 © The Japan Wood Research Society 2002

ORIGINAL ARTICLE

Nobuyasu Hanari · Hiroshi Yamamoto · Ken-ichi Kuroda

Comparison of terpenes in extracts from the resin and the bark of the resinous stem canker of *Chamaecyparis obtusa* and *Thujopsis dolabrata* var. *hondae*

Received: November 22, 2000 / Accepted: February 19, 2001

Abstract A monoterpene and 15 diterpenes were isolated from the ethyl acetate extracts of the bark-glued resin from the resinous stem canker of *Thujopsis dolabrata* var. *hondae* Makino. A monoterpene (nezukone **20**) and 4 diterpenes (acetyl torulosol **5**, acetyl isocupressic acid **8**, acetyl abietinol **11**, and 7α-methoxytotarol **18**) were characteristic constituents of the ethyl acetate extracts but were absent in the *n*-hexane extracts from the resinous stem canker of *T. dolabrata* var. *hondae*. These terpenes were first isolated from *T. dolabrata* var. *hondae* and *T. dolabrata*. The available literature suggests that diterpene **18** is a new compound. The resinous stem canker of *Chamaecyparis obtusa* Endlicher contained larger amounts of manool **1**, *trans*-communic acid **6**, and ferruginol **12** and smaller amounts of isocupressic acid **7** and abietinol **10** than the resinous stem canker of *T. dolabrata* var. *hondae*. The concentration of **18** was less than 2% in the extracts, and the resinous stem canker of *C. obtusa* lacked this compound. The resinous stem cankers of *C. obtusa* and *T. dolabrata* var. *hondae* provided extracts 15.6 and 4.96 times, respectively, heavier than the healthy ones. Large differences in the ratios and compositions of terpenes were also observed between the resinous stem canker and the healthy trees. Terpenes isolated from the extracts contained many kinds of diterpene, especially the labdane-type diterpenes, in these diseased trees. These results suggest that the presence of labdane-type diterpenes is closely associated with the resinous stem canker or the causal fungi of this disease.

Key words Diterpene · Resinous stem canker · *Thujopsis dolabrata* var. *hondae* · *Chamaecyparis obtusa* · Labdane-type diterpene

N. Hanari (✉) · K. Kuroda
Institute of Agricultural and Forest Engineering, University of Tsukuba, Tsukuba 305-8572, Japan
Tel. +81-298-53-4578; Fax +81-298-55-2203
e-mail: s985635@ipe.tsukuba.ac.jp

H. Yamamoto
Faculty of Education, Ibaraki University, Ibaraki 310-8512, Japan

Present address:
[1]National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8569, Japan

Part of this report was presented at the 50th annual meeting of the Japan Wood Research Society, Kyoto, April 2000

Introduction

The resinous stem cankers of *Chamaecyparis obtusa* Endlicher and *Thujopsis dolabrata* var. *hondae* Makino are well known tree diseases that have been observed not only in the areas of high snowfall but all over Japan.[1–3] The affected trees show symptoms such as the resin escaping constantly from the trunk. A prompt solution for the resinous stem canker is required because it lowers the market value of these trees as raw materials. Many reports, anatomical observations,[4–6] and pathological studies[5–8] on the resinous stem canker are available, but few reports have taken note of the extracts from the resinous stem canker.[9]

Our attention has focused on resin flow as a symptom of the resinous stem canker.[10–12] We examined the resinous stem cankers of *C. obtusa* and *T. dolabrata* var. *hondae* and identified a diterpene dimer,[10] 6-(abieta-6′,8′,11′,13′-tetraenyl-12′-oxy)-7-methoxyabieta-8,11,13-trien-12-ol, and methyl esters of higher fatty acids (N. Hanari, H. Yamamoto, and K. Kuroda, unpublished observations, 2001) as characteristic compounds in the healthy barks of *C. obtusa* and *T. dolabrata* var. *hondae*, respectively. The resin and bark of the resinous stem canker of *C. obtusa* contained a large concentration of diterpenes, particularly the labdane-type diterpenes soluble in the *n*-hexane and ethyl acetate extracts.[11,12] The resin of the resinous stem canker of *T. dolabrata* var. *hondae* also contained many diterpenes in the *n*-hexane extracts (Hanari et al., unpublished observations). The resin and bark of the resinous stem cankers of *C. obtusa* and *T. dolabrata* var. *hondae* contained the same diterpenes. We were interested in these findings because the two trees are of different genuses.

