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Identification of benzoin obtained from calli of *Styrax officinalis* by HPLC

Hatice DEMİRAY*, Aylin EŞİZ DEREBOYLU, Zekiye Işın YAZICI, Fatih KARABEY
Section of Biology, Department of Botany, Faculty of Science, Ege University, Bornova, İzmir, Turkey

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Abstract: The stem tissue of *Styrax officinalis* L. distributed in West Anatolia was induced with agents such as boric acid and cocarboxylase (thiamine diphosphate), extensive stimulators of resin channels, in order to increase the amount of benzoin volatile oil. While the benzoin content was 120% in the induction medium to which excess boron and niacin were added, it increased to 231% when cocarboxylase (thiamine diphosphate) was added to the medium. Benzoin content of *Styrax* from petiole calli was 166%. HPLC-DAD results revealed that benzoin resin was present in 90% of the stems of *Styrax officinalis* distributed in West Anatolia. The major components of excess boron- and niacin-induced stem calli were hexane (58.33%), 3-methyl 2-pentene (16.10%), and cyclohexane (8.88%). Hexane (62%), methyl cyclopentane (19.09%), cyclohexane (12.04%), 2-hexanone (0.04%), ethylbenzene (0.03%), and benzene and 1-chloro-2-methylpropyl benzene (propene) were identified by the cocarboxylase application and GC-MS method. With enzyme application, while the percentages of decane and benzyl alcohol decreased, the cyclohexane ratio increased to 12.04%. Acetone (0.03%), ethyl acetate (4.10%), and dichloro methane (0.17%) contents were high as well.

Key words: *Styrax officinalis*, stem calli, benzoin, cocarboxylase, boron

1. Introduction

Styrax officinalis L. (Styracaceae) is a small deciduous tree (up to 4 m) only found around the Mediterranean region and in East and South-Eastern Asia (Fritsch, 1999). When the distribution of this plant in Turkey and the Mediterranean region was compared with world distribution, it was concluded that they originally had the same environmental distribution in North America before the continental drift that occurred at the beginning of the Cretaceous when North America was separated from Europe (Melville, 1967). The presence of a tropical species on both continents has been ascribed to the fact that climate and physiological formations of the Mediterranean region and California are similar. Because the evolution rate of this species was rather slow with a high adaptability to terrestrial habitats, the interregional variations were greater than the inter-continental variations (Vardar & Oflas, 1973). Moreover, the distribution of this species was demonstrated not only in the Mediterranean region, but also in the sub-Mediterranean region and even in terrestrial zones such as Konya, located in the western part of Central Anatolia, and Adıyaman, located in the northern part of South-Eastern Anatolia.

At first glance, it is a lower element of forest areas with a wide distribution and has seeds rich in oil content, making it industrially important. Benzofuran glycosides were isolated from the seeds of *Styrax officinalis* (Anıl, 1980; Akgül & Anıl, 2003), and benzofurans and sterol were isolated from the seeds of Styrax obasia Siebold & Zucc. (Lee et al., 2008). In addition, *Styrax officinalis* is known to produce resinous material usually secreted when the barks and trunks are injured by sharp objects (Pio Correa, 1931). The species, a relict dating from Mesozoic era, has resinous materials such as "Siam benzoe" from Styrax tonkinensis (Pierre) Craib ex Hartwich, "benzoe" from Styrax benzoides Craib, "Sumatra benzoe" from Styrax benzoin Dryand., and "storax" from *Styrax officinalis* L. (Tschirsch, 1925; Perkins, 1932; van Steenis, 1932; McKechnie, 1959; Milne & Milne, 1967). However, species distributed in Turkey do not include resinous material or resinous channels (Zeybek, 1970). Styrax and benzoin balsams have been widely employed since ancient times by the Romans (Gianno et al., 1990; Modugno et al., 2006), Egyptians, and Phoenicians to treat chronic infections of the respiratory tract, due to the therapeutic and pharmacological properties of the species which include disinfectant,

* Correspondence: haticedemus@yahoo.com

expectorant, and vulnerary activities (Modugno et al., 2006). Nowadays, their use is extended to perfumery and fixative agents, whilst their antioxidant and organoleptic properties are valued in the cosmetic and food industries for conservation and improvement of flavour (Fernandez et al., 2003, 2006a, 2006b; Castel et al., 2006). Due to the great economic importance of its resinous benzoin substance, in the present study we have tested different agents such as boric acid and cocarboxylase (thiamine diphosphate), extensive stimulators of resin channels, in order to increase the amount of benzoin volatile oil in in vitro grown stem tissues of *Styrax officinalis*.

