of the plants own defence response mechanisms (Terry & Joyce, 2000). Therefore, plant activators, or simply elicitors, are a class of compounds able to trigger a long lasting, broad spectrum and systemic immunity (systemic acquired resistance, SAR) in plants as well as harvested fruits and vegetables (Iriti et al., 2004, 2005).

One of the most effective synthetic compounds belonging to the plant activator group is benzothiadiazole (BTH), a functional analogue of salicylic acid (SA), a plant hormone-like compound involved as a molecular signal in SAR induction. It was previously demonstrated that pre-harvest BTH treatment suppressed grey mould in harvested strawberry and grape (Iriti et al., 2004; Terry & Joyce, 2000), while enhancing the synthesis of secondary metabolites (resveratol, anthocyanins, proanthocyanidins and melatonin), valuable both for plant defence and human nutrition (Iriti et al., 2004, 2005; Iriti, Rossoni, & Faoro, 2006).

In this work, we controlled the post-harvest grey mould infection in tomato by post-harvest BTH treatments, assaying the effect of elicitation on tomato phytochemistry, namely the variation of lycopene, tocopherol and salicylic acid contents. Finally, a microscopic technique has been set up to visualize and quantify the lycopene crystalloid directly in tomato pericarp tissues, by a dichroic filter and an image analysis system.

2. Materials and methods

2.1. BTH treatments

Ripened red tomatoes (*Lycopersicon esculentum* Mill. cv. *Ciliegino*) of uniform size and colour, assessed with a Chroma Meter CR-400 (Konica Minolta, Japan), were purchased from a commercial greenhouse, washed with water and gently dried with a soft cloth. They were sprayed three times during the course of a week (on days 1, 3 and 5) with a water solution of 0.3 mM BTH [benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester], prepared from a wettable formulation containing 50% (w/w) active ingredient (Bion®). Control tomatoes were sprayed with a solution of the wettable powder alone. Two days after the last spray, some tomatoes were frozen in liquid nitrogen and stored at −80 °C, prior to HPLC analysis, and some were inoculated with *B. cinerea*; another set was used for microscopical observations.

2.2. Fungal inoculation and decay assessment

B. cinerea Pers. was isolated from infected tomatoes and cultured on potato dextrose agar (PDA, Difco, Detroit, USA), in Petri plates. Conidia were collected from the freshly sporulating edge of two week old fungal cultures, grown at 25 °C. To prepare the inoculum, 5 ml of 0.05% (v/v) Tween 20 in sterile distilled water were added to cultures, and the spores were gently dislodged from the plate surface with a sterile bacteriological loop. Conidial suspensions were filtered through three layers of cheesecloth to remove mycelial fragments, counted with a haemocytometer and, then, adjusted to a concentration of 10^5 spores ml^{-1}, after dilution with sterile distilled water.

The inoculation was carried out by injecting 20 μl of a conidial suspension or Tween solution (positive control) 2 mm deep into the equatorial zone of pericarp, using a 25 gauge needle. Unwounded tomatoes were used as negative control. Tomatoes were kept at room temperature, under room lightning and at 99% RH. Developing symptoms were observed daily, and 8 days after inoculations the colony/lesion recorded.

Three experiments were performed, and each set of tomatoes included ten berries.

2.3. Lycopene and tocopherol extraction

Freeze-dried tomato fruits were ground and extracted with a solvent mixture of hexane:acetone:methanol (50:25:25, v/v/v). About 300 mg of fruit samples were extracted by 10 ml of solvent, and kept in a vessel protected from light and closed in a nitrogen atmosphere. The mixture was gently shaken for 30 min; then the supernatant was removed. This procedure was repeated 5–6 times, until

the pulp was completely colourless. The extracts were pooled and, after filtration through a Whatman No. 1 filter paper, evaporated to dryness under nitrogen flow. The residues were dissolved in chloroform and conserved under nitrogen prior to analyses.