The object of this study, in connection with the resinous stem cankers of *C. obtusa* and *T. dolabrata* var. *hondae*, was to isolate and identify characteristic compounds from the ethyl acetate extracts of the resinous stem canker of *T. dolabrata* var. *hondae*. The results were then compared with previous results on the resinous stem cankers of *C. obtusa*[11,12] and *T. dolabrata* var. *hondae* (N. Hanari et al., unpublished observations). The results are instructive for better understanding the resinous stem canker from the point of view of chemical composition.

Experiment

Electron impact-mass spectrometry (EI-MS) spectra were obtained using a JEOL JMS-DX300 spectrometer at an ionizing voltage of 70eV. Gas chromatography-mass spectrometry (GC-MS) was performed using an OV-17 capillary column: 0.32mm i.d. × 15m; column temperature 150°–250°C (16°C/min); injector temperature 250°C; carrier gas He 40ml/min. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were measured with a JEOL EX-270 NMR spectrometer in $CDCl_3$ or dimethylsulfoxide (DMSO)-d_6 or CD_3OD with tetramethylsilane as an internal standard. Elemental analysis datum was measured with a Perkin-Elmer 2400 CHN elemental analyzer. Infrared (IR) spectra were measured with a JASCO FT/IR-430 spectrometer. High-performance liquid chromotography (HPLC) analysis was performed using a JASCO 980 pump with a SIL $C_{18}T$ column (7.2mm i.d. × 25cm; eluent MeOH-H_2O 10:0–6:4 v/v or CH_3CN-H_2O 8:2–5:5 v/v; flow rate 2.5ml/min) and a JASCO 970 ultraviolet (UV) detector (λ 215nm). Preparative HPLC was performed using a JASCO instrument with a Shim-pack PREP-ODS column (20mm i.d. × 25cm; eluent MeOH-H_2O 10:0–9:1 v/v; flow rate 7.5ml/min) or an ODS-80Ts column (column size 7.8mm i.d. × 30cm; eluent MeOH-H_2O 95:5–6:4 v/v or CH_3CN-H_2O 8:2–5:5 v/v; flow rate 2.5ml/min) or an OCTYL-80TS column (column size 7.8mm i.d. × 30cm; eluent MeOH-H_2O 95:5 v/v; flow rate 2.5ml/min).

Extraction of terpenes

The bark from *T. dolabrata* var. *hondae* (kusaate), a planted 40-year-old tree (diameter 17cm), with the resinous stem canker was obtained in Tsurugi-Machi, Ishikawa Prefecture (May 12, 1998). Air-dried bark (560.26g) was crushed for extraction in *n*-hexane (8l × 2) for 4 days at room temperature. Evaporation of the solvent left yellow, sticky extracts (70.8g). Following *n*-hexane extraction, the extracted bark (459.81g) was further refluxed with ethyl acetate (4.5l × 2) for 10h. Evaporation of the solvent left dark red, sticky extracts (84.68g).

Isolation and identification of terpenes from the ethyl acetate extracts

The ethyl acetate extracts (11.9g) were repeatedly separated by column chromatography and preparative HPLC (Fig. 1). The compounds obtained by the first separation procedure were numbered fractions 1 and 2 (Fr. 1, Fr. 2); double figures were used to identify those obtained by the second separation procedure (e.g., Fr. 21, Fr. 22). The com-