2. Materials and methods

2.1. Plant material

Mature seeds of *Styrax officinalis* were collected from naturally growing plants in the vicinity of İzmir (Kemalpaşa, İzmir). Dehusked mature seeds were surface sterilised in 7.5% commercial bleach (20% sodium hypochloride) for 20 min. Three types of modified MS medium (Murashige & Skoog, 1962) were used. Nutritional variants were set: (1) - basic MS supplemented with the addition of 310 mg of H3BO3 and 25 mg of nicotinic acid (Demiray & Dereboylu, 2006); (2) - basic MS medium of Styrax with 0.5 mg of BAP plus 1 mg of NAA and 10 mMol of thiamine pyrophosphate chloride (cocarboxylase) to initiate callus; (3) - basic MS medium supplemented with 0.5 mg of BAP plus 1 mg of NAA as control medium, as a second medium, and as a third medium.

The seeds were germinated in the MS medium supplemented with 20 mg/L GA3. Then explants taken from the nodal buds of the stem tissue were grown in all 3 media mentioned above. Three hundred seeds were sown in each of the media, and experiments were repeated 3 times.

2.2. Extraction of essential oils

The essential oils were extracted by hydro-distillation using an apparatus of Clevenger type 44 in the Ege University Faculty of Sciences (Turkey). The extraction took 2.5 h, mixing 20 g of stem calli tissues in 1400 mL of distilled water. The yellowish oil (0.5 mL) was dissolved in hexane and then dried over anhydrous sodium sulphate. After filtration the solvent was eliminated by pressure distillation and reduced in a rotary evaporator at 35°C, and pure oil was stored at 4°C in darkness until the beginning of analysis.

2.3. HPLC method

Benzoin contents of the calli were identified by HPLC-DAD (Table 1) with benzoin standard (Figure 1), and the composition of essential oils was identified with GC-MS in

Table 1. HPLC calibration method. Buffer: 1.5% acetic acid (v/v): 1.5% ammonium acetate (w/v): water. Instrument: surveyor PDA plus detector. Column: Waters C-18, 4.6 × 250 mm. Sample preparation: each sample was dissolved in 70% ethanol and filtered.

Time (min)	Methanol	Buffer	Flow rate (mL/min)
0.01	90.00	10.00	1.000
25.00	30.00	70.00	1.000
28.00	30.00	70.00	1.000
31.00	90.00	10.00	1.000
40.00	90.00	10.00	1.000

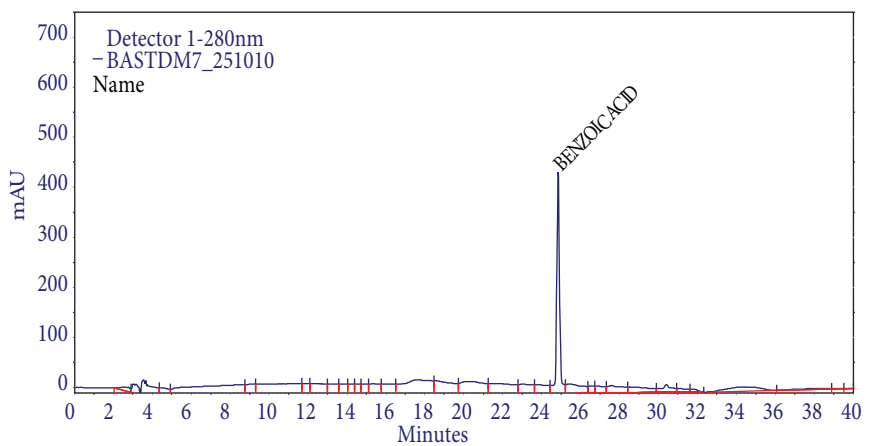

Figure 1. Benzoic acid standard at 280 nm.

the Ege University Centre for R & D and Pharmacokinetics Applications Environment & Food Analysis Laboratories (Bornova, İzmir).

