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Biosynthesis and Accumulation of Isoprenoid Carotenoids and Chlorophylls and Emission of Isoprene by Leaf Chloroplasts

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ABSTRACT. Chloroplasts and other plastid forms possess a genuine biosynthetic pathway for the synthesis of isopentenyl diphosphate (IPP) and isoprenoids: the 1-deoxy-D-xylulose-phosphate/2-C-methylerythritol 5-phosphate pathway, known as DOXP/MEP pathway. This isoprenoid pathway provides the C_5 isoprenoid precursors (IPPs) needed for the light-induced biosynthesis of chlorophylls (C_{20} phytyl side-chain) and of the tetraterpenoid carotenoids (C_{40} isoprenoids) which are essential constituents of photochemically active thylakoids of chloroplasts. The performance of the DOXP/MEP pathway is reviewed and the accumulation and final concentration of chlorophylls and carotenoids in photosynthetically active leaves is provided. Under high-light (> 1000 µmol photons m^{-2} s^{-1}) and high temperature conditions (> 28 °C) many plant leaves emit volatile hemiterpenes at high rates, either isoprene (broadleaf trees) or methylbutenol (American ponderosa pines), both of which are formed via the plastidic DOXP/MEP pathway. Biosynthesis and physiological significance of the emission of isoprene and methylbutenol are briefly discussed. This paper is dedicated to Professor Guivi Sanadze, Tbilisi, Georgia, the pioneer in plant isoprene research, on the occasion of his 80th birthday (July 30, 2009). © *2009 Bull. Georg. Natl. Acad. Sci.*

Key words: *hemiterpenes, isoprenoid, isoprene, methylbutenol.*

INTRODUCTION

Within the plant cells the photosynthetic pigments, chlorophylls and carotenoids, which are responsible for the absorption of light, are bound to the photochemically active thylakoids that perform the photosynthetic light and associated electron transport reactions of chloroplasts [1–4]. The individual chlorophylls and carotenoids are specifically bound to several chlorophyll-carotenoid proteins [5–9] that are functionally integrated into the thylakoid biomembrane, together with the reaction centers of photosystem I and II [10]. Carotenoids are tetraterpenoids, i.e. C_{40} compounds, which are composed of eight active isoprenic C_5 units, whereby one differentiates between carotenes (pure hydrocarbons) and the oxygen-bearing xanthophylls, such as lutein, zeaxanthin, violaxanthin and neoxanthin. All carotenoid carbons are of isoprenoid origin. Chlorophylls, in turn, are mixed prenyllipids [11, 12], their chemical structure consists of a porphyrin ring that is esterified with the C_{20} diterpene phytol.

For many decades it had been accepted that carotenoids and the phytyl chain of chlorophylls are synthesized, just like sterols, from isoprenic C_5 units, i.e.

isopentenyl diphosphate molecules (IPPs), made via the cytosolic acetate/mevalonate pathway of IPP formation [see reviews 13, 14]. Today, however, it is clear that the biosynthesis of carotenoids and the phytyl chain of chlorophylls proceeds via the DOXP/MEP pathway of IPP biosynthesis which is bound to chloroplasts [13 – 15]. This has been demonstrated in the 1990s by a joint collaboration of the working groups of Hartmut Lichtenthaler, Karlsruhe, and Michel Rohmer, Mulhouse/ Strasbourg, via the application of new labeling and detection methods, such as ^{13}C-labeling combined with high resolution NMR spectroscopy and deuterium-labeling combined with mass spectroscopy [13, 16].

Plants can emit various volatile organic compounds (VOCs) found in the atmosphere, such as alkanes, alkenes, alcohols, aldehydes, ethers, esters, carboxylic acids [17] and various types of isoprenoids [18–20]. A large part of these phytogenic volatile compounds is of isoprenoid origin, especially isoprene [17, 18, 21-25]. These isoprenoids primarily consist of the volatile hemiterpene *isoprene*, e.g. in various herbaceous plants and trees, and of its partial oxidation product *2-methylen-3-buten-2-ol* as emitted by several ponderosa pines in the western states of North America [3]. Isoprene, methylbutenol and also certain volatile monoterpenes possess a high impact on atmospheric chemistry and ozone formation [19, 21, 26, 27], since they essentially contribute to the formation of photochemical smog and ozone. This is why their biosynthesis and the causes of their emission from green leaves have been gaining increasing interest.

This report briefly reviews the plastidic DOXP/MEP pathway of IPP biosynthesis, which is responsible for the biosynthesis of chlorophylls (phytyl side-chain) and carotenoids, and differentiates it from the cytosolic acetate/mevalonate pathway of sterol biosynthesis. The light-induced accumulation and final functional concentration of carotenoids and chlorophylls in green leaves is presented. Moreover, the biosynthesis of isoprene and methylbutenol in green leaves and needles via the DOXP/ MEP pathway are outlined as well as their emission under special environmental conditions. In addition, the probable physiological meaning of the emission of isoprene and other isoprenoid volatiles by plants, thus losing high amounts of photosynthetically fixed carbon, is indicated.