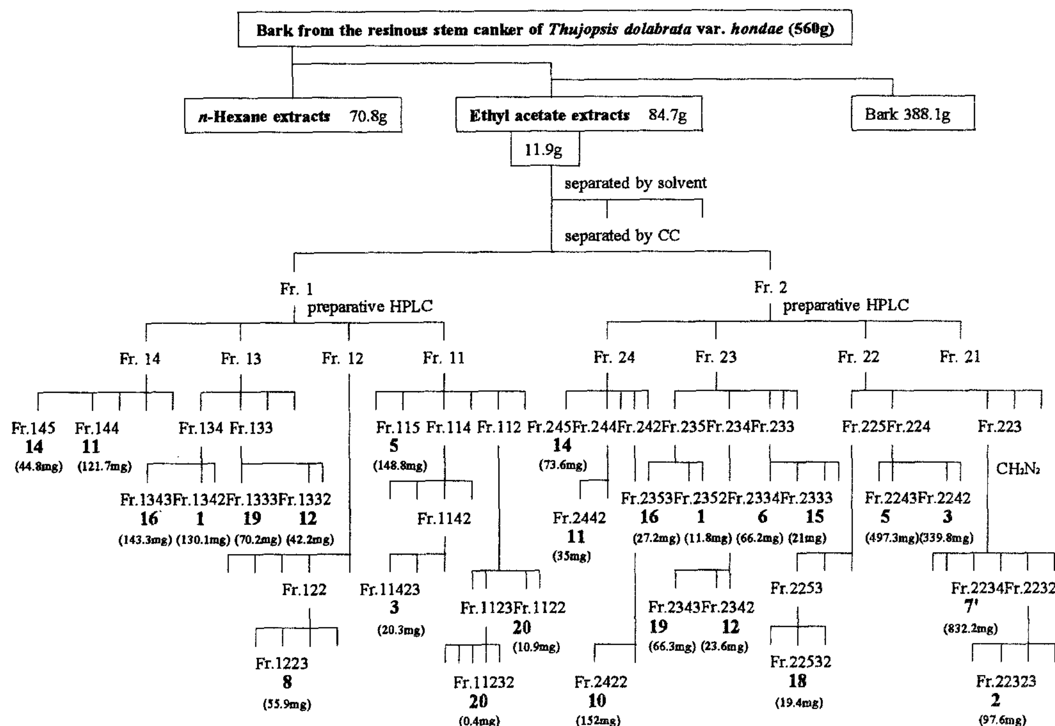

Fig. 1. Separation scheme of terpenes from the ethyl acetate extracts of the bark of the resinous stem canker of *Thujopsis dolabrata* var. *hondae*. The boldface numbers mean the number of isolated terpenes. The structures of compounds **1–20** refer to those in Fig. 2. Apostrophe (′) means a methyl ester of a related acid

pounds obtained by further separation procedures were numbered with three and four figures. Isolated compounds were identified by NMR and MS spectra data analyses and comparisons to known compounds.

Manool: 8(17),14-labdadien-13*R*-ol (**1**)

An 11.9-g aliquot was separated from Fr.1 to Fr.2 by column chromatography (developing solvent *n*-hexane/ethyl acetate 9:1 v/v) on silica gel. Fr.1 (3.0g) was separated from Fr.11 to Fr.14 by preparative HPLC (OCTYL-80TS column; eluent MeOH-H_2O 95:5 v/v; flow rate 2.5ml/min). Fr.13 (725.3mg) was separated from Fr.131 to Fr.134 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 9:1 v/v; flow rate 2.5ml/min). Fr.134 (321.1mg) was further separated from Fr.1341 to Fr.1343 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 8:2 v/v; flow rate 2.5ml/min). Fr.1342 (130.1mg) was identified as manool[10,11] by ^{1}H-NMR and MS analyses (Fig. 2). Fr.2 (7.08g) was also separated from Fr.21 to Fr.24 by preparative HPLC (Shim-pack PREP-ODS column; eluent MeOH; flow rate 7.5ml/min). Fr.23 (579.1mg) was separated from Fr.231 to Fr.235 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 9:1 v/v; flow rate 2.5ml/min). Fr.235 (42.7mg) was further separated from Fr.2351 to Fr.2353 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 8:2 v/v; flow rate 2.5ml/min). Fr.2352 (11.8mg) was also identified as manool: colorless oil, ^{1}H-NMR ($CDCl_3$): δ 0.67 (s, 3H, 20-Me), 0.80 (s, 3H, 19-Me), 0.86 (s, 3H, 18-Me), 1.27 (s, 3H, 16-Me), 4.47 (s, 1H, 17-H), 4.80 (s, 1H, 17-H), 5.06 (dd, 1H, J = 1.3 and 10.8Hz, 15-H), 5.19 (dd, 1H, J = 1.3 and 17.4Hz, 15-H), 5.90 (dd, 1H, J = 10.8 and 17.4Hz, 14-H); DI-MS: m/z (%) 290 (M^+; 2), 272 (29), 137 (100), 81 (78).