2.4. GC-MS method

Measurements were performed on a Shimadzu GCMS-QP 2010 plus gas chromatograph using helium as the carrier gas at a flow rate of 1.40 mL/min. A 2.0 µL aliquot of the analyte was injected onto a Restek CL Pesticides 2 capillary column (20 m, 0.18 mm i.d., 014 µm film thickness). During injection the temperature of the GC oven was kept at 120 °C for 30 s and subsequently heated at a rate of 45 °C/min to 200 °C. Then, the oven was heated at a rate of 15 °C/min to 230 °C. Finally, the oven was heated at a rate of 30 °C/min to 300 °C where it was kept for additional 5 min. The interface was kept at 250 °C. The compounds were ionised by electron impact at 70 eV. The scan mode event time was 0.20 s, and scan speed was 2500. The mass range was m/z 50–450. The data analysis was by Wiley 7 and Pest-EI library.

2.5. Calculating the RI values of volatiles

Retention indices could be estimated by extrapolation, but the greatest accuracy was obtained by bracketing the analyte in question with n-paraffins, as shown in the example. Retention indices were also calculated by using the following equation:

$$I = 100Z + \frac{100[\log t'_R(\dot{I}) - \log t'_R(Z)]}{\log t'_R(Z+1) - \log t'_R(Z)}$$

where t'R(i) is the corrected retention time required to elute the compound of interest from the column, t'R(z) is the corrected retention time for the n-alkane eluted prior to the compound of interest, t'R(z + 1) is the corrected retention time for the n-alkane eluted after the compound of interest, and Z is the carbon number of the n-alkane of retention, t'R(z).

3. Results

Benzoin content was 120% in the induction medium to which excess boron and niacin were added (Figure 2), while the content increased to 231% in another medium to which cocarboxylase was added (Figure 2). The benzoin content of Styrax from petiole calli was 166% (Figure 2). HPLC-DAD results revealed that the amount of benzoin resin in the stems of *Styrax officinalis* was 90% (Figure 3). Figure 3a demonstrated the analyses of methanol extraction in plant stem collected from its natural habitat. The spectrum obtained from the chloroform extraction of the same plant sample was in Figure 3b, and in c option the pure benzoic acid HPLC diagram was shown. All of these spectra have indicated the benzoic acid contents of the plant stem samples collected from their natural habitats in order to compare the results obtained from the nodal calli tissues of stem samples of the same plant materials.

The major essential oil components of excess boron and niacin and also cocarboxylase-enzyme–induced stem calli are compared in Table 2. Hexane (58.33%– 62.19%), cyclohexane (8.88%–12.04%), acetone (0.02%–0.03%), dichloromethane (0.16%–0.17%), and acetonitrile (0.03%–0.04%) were found in both calli induced with media supplemented with enzymes and in those without any enzymes. Increases in the ratio of compounds were seen, as mentioned above. However, 1,1-dimethylcyclopentane (0.56%–0.25%); decanol (0.02%–0.01%); acetic acid, butyl ester, or butyl acetate (0.03%–0.02%); p-xylene (0.03%–0.02%); l-butanol (0.56%–0.42%); and benzyl alcohol (0.04%–0.02%) ratios decreased with enzyme application while they were also present in the 2 different media.

While 3-methyl-2-pentene, ethyl acetate (16.10%); ethyl acetate, acetic acid, ethyl ester (3.98%); propanoic acid, ethyl ester, or ethyl propanoate (0.01%); 4-methyl-2-pentanone (0.06%); benzene, 1,2 dimethyl-xylene (0.02%); and benzyl chloride (0.28%) were only present in calli induced with excess boron and niacin, cyclopentane (1.19%), methylcyclopentane (19.09%), ethyl acetate (4.10%), 2-hexanone (0.04%), propanenitrile (0.01%), and (1-chloro-2 methyl) propylbenzene (0.15%) were only found in excess boron and niacin-with-cocarboxylase–induced Styrax stem calli (Table 2).