2.4. Salicylic acid extraction

Extraction of total salicylic acid was carried out, following the (Venema, Hollman, Janssen, & Katan, 1996). Frozen tomato samples were mixed with 10 ml of NaOH (250 g/l) for 1 h on a rotary shaker at 250 rpm. Extracts were left to stand overnight at room temperature, shaken for 1 h, acidified to pH 1–2 with 10 M HCl and transferred into a liquid/liquid extractor. The extractor was placed onto a heated flask, and diethyl ether was added. The diethyl ether fraction was carefully evaporated until almost dry, and the last drops of ether were evaporated at room temperature, to avoid sublimation of salicylic acid. The residue was dissolved in 25 ml of acetonitrile:water:acetic acid (25:75:5, v:v:v) and sonicated for 5 min. Approximately 2 ml, of each sample extract, were filtered through a 0.45 μm filter for organic solvents (Acrodisc CR) prior to injection into the HPLC system.

2.5. Lycopene content determination

Amounts of fruit extracts in chloroform were diluted in chloroform:methanol (50:50, v:v) to a suitable concentration for HPLC analyses. The HPLC system was a Jasco (Japan) instrument, composed of a pump, online degasser and MD-910 photodiode-array detector. The analytical column was a Chromolith RP-18e (100 mm for 4.6 mm) with a LiChrospher RP-18 5 μm precolumn (4 mm for 4 mm), both purchased from Merck (Germany). The mobile phase was methanol:acetonitrile (90:10, v:v) and flow rate was 1.2 ml/min. Data processing and quantification of lycopene were done by Borwin-PDA software (JMBS, France), in comparison to a calibration curve made with all-*trans*-lycopene standard. All-*trans*-lycopene standard (Fluka, Germany) was freshly prepared by dissolving 1 mg in 2 ml of chloroform. Standard purity check and concentration were spectrophotometrically evaluated after dilution in hexane and $E^{1\%} = 3450$ at 472 nm according to Scott, Finglas, Seale, Hart, and de Froidmont-Gortz (1996). After determination of the precise concentration, standard lycopene was further diluted with chloroform:methanol (50:50, v/v) to 5 μg/ml, for HPLC analyses All operations were carried out in dim light with brown vessels and the presence of nitrogen flow.

2.6. Tocopherol content determination

Amounts of fruit extracts in chloroform were dried under a nitrogen stream, redissolved in hexane, filtered through a 0.2 μm membrane filter and hexane-diluted to a suitable concentration for HPLC analyses. The HPLC system was a Jasco (Japan) instrument composed of a pump, an online degasser and an Uvidec 100-VI detector set at 280 nm. The analytical column was a Chromolith RP-18e (100 mm for 4.6 mm) with LiChrospher RP-18 5 μm precolumn (4 mm for 4 mm), both purchased from Merck (Germany). The mobile phase was methanol:water (95:5, v/v) and flow rate was of 1.2 ml/min. Data processing and tocopherol quantification in samples were performed by Jasco-Borwin 1.5 software (JMBS, France) in comparison to a calibration curve made with tocopherol standards. Stock solutions of standards, α, β, δ and γ, were prepared by dissolving 1 mg of different tocopherols (Merck, Germany) in 1 ml hexane. After dilution in hexane, the standard concentrations were spectrophotometrically determined at 296 nm for α tocopherol, $E^{1\%} = 75.7$; at 297 nm for β tocopherol, $E^{1\%} = 86.4$; at 298 nm for δ tocopherol, $E^{1\%} = 91.2$; at 298 nm for γ tocopherol, $E^{1\%} = 92.8$.