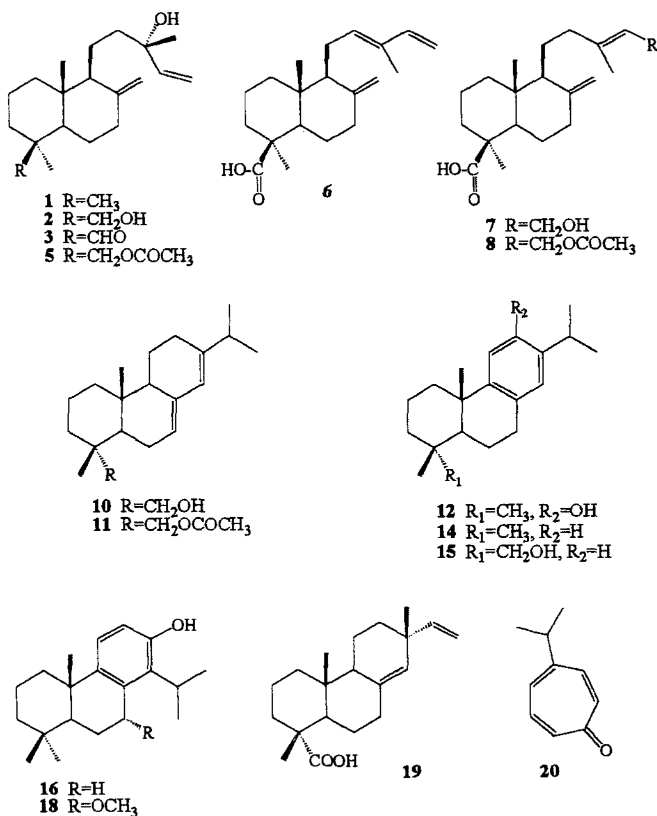

Fig. 2. Structures of diterpenes and nezukone (**20**) from the ethyl acetate extracts of the resinous stem canker of *Thujopsis dolabrata* var. *hondae*

Torulosol: 8(17),14-labdadien-13*R*, 19-diol (**2**)

The fraction Fr.22 (4.60g) was separated from Fr.221 to Fr.225 by preparative HPLC (Shim-pack PREP-ODS column; eluent MeOH-H_2O 95:5 v/v; flow rate 7.5ml/min). Fr.223 (2.18g) was methylated with an ethereal diazomethane solution at room temperature.[13] Methylated Fr.223 (1.90g) was separated from Fr.2231 to Fr.2236 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 8:2 v/v; flow rate 2.5ml/min). Fr.2232 (262.8mg) was further separated from Fr.22321 to Fr.22324 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 7:3 v/v; flow rate 2.5ml/min). Fr.22323 (97.6mg) was identified as torulosol[11,12] by ^{1}H-NMR and MS analyses: colorless oil, ^{1}H-NMR ($CDCl_3$): δ 0.64 (s, 3H, 20-Me), 0.98 (s, 3H, 18-Me), 1.27 (s, 3H, 16-Me), 3.38 (d, 1H, J = 10.9Hz, 19-H), 3.75 (d, 1H, J = 10.9Hz, 19-H), 4.49 (s, 1H, 17-H), 4.81 (s, 1H, 17-H), 5.06 (d, 1H, J = 11.0Hz, 15-H), 5.20 (d, 1H, J = 17.5Hz, 15-H), 5.91 (dd, 1H, J = 11.0 and 17.5Hz, 14-H); DI-MS: m/z (%) 306 (M^+; 1), 291 (2), 288 (12), 275 (2), 81 (100).

Torulosal: 13*R*-hydroxy-8(17),14-labdadien-19-al (**3**)

The fraction Fr.11 (705.4mg) was separated from Fr.111 to Fr.116 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 9:1 v/v; flow rate 2.5ml/min). Fr.114 (119.2mg) was separated from Fr.1141 to Fr.1144 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 75:25 v/v; flow rate 2.5ml/min). Fr.1142 (93.9mg) was further separated from Fr.11421 to Fr.11423 by preparative HPLC (ODS-80Ts column; eluent CH_3CN-H_2O 65:35 v/v; flow rate 2.5ml/min). Fr.11423 (20.3mg) was identified as torulosal[11] by ^{1}H-NMR and MS analyses. Fr.224 (969.3mg) was also separated from Fr.2241 to Fr.2244 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 9:1 v/v; flow rate 2.5ml/min). Fr.2242 (339.8mg) was also identified as torulosal: colorless oil, ^{1}H-NMR ($CDCl_3$): δ 0.57 (s, 3H, 20-Me), 1.00 (s, 3H, 18-Me), 1.27 (s, 3H, 16-Me), 4.56 (s, 1H, 17-H), 4.89 (s, 1H, 17-H), 5.06 (dd, 1H, J = 1.3 and 10.6Hz, 15-H), 5.20 (dd, 1H, J = 1.3 and 17.2Hz, 15-H), 5.90 (dd, 1H, J = 10.6 and 17.2Hz, 14-H), 9.74 (s, 1H, 19-H); DI-MS: m/z (%) 286 (M^+-H_2O; 7), 275 (1), 81 (100).