4. Discussion

Tissue culture is one of the biotechnological methods applied for the production of volatile compounds such as essential oils, flavours, and volatile isolates in addition to volatile aldehydes and alcohols that are more easily produced by cultured, genetically modified microorganisms (bacteria, algae, and fungi, including yeast) (Guanaris, 2010; Namdjoyan et al., 2012; Yamaner et al., 2013). Benzoin is a hydroxy ketone attached to 2 phenyl groups. It appears as off-white crystals and has a light camphor-like odour. It is synthesised from benzaldehyde in the benzoin condensation. Benzoin is not a constituent of benzoin resin obtained from the benzoin tree (Styrax) or tincture of benzoin. The main component in these natural products is benzoic acid (Adams & Marvel, 1941).

It is indicated that even under the optimum induction conditions the yield of essential oil by in vitro plant tissues and cells was generally less than that achieved by the intact untreated plant, and the inability of cultured plant cells and calli to accumulate significant amounts of monoterpenes could be due to the combined effect of lower enzymatic activity and their higher catabolic rate (Falk et al., 1990). Because they are the enzymes of the volatile aldehyde and alcohol synthesis path, the activity of lipoxygenase and hydroperoxide lyase has been found in in vitro-cultured

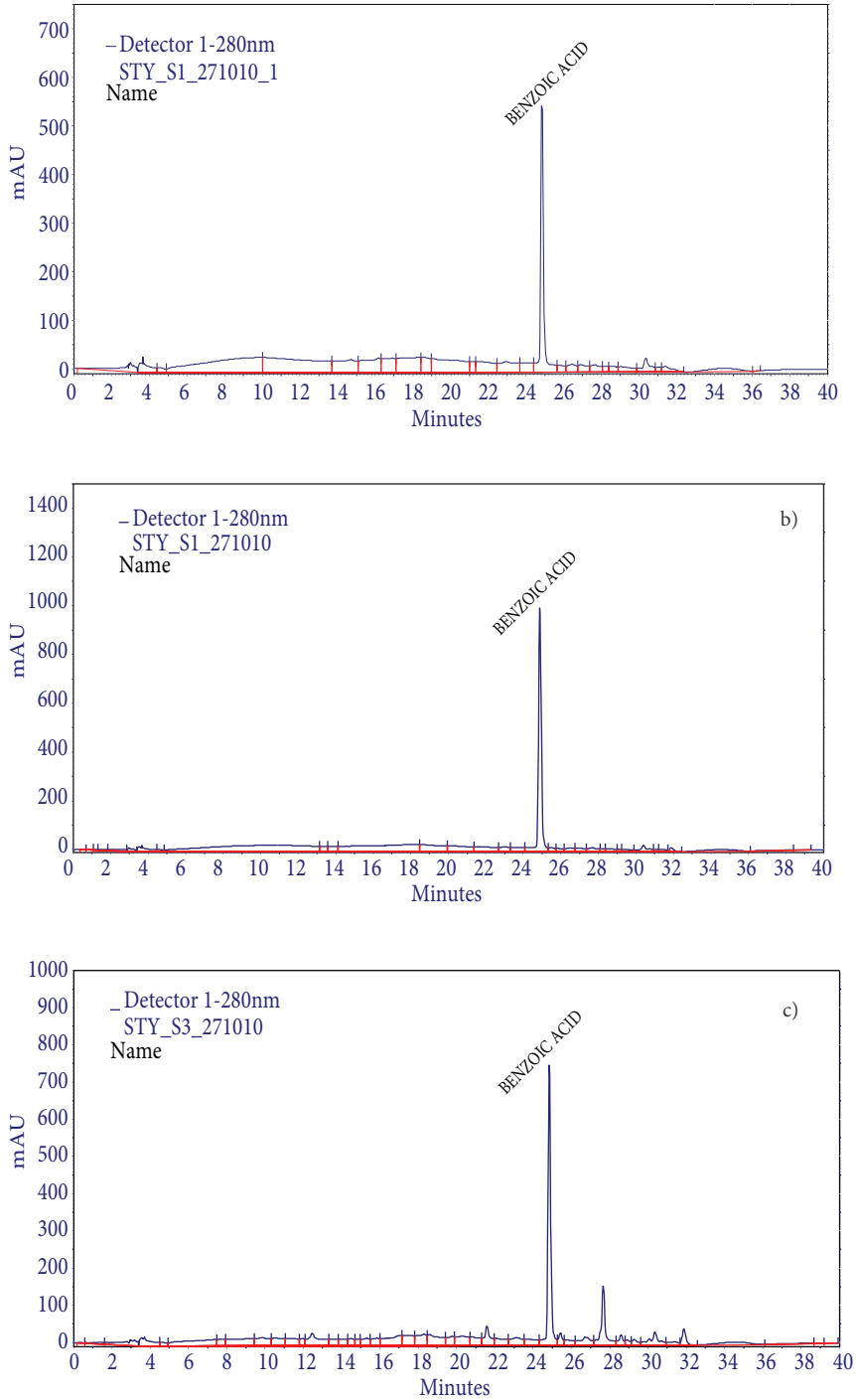

Figure 2. Benzoin diagrams of Styrax HPLC diagrams of benzoin obtained from a- shoot calli of Styrax not treated with enzymes, b- shoot calli of *Styrax* treated with enzymes in enzyme media, and c- petiole calli of Styrax not treated with enzymes.