2.7. Salicylic acid content determination

The HPLC system consisted of a Shimadzu LC-10ADvp, SIL-10ADvp high-performance liquid chromatograph apparatus, with a SPD-10Avp, RF-10Axl detector. The HPLC pumps, autosampler and detectors were controlled via Class vp 3.4 software. A Lichrospher LiChroCART (Merck, Germany) Li 100 RP-18 column (4.6 mm for 250 mm, i.d.; 5 μm), protected with a guard column LiChroCART (4 mm for 4 mm) of the same material, was employed for all the analysis at room temperature (24 °C) and 1 ml/min of flow-rate.

Fluorimetric detection was carried out with a fluorescence detector (Merck Hitachi) with excitation wavelength at 300 nm and emission wavelength at 400 nm. Standards of salicylic acid (Sigma), were dissolved in acetonitrile/acetic acid (99:1, v:v) to a concentration of 1 g/l. Calibration curves were constructed in the range of 10–300 μg/l.

2.8. Microscopy

Tomato skin was peeled off and a 0.5 mm thick tangential section of the pericarp was cut with a vibratome (Intracell, UK). The section was laid on a glass slide with a drop of 50% solution of glycerol in water and gently squashed with a cover slide to disaggregate pericarp cells, without damaging them. Samples were immediately examined with an Olympus BX 50 light microscope (Olympus, Japan), equipped with epifluorescence (excitation at 520–550 nm), differential interference contrast and dichroic mirror.

To roughly quantify lycopene crystalloids, that appeared as brilliant silver rods, micrographs of apparently undamaged cells were taken from each sample at 200 magnifications, using a Pixera 120es high-resolution digital camera (Pixera Co, USA). Micrographs (400 pixels per inch) were processed with Global Lab™ (Datatranslation, USA) and lycopene content was expressed as percentage of silver pixels forming the crystalloids, in respect to the total pixels constituting the whole cell.

2.9. Presentation of results

B. cinerea lesion diameter was analyzed by using MSTAT-C software (Michigan State University, USA). Extraction of metabolites, spectrophotometric and HPLC analyses were repeated at least three times for each replicate and median values ± SD were given, if not otherwise stated. Image analysis was carried out on 30 randomly chosen pericarp cells from each sample, in triplicate.

3. Results

3.1. Tomato decay

BTH treatments had no phytotoxic effects on tomatoes and no apparent morphological changes were observed between control and BTH-sprayed fruits. Regarding infection, in untreated tomatoes, fungal mycelium developed concentrically from the inoculation site after incubation for 48 h, becoming clearly evident, under our conditions, between 48 h and 72 h after inoculation. Disease markedly increased after the 4th day (Fig. 1), with a maximum at the 8 th day. In BTH-treated tomatoes, the reduction of mycelium spread, measured at the 8 th day, was of 71 ± 7.5%, though, in many cases, fungal growth was completely inhibited.

In tomatoes sprayed with a solution of wettable powder, no resistance was induced, whereas tomatoes pricked with a Tween solution alone appeared symptomless.

3.2. Lycopene, tocopherol and salicylic acid content

As shown in Table 1, HPLC analysis showed an increase of lycopene contents in treated vs. control tomatoes by 15.7% (Fig. 2), while tocopherol and SA concentration did not vary appreciably. In particular, among the four tocopherol analogues, only α-tocopherol was detectable, with concentrations of 0.0684 and 0.0674 mg/g fresh weight in the control and BTH treated tomatoes, respectively. Instead, SA concentration was 11.7 mg/g fresh weight in BTH-treated tomatoes and of 11.3 mg/g fresh weight in untreated control.

Table 1
Contents of lycopene, α-tocopherol and salicylic acid in tomato berries after treatment with benzothiadiazole

	Lycopene	α-Tocopherol	Salicylic acid
Control	1.88 ± 0.057 mg/g	0.0684 ± 0.00 mg/g	11.7 ± 0.33 mg/g
BTH	2.18 ± 0.230 mg/g	0.0674 ± 0.00 mg/g	11.3 ± 0.62 mg/g

Data, expressed as mg/g of fresh weight, represent means ± SD from three independent experiments.