THE TWO CELLULAR PATHWAYS OF ISOPRENOID BIOSYNTHESIS

The DOXP/MEP pathway of chloroplasts

The DOXP/MEP pathway of IPP biosynthesis, detected in the mid - 1990s only [3, 13-16], proceeds in chloroplasts and starts from pyruvate and glyceraldehyde-3-phosphate (GA-3-P), whereby 1-deoxy-D-xylulose 5-phosphate (DOXP) is formed as the first C_5 product. In 6 further enzymatic steps DOXP is transferred to the C_5 compounds DMAPP and IPP (Fig. 1). The volatile hemiterpene isoprene, a C_5 compound, is synthesized from DMAPP in one step via the action of isoprene synthase. By head-to-tail condensation DMAPP and IPP yield the monoterpene C_{10} compound geranyl diphosphate (GPP), from which other monoterpenes are derived in specific plants. The diterpene geranygeranyl diphosphate (GGPP) is formed by adding two more C_5 carbon skeletons of IPP. Via partial saturation of GGPP the C_{20} compound phytol diphosphate is obtained, the isoprenoid side-chain of the two chlorophylls a and b. The C_{20} phytyl chain is also used for the biosynthesis of phylloquinone, a phytyl-methyl-1,4-naphthoquinone (vitamin K1), as well as for that of α-tocopherol (vitamin E) and its oxidation product α-tocoquinone. By tail-to-tail condensation of two GGPPs the first linear C_{40} chain is formed which, by successive desaturation (introduction of conjugated double bonds) and the introduction of two ionone rings, yields the tetraterpenoid carotenoids. A further elongation of the C_{20} geranylgeranyl chain by 5 IPP units yields the nonaprenyl chain of plastoquinone-9, another electron carrier of the photochemically active thylakoid membranes. All these steps, including the formation of particular mono- and diterpenes, e.g. as components of etheric oils, proceed in chloroplasts, whereby other plastid forms as well possess a fully functional DOXP/MEP pathway of IPP biosynthesis. Under photosynthetic conditions an active export of isoprenoid C_5 units proceeds from chloroplasts to the cytosol, a process that has been reviewed in detail [3]. The DOXP/MEP pathway can specifically be inhibited by 5-*ketochlomazone* that blocks DOXP synthase, the first enzyme of the pathway [28, 29]. A second inhibitor is *fosmidomycin* that specifically blocks the second enzyme DOXP reductoisomerase, as has been shown both for plants [30, 31 and for eubacteria [32], which possess the DOXP/MEP pathway as well. Fosmidomycin is a structural analogue of 2C-methylerythrose 4-phosphate, the intermediate in the enzymic conversion of DOXP to its product 2C-methylerythritol 4-phosphate (MEP) [28, 30].

The cytosolic acetate/mevalonate pathway

In contrast to the DOXP/MEP pathway, the cytosolic acetate/mevalonate (MVA) pathway starts from 3 acetyl CoA and provides IPP and DMAPP for isoprenoid biosynthesis in the cytosol (Fig. 1). By head-to-tail condensation of three C_5 units it delivers the C_{15} isoprenoid

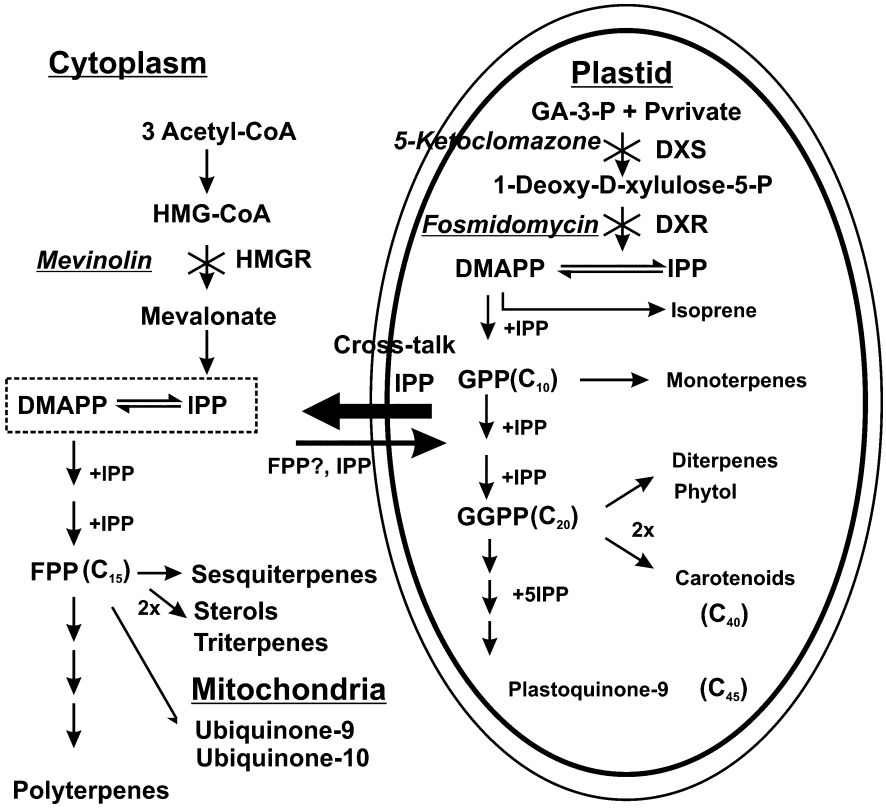


Fig. 1. Compartmentation of the two isoprenoid biosynthesis pathways in the plant cell: (1) The classical cytosolic acetate/mevalonate pathway synthesizes the isopentenyl diphosphates (IPPs) for the biosynthesis of sterols, sesquiterpenes, triterpenes and polyterpenes and also for the nonaprenyl and decaprenyl side-chains of the mitochondrial ubiquinones Q_9 and Q_{10}. (2) The DOXP/MEP pathway is localized in chloroplasts and provides the active C_5-units (IPP) for the biosynthesis of chlorophylls (phytyl side-chain), carotenoids, prenylquinones (isoprenoid side-chains), isoprene methylbutenol as well as mono- and diterpenes. The cytosolic acetate/mevalonate pathway is specifically inhibited by *mevinolin* (target: HMG-CoA reductase = HMGR). For the DOXP/MEP pathway in chloroplasts two inhibitors exist: i) *5-ketoclomazone* (target: DOXP-synthase = DXS) and ii) *fosmidomycin* (target: DOXP-reductoisomerase = DXR). The cross-talk between the two cellular biosynthetic isoprenoid pathways primarily consists of an export of active isoprenoid C_5-units from chloroplasts to the cytosol [3]. The scheme shown is based on the papers of Lichtenthaler's group [3, 13, 28, 29, 31]. Abbreviations used: DMAPP = dimethylallyl diphosphate, IPP = isopentenyl diphosphate, FPP = farnesyl diphosphate, GPP = geranyl diphosphate, GGPP = geranylgeranyl diphosphate, HMG-CoA = hydroxymethylglutaryl-coenzyme A.

chain farnesyl diphosphate (FPP) from which, depending on the plant, several sesquiterpenes can be formed. A dimerization of two FPPs (tail-to-tail condensation) yields the triterpene squalene from which the sterols, components of cytosolic plasma biomembranes, are formed. Further addition of the carbon skeletons of IPP molecules results in the formation of polyterpenes that show up in the white isoprenoid latex of many plant families. Mitochondria do not possess their own isoprenoid biosynthesis machinery. The nonaprenyl and tetraprenyl side chains of their ubiquinones Q_9 and Q_{10} are formed from IPP units synthesized via the cytosolic acetate/mevalonate pathway . The cytosolic isoprenoid pathways can specifically be inhibited by *mevinolin* and other statins (target: HMG-CoA reductase) [33, 34]. Although there exists a "cross-talk" between both cellular isoprenoid pathways, the main direction is the export of active isoprenoid C_5 units, possibly as IPP, from chloroplasts to the cytosol as reviewed by Lichtenthaler [3].