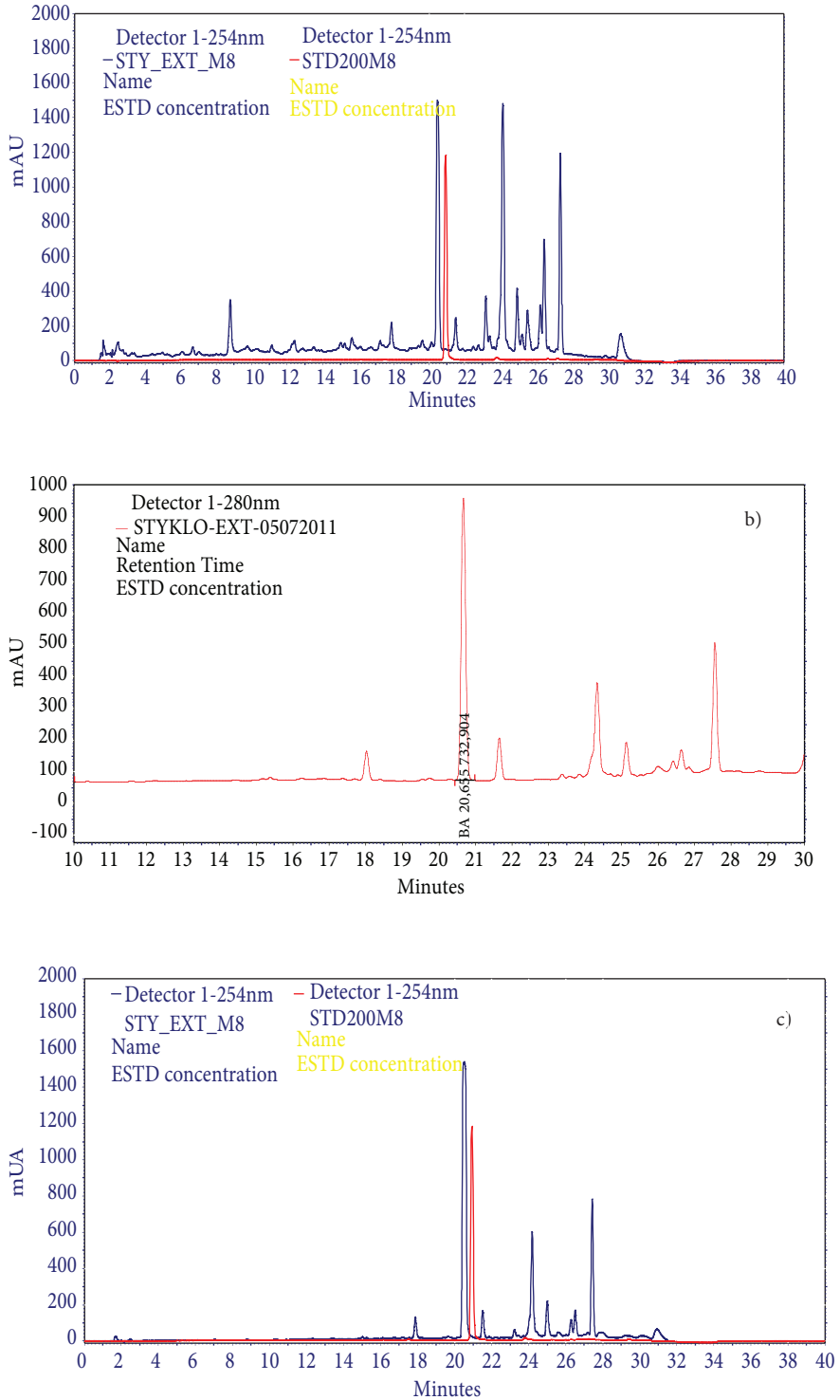

Figure 3. Benzoic acid standards of a- MeOH and b- CHCl3 extracts of *Styrax* shoots. c- benzoin diagram of *Styrax* shoots.

plant tissues (Matsui et al., 1996; Williams & Hardwood, 1998; Fauconnier et al., 2001). Therefore, we obtained successive increases with the applied induction media, one with excess boron plus niacin (120%) and the other with cocarboxylase enzyme (231%), depending on the intact plant benzoin content (90%).

Table 2. GC-MS values of stem calli of *Styrax officinalis* with and without cocarboxylase enzyme; nd: not determined, RI[a]: polar retention index value; RI[b]: apolar retention index value.

Compounds	Nodal bud calli	Nodal bud calli + cocarboxylase enzyme (replicated 3 times)	Identification methods	RI[a]	RI[b]
Hexane	58.33	62.19	GC-FID, GC-MS	599	572
Cyclopentane	-	1.19	GC-FID, GC-MS	580	575
Methylcyclopentane	-	19.09	GC-FID, GC-MS	575	571
3-Methyl-2-pentene	16.10	-	GC-FID, GC-MS	nd	nd
1,1-Dimethylcyclopentane	0.56	0.25	GC-FID, GC-MS	714	709
Cyclohexane	8.88	12.04	GC-FID, GC-MS	647	639