3.3. Microscopy

Lycopene crystalloids were easily recognizable with the aid of epifluorescence and a dichroic mirror mounted on the microscope, and appeared as brilliant silver rods (Fig. 3). In BTH-treated tomatoes, the percentage of cell area occupied by crystals was higher (27.55% ± 1.74) than in cells from untreated control (24.85% ± 3.39), though this difference was not significant, due to the high-variability among different samples. In any case, crystalloids often appeared larger and more brilliant in BTH-treated cells, than in untreated control ones (Fig. 3).

4. Discussion

Tomato is a major crop, worldwide, because of its high-consumption, either raw or after processing, large content of nutraceuticals and year-round availability. Besides, tomatoes constitute almost the sole available source of lycopene, an antioxidant and anticarcinogenic carotenoid associated with a reduced risk of developing different types of cancer (Mayne, 1996; Riso, Pinder, Santangelo, & Porini, 1999).

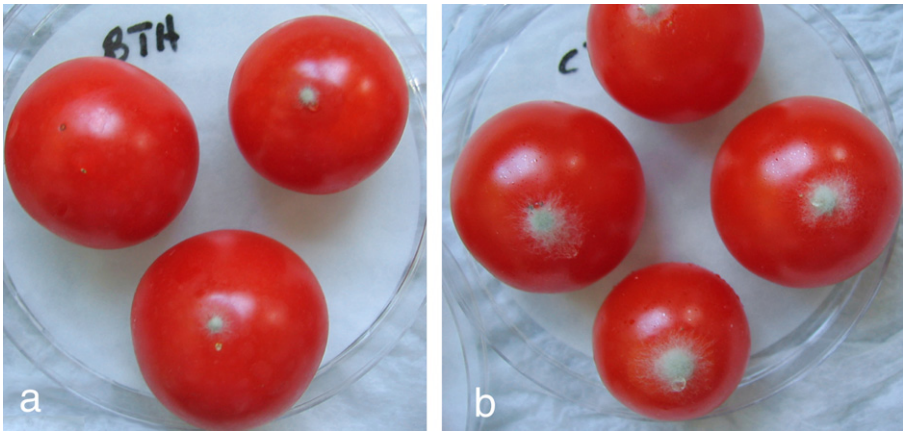

Fig. 1. *Botrytis cinerea* infection on tomatoes treated three times within a week with 0.3 mM benzothiadiazole (BTH) (a) and untreated tomatoes (b), four days after inoculation with conidial suspension: the reduction of mycelium spreading from the inoculation site is significant in treated berries, in comparison with the untreated ones.