LEVELS OF CHLOROPHYLLS AND CAROTENOIDS IN LEAF CHLOROPLASTS

Chlorophyll and carotenoid levels in etiolated and green leaf tissue

Plant seedlings germinating in the dark (etiolated seedlings) do not form chlorophylls, however, they synthesize low amounts of carotenoids. They possess trace amounts of proto-chlorophyllide that, upon illumination and induction of chloroplast formation, is quickly transformed in a photoreduction step to chlorophyllide and esterified with phytol (phytyl side chain of chlorophylls). Although the type of carotenoids of such etiolated seedlings are the same as in green leaf tissue, their level is, however, completely different from that of green seedlings. Thus, among total carotenoids ß-carotene is present in very low relative amounts of < 10 % of the total carotenoids as shown for barley seedlings in Table 1. In contrast, in green leaf tissue, where ß-caro-

tene is bound to the two photosynthetic reaction centers PSI and PSII, its level ranges from 30 % to 37 % of the total carotenoid content. It is of interest that the percentage of the xanthophyll cycle carotenoids zeaxanthin + antheraxanthin and violaxanthin (Z + A + V) is rather high with 27 % in etiolated barley seedlings, whereas it usually only ranges from 10 % to 15 % in green barley and other herbaceous leaf tissue. Lutein is the major xanthophyll in etiolated tissue (range: 58 % to 65 %) and also in green leaf tissue (range: 49 % to 55 %, depending on growth conditions). In the first hours of light-induced chloroplast and thylakoid biogenesis particularly those photosynthetic pigments are formed that have either not been present in etiolated tissue or only in low amounts. These are, besides chlorophyll *a* and *b*, particularly ß-carotene, whereas the level of lutein and xanthophyll cycle carotenoids remain initially at the same level and only increase later, as has firstly been shown in detail by Lichtenthaler [35].

Table 1

Relative level of total chlorophylls (a+b) and total carotenoids (x+c) and of pigment ratios in the shoots of 9-day old green and etiolated barley seedlings. The values are indicated as µg pigment in 50 shoots consisting mainly of the primary leaf. The data are mean values and based on [12, 35–37].

	Green plants	Etiolated plants	Green/ etiolated
Pigment levels			
Chlorophyll a	7450	0	
Chlorophyll b	2460	0	
ß-Carotene (c)	490	12	40.8 x
Lutein	840	76	11.2 x
Zeaxanthin (Z)	60	21	2.9 x
Antheraxanthin (A)	8	3	2.7 x
Violaxanthin (V)	166	11	15.1 x
Neoxanthin	62	5	12.4 x
Total xanthophylls (x)	1136	116	9.8 x
Pigment ratios*			
a/b	3.0	0	
(a+b)/(x+c)	6.1	0	
(x/c)	2.3	9.7	
% Proportions**			
% ß-c of (x+c)	30.1	9.4	
% (Z+A+V) of (x+c)		14.4	27.3

* The pigment ratios (weight ratios) comprise chlorophyll a/b, total chlorophylls to total carotenoids (a+b)/(x+c) and xanthophylls/ ß-carotene (x/c).

** Percentage proportions of ß-carotene (% ß-c) and of xanthophyll cycle carotenoids, % (Z+A+V), of the total carotenoid content (x+c).

The ratio of chlorophylls a/b in green leaf tissue principally ranges from 2.6 to 3.2, depending on the photon flux density during plant and leaf growth [9] as is indicated in more detail in the next paragraph. In addition, the photosynthetic apparatus is characterized by specific values for the weight ratio of total chlorophylls to total carotenoids (a+b)/(x+c) from 5.3 – 7.0 in shade exposed plants, and from 4.1 – 5.3 in sun exposed plants. All the prenyl chains needed for chlorophyll and carotenoid biosynthesis are made from IPP molecules synthesized via the DOXP/MEP pathway of chloroplasts which is directly fed from intermediates of the Calvin cycle, such as glyceraldehyde-3-phosphate (GA-3-P) and pyruvate, whereby pyruvate can be directly synthesized in chloroplasts from phosphoglyceric acid, the first substance being formed in the Calvin cycle.

LEAF CHARACTERISTICS AND PIGMENT LEVELS OF SUN AND SHADE LEAVES

Leaf water content and specific leaf area

Sun leaves of trees and leaves from plants grown at high-light conditions are smaller and thicker, have thicker cell walls and show a lower water content (range: 54 – 57 %) as compared to shade leaves or leaves from plants grown at low-light conditions (64 – 70 %) as is indicated in Table 2. This has also been described for other plants [9, 38]. As a consequence sun leaves exhibited a significantly smaller specific leaf area (SLA range: 106 – 117 cm^2g^{-1} dw) as compared to shade leaves (SLA range: 317 – 385 cm^2g^{-1}dw) as is shown in Table 2. These differences between sun and shade leaves are also documented in higher values for the specific leaf weight (SLW range: 8.5 – 9.4 in sun leaves) and shade leaves (SLW range: 3.0 – 3.7 mg dw cm^{-2}) as summarized in Table 2.

Chlorophyll (Chl) and carotenoid levels

Due to the differences in morphology, thickness and water content, the levels of total chlorophylls of sun and shade leaves considerably differ depending on the reference system applied. On a leaf area basis the levels of total Chl a+b of sun leaves were significantly higher (range: 448 - 478 mg Chl a+b m^{-2}) than those of shade leaves (336 – 378 mg Chl a+b m^{-2}) (Table 2). However, on a dry weight (mg g^{-1}dw) and on a fresh weight basis

(mg g^{-1}fw) the chlorophyll a+b levels of sun leaves are much lower than those of shade leaves (Table 2). Similar relationships show up for the total carotenoid x+c content of green photosynthetic leaf tissue. On a leaf area basis sun leaves of trees and sun-exposed leaves of herbaceous plants exhibit much higher levels of carotenoids than shade leaves and leaves of low-light plants (Table 2). In contrast, on a leaf dry weight and leaf fresh weight basis shade and low-light leaves possess higher carotenoid levels than sun and sun-exposed leaves of trees and herbaceous plants.