Methylcyclohexane	0.02	0.02	GC-FID, GC-MS	656	651
Acetone	0.02	0.03	GC-FID, GC-MS	459	454
Ethyl acetate	-	4.10	GC-FID, GC-MS	589	583
Ethyl acetate, acetic acid, ethyl ester	3.98	-	GC-FID, GC-MS	nd	nd
Dichloromethane	0.16	0.17	GC-FID, GC-MS	415	408
Benzene	0.02	0.02	GC-FID, GC-MS	638	635
Propanoic acid, ethyl ester, or ethyl propanoate	0.01	-	GC-FID, GC-MS	nd	nd
Decanol	0.02	0.01	GC-FID, GC-MS	1272	1743
2-Hexanone	-	0.04	GC-FID, GC-MS	805	760
4-Methyl-2-pentanone	0.06	-	GC-FID, GC-MS	nd	nd
Acetonitrile	0.03	0.04	GC-FID, GC-MS	466	461
Toluene	0.25	-	GC-FID, GC-MS	747	743
Propanenitrile	-	0.01	GC-FID, GC-MS	nd	nd
Acetic acid, butyl ester, or butyl acetate	0.03	0.02	GC-FID, GC-MS	1492	1480
Ethylbenzene	-	0.03	GC-FID, GC-MS	nd	nd
p-xylene	0.03	0.02	GC-FID, GC-MS	844	842
Benzene, 1,2-dimethyl-xylene	0.02	-	GC-FID, GC-MS	nd	nd
1-Butanol	0.56	0.42	GC-FID, GC-MS	558	553
Benzyl chloride	0.28	-	GC-FID, GC-MS	nd	nd
1-Chloro-2-methylpropyl benzene	-	0.15	GC-FID, GC-MS	nd	nd
Benzyl alcohol	0.04	0.02	GC-FID, GC-MS	1034	1032
Not identified	10.59	-	GC-FID, GC-MS	nd	nd