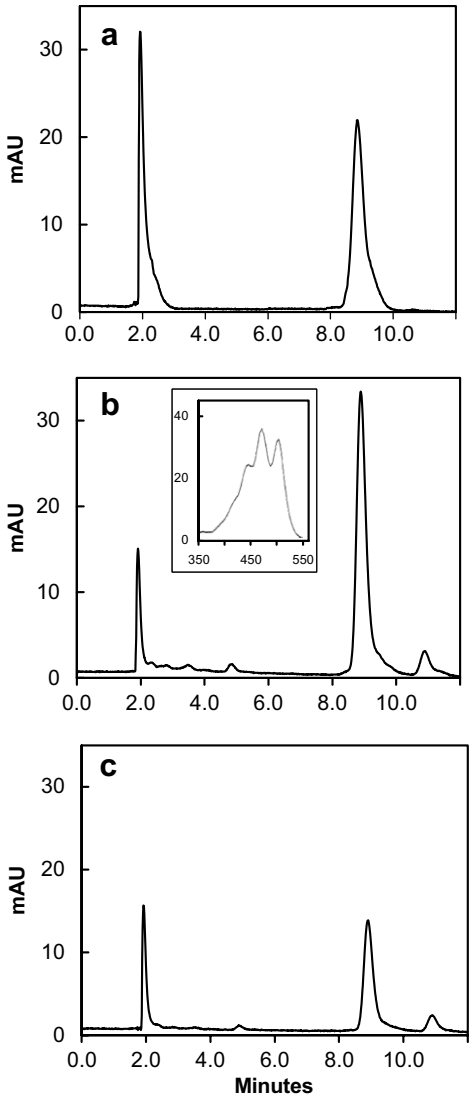

Fig. 2. HPLC elution profiles recorded at 472 nm of (a) standard all-*trans*-lycopene, (b) tomato fruits sprayed with BTH, (c) control tomato fruits sprayed with wettable powder. The insert in panel (b) represents the spectrum of peak eluted at 8.9 min corresponding to lycopene.

In this work, it has been shown that chemical-induced post-harvest resistance modified lycopene profile in tomato, while α-tocopherol and SA remained unchanged.

In plants, the carotenoid biosynthetic pathway is regulated by reactive oxygen species (ROS) produced in response to a plethora of stress conditions. Therefore, ROS generated from plastids and mitochondria can induce carotenogenic gene mRNAs expression (Bouvier, Backhaus, & Camara, 1998). It was previously reported that BTH could inhibit catalases (CAT) and ascorbate peroxidases (APX), major scavenging enzymes located in peroxisomes and plastids, respectively, thus rising ROS level in treated tissues (Wendehenne, Durner, Chen, & Klessig, 1998). Furthermore, it has recently been discovered that BTH inhibits the NADH:ubiquinone oxidoreductase activity of mitochondrial complex I, in cultured tobacco cells, increasing ROS level (Van der Merwe & Dubery, 2006). From this point of view, in our model, BTH treatment could raise the concentration of endogenously-generated ROS to a threshold able to induce carotenogenic gene mRNAs expression, besides stimulating an array of the plant's own defence mechanisms, such as direct toxicity for the pathogen, cell wall strengthening, and synthesis of pathogenesis-related proteins.

BTH is a functional analogue of the plant hormone-like molecule, SA, arising either from the chorismate or phenylpropanoid pathway (Wildermuth, Dewdney, Wu, & Ausubel, 2001). SA is involved in plant defence against pathogens, though it is a less powerful inhibitor of CAT and APX than is BTH. Moreover, it seems that BTH acts downstream of, or in correspondence, with the SA action site (Gozzo, 2003). The fact that BTH treatments did not modify the endogenous synthesis of SA in tomato, is in agreement with this view.

BTH treatment failed to improve α-tocopherol synthesis in tomato, as well. Tocopherols derive from the prenylation of homogentisic acid, a tyrosine derivative, via a 4-hydroxyphenyl-pyruvate intermediate (Fig. 4), thus involving the isoprenoid and shikimate/chorismate (or aromatic amino acid) pathway (Della Penna & Pogson, 2006). BTH could then orchestrate a tidy regulation between the isoprenoid/tetraterpene and shikimate/chorismate pathways, improving the geranylgeranyl pyrophosphate (GGPP) pool and diverting cellular metabolic resources toward lycopene synthesis (Fig. 4). Although GGPP is a precursor of tocophe-