Reference systems for pigment determination

Concerning the question, which reference system should be used for the determination of chlorophylls and carotenoids, the answer is clearly as follows: Best is the simultaneous use of two separate reference systems, i.e. the determination of pigment levels per leaf area unit (mg m^{-2}) and on a dry weight basis (mg g^{-1} dw). The use of fresh weight as a reference system should be avoided because it is not a reliable reference system. The water content always changes and is different between two plant groups, e.g. the control plants and treated plants. In fact, the difference between both groups is often only the water content, and, when referring pigments levels to the fresh weight, pseudo-differences in pigment levels showed up that were exclusively a result of the differing water content of control and treated plants. With respect to photosynthetic investigations it is always essential and absolutely necessary to determine chlorophyll and carotenoid levels on a leaf area basis. Several other photosynthetic parameters are always determined on a leaf area basis. These are photosynthetic CO_2 fixation rates (P_N), the different chlorophyll fluorescence parameters, the light exposure of leaves known as photosynthetic photon flux density PPFD. With the knowledge of the chlorophyll content per leaf area unit and the P_N rate per leaf area [mmol CO_2 m^{-2} s^{-1}] one can easily determine the photosynthetic rates as P_N per chlorophyll unit [e.g. mmol CO_2 mg Chl(a+b)$^{-1}$ h^{-1}] as given in [38]. This is an essential photosynthetic rate reference parameter that is independent of the chlorophyll content of a leaf.

Differences in pigment ratios

Due to either the high-light or shade adaptation response of leaves and chloroplasts [9] the relative amounts of chlorophylls and carotenoids are different in sun leaves and high-light plants as compared to shade leaves and low-light plants. Thus, the ratio of chloro-

Table 2

Differences in the specific leaf area (SLA), specific leaf weight (SLW), percentage of water content (% H_2O), as well as chlorophyll a+b levels (using different reference units: leaf area, dry weight, fresh weight), carotenoid x+c levels and the pigment ratios Chl a/b, chlorophylls to carotenoids, (a+b)/(x+c), and xanthophylls to carotenes, x/c, between fully developed green sun and shade leaves of plane tree (*Platanus*), poplar (*Populus*) and linden tree (*Tilia*). The values were taken at the beginning of July before the start of the hot and dry summer period (mid-July to end of August). For better comparison the values of sun leaves are shown in bold face. The photosynthetic pigments, chlorophylls a and b, as well as total carotenoids x+c were determined spectrophotometrically [2, 85] and the individual carotenoids via HPLC [44].
Mean values of 5 to 7 determinations. The differences between sun and shade leaves are highly significant: * $p < 0.01$, ** $p < 0.001$. The standard deviation in pigment ratios ranges from 2.5 % to 4.5 % for Chl a/b and x/c, and from 3 % to 6 % for the ratio (a+b)/(x+c).

	Platanus acerifolia		*Populus nigra*		*Tilia cordata*	
Parameter	Sun	Shade	Sun	Shade	Sun	Shade
H_2O (% of fw)*	**56.4** ± 1.7	64.5 ± 1.9	**56.1** ± 2.1	67.4 ± 1.6	**54.2** ± 1.7	69.1 ± 1.5
SLA (cm^2 g^{-1} dw)*	**106.3**	385.2	**112.1**	328.1	**117.3**	317.1
SLW (mg cm^{-2})*	**9.41**	3.28	**8.93**	3.05	**8.53**	3.74
Chl a+b (mg m^{-2})**	**448** ± 21	336 ± 17	**478** ± 38	369 ± 29	**471** ± 35	378 ± 23
Chl a+b (mg g^{-1}dw)**	**4.76**	12.9	**5.35**	12.1	**5.52**	12.0
Chl a+b (mg g^{-1}fw)**	**2.08**	4.59	**2.35**	3.94	**2.47**	3.71
Carotenoids (mg m^{-2})**	**98**±6	63 ± 4	**107** ± 8	68 ± 6	**111** ± 7	70 ± 5
Chl a/b**	**3.25**	2.78	**3.15**	2.65	**3.35**	2.74
(a+b)/(x+c)**	**4.57**	5.33	**4.46**	5.43	**4.24**	5.40
x/c**	**1.8**	2.9	**1.9**	3.1	**1.7**	3.0

phyll a/b exhibited higher values in sun leaves (3.15 – 3.35) than in shade leaves (2.65 – 2.78) (Table 2). In addition, sun leaves possess a relatively higher level of carotenoids with respect to total Chl a+b as compared to shade leaves. As a consequence, this is documented in different values for the ratio chlorophylls/carotenoids, (a+b)/(x+c), which are lower in sun leaves (4.24 – 4.57) than in shade leaves (5.33 – 5.43). Moreover, with respect to xanthophylls sun leaves exhibit relatively higher ß-carotene levels than shade leaves, and this is indicated by lower values for the ratio xanthophylls to ß-carotene, x/c, in sun leaves (1.7 – 1.9) as compared to shade leaves (2.9 – 3.1) (Table 2). In fact, these differences in pigment ratios are typical for the light adaptation of the photosynthetic apparatus, and the ones shown in Table 2 are quite similar to those found in sun and shade leaves of beech and ginkgo [38] and of various other trees [9, 39].

The differences in the relative levels of individual pigments and pigment ratios, such as Chl a/b, and chlorophylls/carotenoids and x/c between sun and shade leaves, are due to the fact that the photosynthetic pigment apparatus of sun leaves exhibits a much lower quantity of light-harvesting Chl a/b proteins (LHCII) and a greater number of reaction centers on a total chlorophyll basis as compared to shade leaves (Lichtenthaler et al., 1982a). As a consequence, the lower level of LHCII proteins with their low Chl a/b ratio of 1.1 to 1.3 in sun leaves results in higher values for the ratio Chl a/b. In contrast, shade leaves possess a higher proportion of LHCII proteins and lower Chl a/b ratios. Furthermore, also the lower values for the ratio (a+b)/(x+c) in shade leaves as compared to sun leaves is explained by their higher LHCII content. The different Chl a/b pigment proteins of LHCII (the LHCPs) exhibit higher values for the ratio (a+b)/(x+c) ranging from 7 to 13 [6] in comparison to the reaction center chlorophyll proteins CPa and CPI. The higher levels of LHCII in shade plants and shade leaves emphasize their priority investing in the light-harvesting pigment antenna because these plants lack incident light and therefore they try to absorb all the incoming light. In contrast, the chloroplasts of sun leaves (that always receive enough incident light) primarily invest in a higher number of photosynthetic reaction centers PSI and PSII, in order to guarantee a high rate of photosynthetic quantum conversion which finally results in higher photosynthetic rates. In this respect it is of interest that higher levels of LHCII in shade leaves and low-light plants are associated with higher and broader grana thylakoid stacks and a higher membrane stacking degree, also known as a higher proportion of appressed thylakoid membranes [6, 40-43].