Because the species of the genus Styrax were not commonly studied for their essential oil and resin contents, it is not easy to compare our results with the results of other studies. To date, all of the studies have been performed on the leaves of *Styrax officinalis*. The volatile oil contents of Styrax japonica were determined by Kim and Shin (2004) and those of *Styrax officinalis*, known to be rare in France (they are found only in a few locations in the south-east of France) were reported by Tayaub et al. (2006). The oil composition obtained by steam distillation of leaves exhibited high levels of 2-hexenal (64%), n-hexanal (4.6%), nerol (4.6%), 3-hexen-1-ol (4.3%), and trans-2-heptenol

(2.6%). Only one paper concerned with essential oil composition of stem of Styrax from south-eastern France indicated that oxygenated monoterpenes were prominent in all of the plant organs. The major compounds of the essential oils of the leaves were 2-hexenal (17.6%), linalool (11.9%), and geraniol (5.5%). While linalool was the major compound (26.4%) of the volatile oils of the flowers, tridecanal (9.8%), dodecane (9.6%), α-terpineol (17%), and eugenol (9.9%) were also present (Tayaub et al., 2006).

Natural essential oils are usually mixtures of terpenoids (mainly monoterpenoids and sesquiterpenoids), aromatic compounds, and aliphatic compounds. Styrax is an aromatic plant that produces high levels of essential-oil–containing aliphatic aldehydes. Decanol, the dominant aldehyde that contributes to the flavour of the benzoin tree, was identified as a natural source of aliphatic aldehydes, which could be useful as food additives and in the perfume industry. Benzyl alcohol, one of the better-known aromatic alcohols, which occurs in storax (a resin obtained from the *Styrax officinalis* tree) and also in balsam of Peru and balsam of Tolu—either in the free state or as an ester in combination with cinnamic or benzoic acid (Maki & Takeda, 2000)—was found in the stem tissue of calli, with or without applied enzymes. Ethylbenzene and propylbenzene (in enzyme-applied calli) and p-xylene (in calli with or without applied enzymes) occurred as aromatic hydrocarbons. Methylcyclopentane and 1,1-dimethylcyclopentane were found as monocyclic terpene cyclopentane derivatives (Crane, 1955) in enzyme-applied calli and calli with or without enzymes applied, respectively. In addition to these compounds, methylcyclohexane and cyclohexane were the monocyclic terpene hydrocarbons determined in Styrax stem tissues with or without enzymes applied.

In this preliminary tissue culture study of *Styrax officinalis* from West Anatolia, the major essential oil components of excess boron- and niacin-induced stem calli were hexane (58.33%), 3-methyl-2-pentene (16.10%), and cyclohexane (8.88%). The volatile oil composition was changed by cocarboxylase enzyme application, and the compounds hexane (62%), methylcyclopentane (19.09%), cyclohexane (12.04%), 2-hexanone (0.04%), ethylbenzene (0.03%), and 1-chloro-2-methylpropyl benzene (propene) were provided. Cyclohexane was found in each of the different calli tissues, but its ratio increased to 12.04% with enzyme application; acetone (0.03%), ethyl acetate (4.10%), and dichloromethane (0.17%) contents were high as compared with the results of boron-plus-niacin–induced calli tissues. Decane and benzyl alcohol were present in the 2 different calli, but percentages decreased with enzyme application.

Apart from their nonoxidative and oxidative decarboxylation of 2-ketoacids, the formation of chiral 2-hydroxy ketones has been established for thiamine diphosphate dependent enzymes (Pohl et al., 2004). The cause of the increase in benzoin resin content is thiamine diphosphate enzyme. Styrax benzoin resins can only be produced after deep incisions have been made into the bark of the trees belonging to the genus Styrax (family Stracaceae), which are endemic in numerous East Asian countries such as Indonesia (Sumatra and Java), Laos, Thailand, and Vietnam. For the first time on record benzoin was identified in *Styrax officinalis* distributed in Turkey (Kemalpaşa), and the benzoin content of in vitro grown plant materials increased both excess-boron-plus-niacin and cocarboxylase treatments.

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