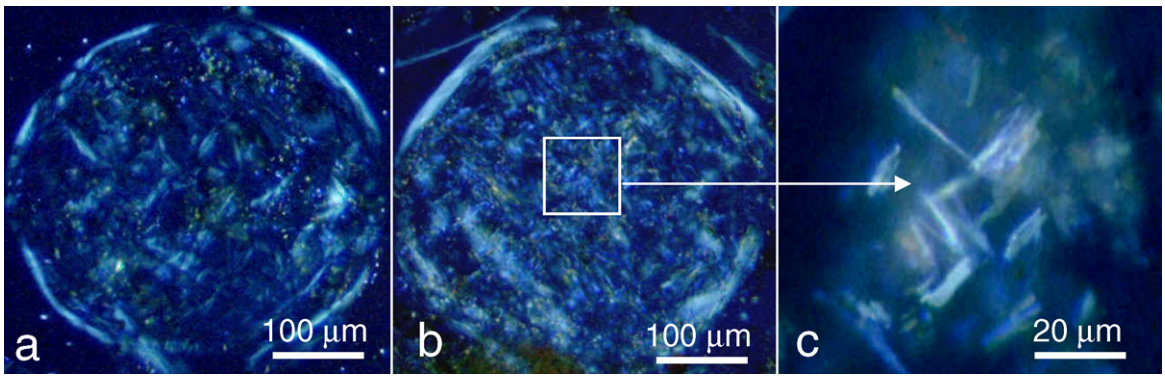

Fig. 3. Lycopene crystalloids in pericarp cells of benzothiadiazole (BTH)-treated (a) and untreated (b) tomatoes, observed in epifluorescence with a dichroic mirror: note that crystalloids are visible as silver brilliant rod-shaped structures (c).