Differences in the level of individual carotenoids of sun and shade chloroplasts

Sun leaves possess ß-carotene levels (on a leaf area basis) that are 2.2 to 2.35 times higher than shade leaves, whereas the total xanthophyll content is only 1.33 to 1.49 times higher (Table 3). Also, the total chlorophyll content of sun leaves is 1.3 to 1.5 times higher as compared to shade leaves (Table 2). These differences in carotenoid levels are best shown in the percentage composition of the individual carotenoids. The higher percentage of ß-carotene (34 % to 37 %) in sun leaves as compared to shade leaves is associated with a higher percentage of the xanthophyll cycle carotenoids zeaxanthin + antheraxanthin + violaxanthin, Z +A + V, of 13 % and 17 % (Table 3). The latter are known to function in the non-photochemical dissipation of absorbed light energy [9, 44, 45] and are required at high photon flux densities to protect the photosynthetic apparatus against photo-oxidative damage. In contrast, lutein and neoxanthin, the main xanthophylls of the light-harvesting pigment proteins of LHCII, exhibit their highest percentage (49 % and 55 % and 10 % to 11 %, respectively) in shade leaves (Table 3). In shade leaves the xanthophyll cycle carotenoids are mainly present in the epoxidated form as violaxanthin, and the de-epoxidated form zeaxanthin does not show up. In contrast, sun leaves always contain zeaxanthin, and the amount of zeaxanthin present depends on the irradiance and exposure time. After a few hours of sunshine, e.g. at mid-day of a sunny day, violaxanthin is indeed de-epoxidated between 75 % to almost 90 % of zeaxanthin, and the level of the intermediate antheraxanthin remains always rather low. On cloudy days de-epoxidation of violaxanthin to zeaxanthin does not occur, whereas on sunny days this process proceeds at full rates [44]. The performance of the xanthophyll cycle also depends on the stage of leaf development and an early or late greening of leaves [46].

BIOSYNTHESIS AND EMISSION OF ISOPRENE FROM PLANTS

Occurrence of isoprene emission in plants

Isoprene (C_5H_8, 2-methyl-1,3-butadiene, see Fig. 2), a volatile hemiterpene, is emitted by many green plants, including mosses, ferns, gymnosperms and angiosperms at high irradiance conditions [3, 18, 20, 23, 24, 47–49]. Its release from plants amounts to hundreds of millions of metric tons to the global atmosphere, whereby the estimations range from 180 to 450 x 10^{12} g carbon per

Table 3

ß-Carotene and xanthophyll levels (mg m^{-2} leaf area) and percentage composition of carotenoids between fully developed green sun and shade leaves of beech (*Fagus*), poplar (*Populus*), and linden tree (*Tilia*). The values were taken at the beginning of July before the start of the hot and dry summer period (mid-July to end of August). For better comparison the values of sun leaves are shown in bold face.

Mean values of 5 to 8 determinations. The differences between sun and shade leaves are highly significant: $p < 0.001$. The standard deviation in carotenoid levels ranges from 5 % to 8 %. Z + A + V is the sum of xanthophyll cycle carotenoids: zeaxanthin + antheraxanthin + violaxanthin. * The values for *Fagus* were taken from [3]. The individual carotenoids were determined via HPLC [44].

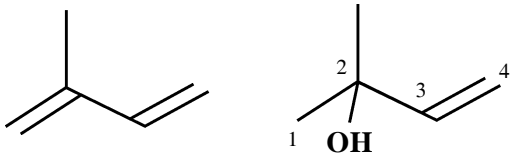
	*Fagus sylvatica**		*Populus nigra*		*Tilia cordata*	
Parameter	Sun	Shade	Sun	Shade	Sun	Shade
Levels (mg m^{-2})						
ß-Carotene	**45.4**	20.0	**36.9**	16.6	**41.1**	17.5
Xanthophylls	**80.6**	54.0	**70.1**	51.4	**69.9**	52.5
% Composition						
ß-Carotene	**36**	28	**34**	24	**37**	25
Lutein	**39**	49	**47**	56	**42**	55
Neoxanthin	**7**	10	**6**	11	**5**	10
Z + A +V	**17**	13	**13**	9	**16**	10
Zeaxanthin (Z)	5	0	6	0	6	0
Antheraxanthin (A)	2	1	1	1	2	1
Violaxanthin (V)	10	12	6	8	8	9

year worldwide. An essential observation is the fact that more organic carbon is lost from plants as isoprene than any other volatile plant molecule [27]. Isoprene emission from leaves is light and temperature dependent and preferably occurs at high rates at temperatures above 28°C and at high irradiances (photon flux density of > 1000 µmol m^{-2} s^{-1}). Thus it proceeds, in full sun light, when the photosynthesis process with its photosynthetic light reactions and associated electron transport reactions is completely light saturated. Under such conditions isoprene is instantaneously formed *de novo*.

Although more than 250 plants have been found to emit isoprene, including many herbaceous plants, trees seem to possess the highest rates of isoprene emission of green leaves. *Acacia nigrescens* (African acacia), *Eucalyptus globulus* (blue gum), *Liquidambar styraciflua* (sweet gum), *Populus nigra* (black poplar) and other *Populus* species, *Quercus robur* (European oak, pedunculate oak), *Quercus coccinea* (scarlet oak), *Salix babylonica* (weeping willow) belong to that category. But also other plants, such as *Arundo donax* (giant reed), *Myrtus communis* (myrtle), *Pueraria* spp. (kudzu), exhibit similar, high emission rates of 30 to 300 µg isoprene g^{-1} dw h^{-1} [18, 50].

Detection of isoprene emission

Isoprene emission by plants was discovered by Guivi Sanadze in the mid-1950s in his laboratory in Tiflis, Georgia [51-53]. He detected the release of isoprene into the atmosphere after the exposure of leaves from acacia (*Robinia pseudoacacia* L.), poplar (*Populus nigra* L.), willow (*Salix alba* L.), oak (*Quercus iberica* Stev,) box tree (*Buxus sempervirens* L.) and several other plants [see review 20, 24]. Around the same time Fritz Went detected, in an independent research, that the "blue haze", often formed over forested areas in the western states of North America, consisted of isoprene as its main volatile organic substance [21, 54]. However, it was Guivi Sanadze who was the first to more closely investigate the conditions for isoprene formation. He found that the isoprene emission rate is strongly dependent on leaf temperature and a high photosynthetic photon flux density [24, 55, 56]. He also localized isoprene biosynthesis in poplar leaf chloroplasts [57, 58] and realized that isoprene was apparently synthesized from freshly fixed carbon in the Calvin cycle [58, 59].



Isoprene **2-Methyl-3-buten-2-ol**

Fig. 2. Chemical structure of the volatile hemiterpenes isoprene, emitted by many green plants, and its oxidation product 2-methylen-3-buten-ol emitted by ponderosa pine needles.

This was later verified by other laboratories, e.g. via the rapid appearance of ^{13}C-labeling in isoprene from photosynthetically fixed $^{13}CO_2$ [60, 61] indicating that the isoprene biosynthesis must be closely connected to intermediates of the Calvin-Benson cycle. In fact, Guivi Sanadze was the pioneer of plant isoprene research, and with his original findings he essentially stimulated the research in laboratories of other parts of the world where his findings were confirmed and further extended.

Biosynthesis of isoprene

After the detection of the plants' DOXP/MEP pathway of IPP biosynthesis in chloroplasts [13–15] we immediately studied the biosynthesis of isoprene. For this purpose we developed a simple photometric UV-cuvette test system that allows the spectrophotometrical tracking of the successive isoprene emission from leaf pieces in a closed system [62]. This is possible because isoprene with its two conjugated double bonds has a typical UV spectrum with two peaks and a shoulder (similar to the spectrum of carotenoids in the blue spectral region). In this or in larger closed systems one can collect the emitted isoprene gas by the solid phase micro extraction (SPME) technique and inject it into a gas chromatography system combined with a mass spectrometer (GC-MS) that allows the detection of the incorporation of labeled precursors fed to the leaves into the emitted isoprene [62]. Such investigations were carried out at high irradiance exposure (> 2000 μmol $m^{-2}s^{-1}$) and a temperature > 30 °C (e.g. water bath).

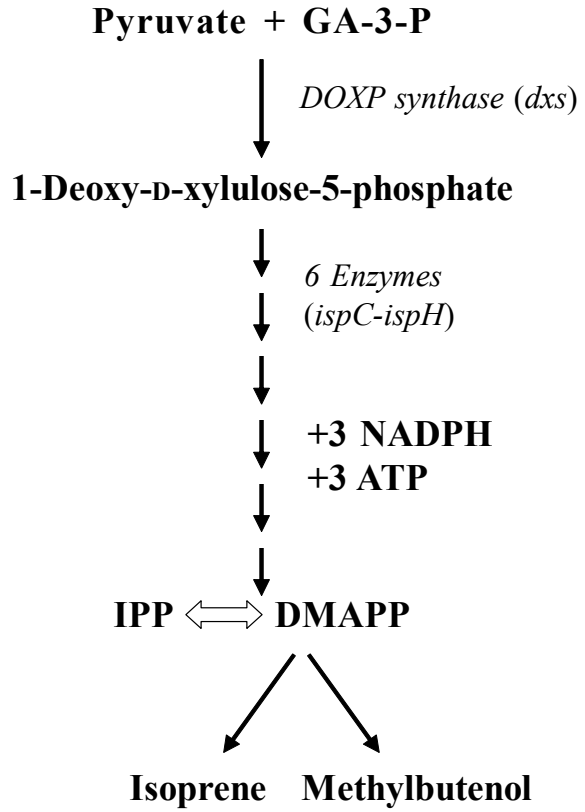


Fig. 3. Biosynthesis of the two volatile hemiterpenes, isoprene and methylbutenol (MBO), from pyruvate and glyceraldehyde-3-phosphate (GA-3-P) via the DOXP/MEP pathway of chloroplasts. The biosynthesis of the active isoprenoid C_5 unit IPP is catalyzed by seven enzymes (7 genes: *dxs*, and *ispC* to *ispH*) and requires three NADPH and three ATP as co-factors. Both hemiterpenes are set free from the IPP-isomer dimethylallyl diphosphate (DMAPP) in one step through the action of the two specific plastidic hemiterpene synthases: isoprene synthase and MBO synthase.

In fact, in 1997 we could show that isoprene is synthesized via the DOXP/MEP pathway from GA-3-P and pyruvate [63, 64], a biosynthetic sequence that requires 3 ATP and 3 NADPH as shown in Fig. 3. This was verified by the specific incorporation of deuterium-labeled 1-deoxy-D-xylulose (^2H-DOX) in the form of its xyluloside into isoprene, as proven by i) gas chromatography in combination with mass spectrometry (GC-MS) and ii) via high resolution NMR spectroscopy [63, 64]. Further proof was the observation that biosynthesis and emission of isoprene from illuminated plant leaves is efficiently blocked by the two inhibitors of the DOXP/MEP pathway, by 5-ketoclomazone (target: DOXP synthase) and by the herbicide fosmidomycin that inhibits the DOXP reductoisomerase of the DOXP/MEP pathway of IPP formation (compare Fig. 1) [31, 65]. The substrate GA-3-P directly derives from the photosynthetic carbon reduction cycle known as Calvin cycle. Pyruvate can be formed in the chloroplast, at least in spinach, from 3-phosphoglyceric acid, also an intermediate of the Calvin cycle [66, 67]. Another source of pyruvate is the cytosol from where it can be imported into the chloroplast. There also exists a third source of pyruvate: it is formed as a byproduct of the ribulosebisphosphate carboxylase/oxygenase activity [68]. One could expect that at higher temperatures and some water stress with a certain shortage of CO_2, the ribulosebisphosphate carboxylase/oxygenase of chloroplasts may exhibit higher rates of pyruvate which stimulates isoprene formation. This possible relationship has, however, yet to be investigated.