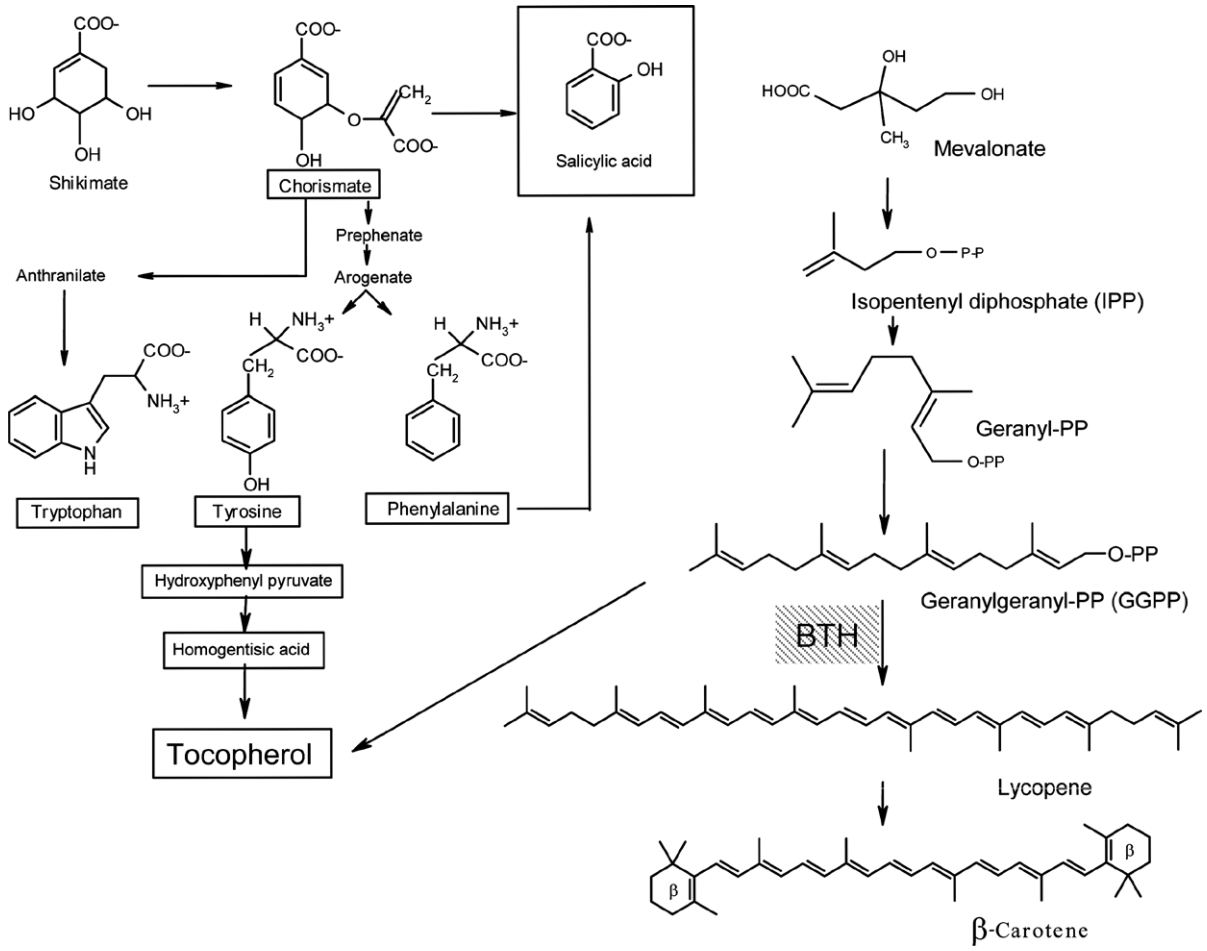

Fig. 4. Lycopene, tocopherol and salicylic acid biosynthesis from mevalonate and shikimate: it has been suggested that the diversion of the geranylferanyl pyrophosphate (GGPP) pool towards the lycopene synthesis is as a result of the benzothiadiazole (BTH) treatment.

rols as well, the synthesis of homogentisic acid could represent a limiting step in α-tocopherol synthesis, thus explaining the unmodified level we found. In the same way, it could also explain the unaffected level of SA, in default of the aromatic amino acid pathway elicitation (Fig. 4).

With regard to grey mould, a major point pertains to the putative antioxidant role of lycopene in tomato resistance. In fact, *B. cinerea* infection is associated with enhanced ROS generation, as a main component of the pathogen armamentarium (Tiedmann, 1997) and as a consequence of fungal sugar oxidases activity and/or decrease of the plant's own antioxidant potential (Edlich, Lorenz, Lyr, Nega, & Pommer, 1989). In this regard, the detrimental effect of *Botrytis* infection on the ascorbate–glutathione cycle has been extensively described in tomato leaves (Kuźniak & Skłodowska, 1999; Kuźniak & Skłodowska, 2001). It would be reasonable then to assert that lycopene functions as the major antioxidant in tomato fruits, as do ascorbate and glutathione in tomato leaves, and its significant increase following BTH treatment is possibly responsible for the enhanced resistance to grey mould.

BTH was already employed to induce resistance against *B. cinerea* in tomato plants (Małolepsza, 2006). In that study, the induced resistance was correlated with an oxidative burst occurring in leaf tissues prior to fungal inoculation. Thus, in spite of a generic oxidative burst on the basis of BTH action, it seems that different resistance mechanisms predominantly operate in leaves and berries.

Lycopene bodies were earlier described in chromoplasts of tomato fruit, as crystalloid rod-shaped structures enveloped by a thylakoid membrane (Ben-Shaul & Naftali, 1969). By exploiting dichroism of some crystals, i.e., the property of exhibiting two different colours when viewed along different axes, we were able to visualize lycopene crystalloids in tomato pericarp cells. Moreover, in BTH-treated samples, the increased amount of lycopene crystalloids, assessed by image analysis, though not significant, was in agreement with the enhanced lycopene content.

5. Conclusions

The post-harvest induced resistance represents a relatively novel strategy to control post-harvest crop diseases (Iriti et al., 2004; Terry & Joyce, 2000). This approach may circumvent some of the side effects due to the application of synthetic fungicides, foremost the development of

resistant fungal strains (LaMondia & Douglas, 1997). Furthermore, plant activators could represent a valid tool to improve the health benefits of plant foodstuffs, because of their stimulating effect on plant secondary metabolism (Iriti et al., 2005, 2006). It is noteworthy, that compounds involved in plant defence, namely phytoalexins, include a variety of phytochemicals involved in prevention of major human chronic diseases (Fumagalli et al., 2006; Iriti & Faoro, 2004), besides being more safe for the environment, operators and consumers.

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