Regulation of isoprene formation

Isoprene is set free from DMAPP in a single enzymatic step via the plastidic isoprene synthase. This enzyme exists in a thylakoid-bound form [69] and also in stromal isoforms in the chloroplast [70]. The relative contribution to or the cooperation of these forms with isoprene biosynthesis is not yet clear. Recently it has been shown that the enzyme isoprene synthase is related to monoterpene synthases found in other plants [72]. Its biochemical characteristics have been assayed in some detail [73] and revealed that its K_m is 10 to 100-fold higher

for its C_5 substrate DMAPP than related monoterpene synthases for their C_{10} substrate geranyl diphosphate. The regulation of the light and temperature dependent isoprene emission possibly proceeds via the concentration of DMAPP [73, 80], which may rise at high irradiances and heat stress conditions. However, it has also been shown that in grey poplar leaves the enzymes isoprene synthase and 1-deoxyxylulose 5-phosphate DOXP reductoisomerase, the second enzyme of the DOXP/MEP pathway, show distinct seasonal patterns peaking in summer [74]. This observation suggests that under isoprene emission conditions there is a close coordination between the metabolic carbon flux through the DOXP/MEP pathway and the isoprene synthase activity. Recent studies of the natural ^{13}C-carbon isotope composition of isoprene in several plants confirmed that isoprene is synthesized *de novo* from freshly assimilated CO_2 [75]. This investigation also demonstrated that a low percentage of carbon came from another cellular carbon source, possibly from cytosolic pyruvate. Moreover, at an inhibition of photosynthetic carbon fixation by CO_2-free air, the contribution of this alternative cellular carbon source increased. It has been demonstrated by labeling techniques that several other leaf-internal carbon pools can support isoprene biosynthesis. Thus, e.g. starch or xylem-fed labeled glucose can be used as alternative carbon sources for isoprene emission, when, e.g. after abscisic acid application, the stomata close and CO_2 for photosynthetic carbon fixation is missing [61].

BIOSYNTHESIS AND EMISSION OF THE VOLATILE HEMITERPENE METHYLBUTENOL

Green needles of several ponderosa pines (*Pinus ponderosa, P. contorta, P. sabiniana*) in western North America do not emit isoprene but instead its partially oxidized form, the hemiterpene 2-methylen-3-buten-2-ol (MBO) (Fig. 2). Biosynthesis and emission of the volatile MBO proceeds in a high-light and high temperature-dependent manner [76, 77] and goes on also under water stress conditions. Like isoprene, the isoprenoid C_5 structure of MBO is formed in chloroplasts via the DOXP/MEP pathway from intermediates of the photosynthetic carbon fixation cycle (Calvin cycle). This has been demonstrated by a high-rate incorporation of deuterium-labeled deoxy-D-xylulose (^2H-DOX) into MBO as verified by mass spectrometry [65, 78]. In contrast, when offering ^{13}C-labeled MVA to ponderosa pine needles we did not find any incorporation of the ^{13}C label into MBO as checked using mass spectrometry [65]. MBO formation also requires DMAPP which is converted to MBO in a single step by the enzyme MBO synthase. The ponderosa pines emitting MBO can not produce isoprene. During evolution they seem to have modified their isoprene synthase to a MBO synthase. Both hemiterpene synthases start from the substrate DMAPP, however, the chemical mechanism for cleavage of the diphosphate from the C_5 carbon skeleton of DMAPP is different from that of isoprene synthase [3]. The biosynthesis of MBO is inhibited via 5-ketoclomazone blocking the DOXP synthase [28] as well as by fosmidomycin [31] that specifically blocks the DOXP reductoisomerase as shown in Fig. 1.

The conditions for MBO emission have been studied in detail [79]. It has been shown that photosynthetic rates and MBO emission increased with light intensity whereby neither process showed light saturation, not even at a PPFD of 2000 μmol m^{-2} s^{-1}. When, during water stress, the stomata closed and the photosynthetic carbon fixation stagnated, the MBO emission was not affected. Like isoprene, the carbon source for biosynthesis of MBO at water stress conditions seems to come from the breakdown of starch. In any case, the rather high amounts of MBO, emitted at higher irradiances and elevated summer temperatures by ponderosa pines, derive from a spontaneous *de novo* biosynthesis.

POSSIBLE FUNCTION OF ISOPRENE AND METHYLBUTENOL EMISSION

The physiological meaning of the emission of isoprene and methylbutenol (MBO) as volatile plant hemiterpenes is not yet really understood. The biosynthesis of both C_5 isoprenoids depends on the synthesis of DMAPP in the DOXP/MEP pathway. As expected, plant species with the highest capacity for isoprene and MBO production also possess enhanced rates of light-dependent synthesis of DMAPP [80]. Several more recent observations show that biosynthesis and emission of isoprene can protect the photosynthetic biomembranes against damage and photo-oxidation. Thus, early observations indicated that isoprene might protect the leaves by providing them with a certain thermotolerance against heat damage [81]. Isoprene and methylbutenol can apparently also function as a potential scavenger of radicals in the chloroplast and its thylakoids and thus protect thylakoid lipids and other chloroplast components from ozone and further reactive oxygen species, as has been shown more recently [e.g. 75, 82, 83]. The biosynthesis of one isoprene and one MBO requires 3 ATP and 3 NADPH, which are formed in the photosynthetic light reactions. Hence, the continuous synthesis and emission of isoprene and MBO, at high irradiance condi-

tions and heat stress of leaves, signify a permanent consumption of ATP and NADPH that keeps the two photosynthetic photosystems and the associated electron transport reactions in full function. Via this process overreduction and photo-oxidative damage of the photosynthetic apparatus is avoided.

Thus, the emission of isoprene and MBO by plants is apparently a mechanism similar to the photo-respiration of plants. The latter is known to produce CO_2 from previously fixed carbon that is immediately re-assimilated in the photosynthetic process. Also, this photo-respiration process keeps the two photosynthetic light reactions and the photosynthetic quantum conversion intact, and thus protects the photosynthetic apparatus. There is another ATP and NADPH consuming mechanism that keeps the photosynthetic light conversion apparatus going at high light and heat stress conditions. This is the continuous *de novo* biosynthesis of ß-carotene and xanthophyll cycle carotenoids (accumulation of zeaxanthin), which functions in protection of the photosynthetic pigment apparatus, as recently reviewed in detail by Lichtenthaler [3]. The biosynthesis of these additional carotenoids, which are tetraterpenoids, requires the amount of 8 IPP molecules per molecule and that means the consumption of 24 ATP and 24 NADPH. In addition, one also has to consider the high export rates of IPP under photosynthetic light conditions from chloroplasts to the cytosol for sterol formation [84, as also reviewed in 3] requiring additional ATP and NADPH. All these processes proceeding at high light and heat stress conditions protect the photosynthetic pigment apparatus with its photosystems PSI and PSII, the chlorophyll *a* proteins (CPa and CPI) and the light-harvesting pigment-proteins of LHCII [7, 39, 40]. In view of this, the emission of isoprene and MBO appears to be a 'safety valve' to protect the photosynthetic pigment apparatus with its photosystems and chlorophyll *a* proteins from photoinhibition and photooxidation. Although this is a waste of previously assimilated reduced carbon, it has the great advantage that it keeps the photosynthetic pigment and quantum conversion going and intact. Thus, as soon as the high light and heat stress conditions are over, the photosynthetic apparatus is still intact and can immediately switch to a normal photosynthetic CO_2 fixation.

CONCLUDING REMARKS

For the biosynthesis of isoprenoids and their biosynthetic C_5 intermediate IPP, plants possess two biochemically separate pathways: one in chloroplasts, the DOXP/MEP pathway, and another one, the acetate/MVA pathway in the cytosol. Both IPP yielding pathways operate independently of each other; however, cross-talk appears to be possible, although it works primarily in a chloroplast-to-cytosol direction. The acetate/MVA pathway provides the IPP molecules for the biosynthesis of cellular sterols, sesquiterpenes and polyterpenes. In contrast the DOXP/MEP pathway supplies the IPP C_5 units necessary for the synthesis of carotenoids, the prenyl side-chains of chlorophylls and prenylquinones, but also for the biosynthesis of hemiterpenes (isoprene, methylbutenol), monoterpenes and diterpenes. The activity of the DOXP/MEP pathway and the use of its IPP molecules for a specific of several possible isoprenoid end products very much depends on the physiological conditions of the plant leaves. During leaf and chloroplast development and at favorable conditions for full photosynthesis it supports chlorophyll (phytyl side-chain), carotenoid and prenylquinone biosynthesis and can contribute, via the export of isoprenoid C_5 units, to cytosolic sterol biosynthesis. The DOXP/MEP pathway also supports the chloroplast adaptation response to high-light or low-light conditions as seen in the formation of sun- and shade-type chloroplasts that are characterized by differential values of the chlorophyll a/b ratio and of the weight ratios of chlorophyll to carotenoids (a+b)/(x+c).

In contrast, at excess high-light and heat stress conditions the DOXP/MEP pathway predominantly operates in the *de novo* biosynthesis and accumulation of additional ß-carotene and xanthophyll cycle carotenoids (zeaxanthin, antheraxanthin) that protect the photosynthetic apparatus from photoinhibition and photooxidation. Moreover, it serves the biosynthesis and emission or accumulation of volatile hemiterpenes, such as isoprene and methylbutenol. As long as the photosynthetic carbon fixation (Calvin cycle) is active the biosynthesis of isoprene is supported by the flow of freshly fixed photosynthetic carbon (Calvin cycle intermediates) into isoprene and methylbutenol. At partial or full water stress condition with CO_2 shortage other cellular processes (e.g. starch breakdown, import of pyruvate from the cytosol) function as carbon source for the isoprene and methylbutenol biosynthesis. The use of IPP in the synthesis of different end products requires a fine tuning of the DOXP/MEP pathway of chloroplasts which presently is not yet clearly understood. So far the physiological significance of isoprene and methylbutenol emission by plants, which continuously consume photosynthetically formed ATP and NADPH, seems to be a 'safety valve' in order to avoid over-reduction, photoinhibition and photooxidation of the photosynthetic apparatus and its pigment systems PSI and PSII.

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იზოპრენოიდების, კაროტინოიდების და ქლოროფილების ბიოსინთეზი და დაგროვება და ფოთლის ქლოროპლასტების მიერ იზოპრენის გამოყოფა

ჰართმუთ კ. ლიჰთენთალერი

ბოტანიკის ინსტიტუტი (მცენარეთა მოლეკულური ბიოლოგია და ბიოქიმია)
კარლსრუეს უნივერსიტეტი, გერმანია

(წარმოდგენილია აკადემიკოს თ. ბერიძის მიერ)

ქლოროპლასტებსა და სხვა პლასტიდებში არის იზოპენტენილპიროფოსფატისა და იზოპრენოიდების სინთეზის ბიოქიმიური გზები: 1-დეზოქსი-D-ქსილულოზო-ფოსფატ/2-C მეთილერითრიტოლ 5-ფოსფატური გზა, ცნობილი როგორც DOXP/MEP გზა. ეს იზოპრენოიდული გზა იძლევა იზოპრენოიდების C_5 წინამორბედებს (IPP), რომლებიც აუცილებელია ქლოროფილების (C_{20} ფიტოლის გვერდითი ჯაჭვი) და ტეტრატერპენოიდური კაროტინოიდების (C_{40} იზოპრენოიდები) სინათლეზე დამოკიდებული სინთეზისათვის, რომლებიც ქლოროპლასტების ფოტოქიმიურად აქტიური თილაკოიდების აუცილებელ შემადგენელ ნაწილს წარმოადგენენ. სტატიაში მოცემულია DOXP/MEP ბიოქიმიური გარდაქმნების გზის მიმოხილვა, აგრეთვე მოყვანილია ფოტოსინთეზურად აქტიურ ფოთლებში კაროტინოიდებისა და ქლოროფილების, როგორც მეტაბოლიზმის საბოლოო პროდუქტების, კონცენტრაციათა სიდიდეები. მაღალი განათებულობისა (>1000 μmol ფოტონი m^{-2} s^{-1}) და ტემპერატურის (>28°C) დროს მრავალი მცენარის ფოთოლი გამოყოფს მაღალი სიჩქარით აქროლად ჰემიტერპენებს; იზოპრენს (ფოთლოვანი ხეები) ან მეთილბუთენოლს (ამერიკული ფიჭვი), რომლებიც წარმოიქმნებიან DOXP/MEP ბიოსინთეზის გზით. განიხილება იზოპრენისა და მეთილბუთენოლის სინთეზის ბიოქიმია და გამოყოფის ფიზიოლოგიური მნიშვნელობა. ეს ნაშრომი მიძღვნილია პროფესორ გივი სანაძისადმი, თბილისი, საქართველო, მცენარეული იზოპრენის შესწავლის პიონერისადმი, მისი დაბადების 80 წლისთავის აღსანიშნავად (30 ივლისი, 2009).

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