Acetyl torulosol: 19-acetoxy-8(17),14-labdadien-13*R*-ol (**5**)

The fraction Fr.115 (148.8mg) was identified as acetyl torulosol[12] by ^{1}H-NMR and MS analyses. Fr.2243 (497.3mg) was also identified as acetyl torulosol: colorless oil, ^{1}H-NMR ($CDCl_3$): δ 0.50 (s, 3H, 20-Me), 1.18 (s, 3H, 18-Me),

1.27 (s, 3H, 16-Me), 2.03 (s, 3H, OAc), 3.86 (d, 1H, J = 11.0Hz, 19-H), 4.22 (d, 1H, J = 11.0Hz, 19-H), 4.48 (s, 1H, 17-H), 4.83 (s, 1H, 17-H), 5.06 (dd, 1H, J = 1.0 and 10.6Hz, 15-H), 5.20 (d, 1H, J = 17.5Hz, 15-H), 5.91 (dd, 1H, J = 10.6 and 17.5Hz, 14-H); DI-MS: m/z (%) 330 (M^+-H_2O; 8), 315 (5), 288 (2), 257 (24), 135 (82), 81 (100).

trans-Communic acid: 8(17),12,14-labdatrien-19-oic acid (**6**)

The fraction Fr.233 (168.8mg) was separated from Fr.2331 to Fr.2334 by preparative HPLC (ODS-80T_s column; eluent MeOH-H_2O 8:2 v/v; flow rate 2.5ml/min). Fr.2334 (66.2mg) was identified as *trans*-communic acid[10–12] by ^{1}H-NMR and MS analyses: colorless oil, ^{1}H-NMR ($CDCl_3$): δ 0.65 (s, 3H, 20-Me), 1.27 (s, 3H, 18-Me), 1.75 (s, 3H, 16-Me), 4.48 (s, 1H, 17-H), 4.84 (s, 1H, 17-H), 4.88 (d, 1H, J = 10.6Hz, 15-H), 5.04 (d, 1H, J = 17.6Hz, 15-H), 5.41 (t, 1H, J = 6.6Hz, 12-H), 6.33 (dd, 1H, J = 10.6 and 17.6Hz, 14-H); DI-MS: m/z (%) 302 (M^+; 45), 287 (25), 257 (9), 81 (100).

Methyl isocupressate: methyl-15-hydroxy-8(17),13-labdadien-19-oate (**7′**)

The fraction Fr.2234 (832.2mg) was identified as methyl isocupressate[12,14] by ^{1}H-NMR and MS analyses: colorless oil, ^{1}H-NMR ($CDCl_3$): δ 0.51 (s, 3H, 20-Me), 1.18 (s, 3H, 18-Me), 1.67 (s, 3H, 16-Me), 3.61 (s, 3H, OMe), 4.14 (d, 2H, J = 6.9Hz, 15-H), 4.52 (s, 1H, 17-H), 4.86 (s, 1H, 17-H), 5.38 (t, 1H, J = 6.9Hz, 14-H); DI-MS: m/z (%) 334 (M^+; 2), 316 (47), 301 (36), 274 (12), 121 (100), 81 (66); IR ν_{max} (KBr) cm^{-1}: 1725, 1154, 989, 888.

Acetyl isocupressic acid: 15-acetoxy-8(17),13-labdadien-19-oic acid (**8**)

The fraction Fr.12 (998.1mg) was separated from Fr.121 to Fr.125 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 95:5 v/v; flow rate 2.5ml/min). Fr.122 (283.2mg) was separated from Fr.1221 to Fr.1224 by preparative HPLC (ODS-80Ts column; eluent CH_3CN-H_2O 7:3 v/v; flow rate 2.5ml/min). Fr.1223 (55.9mg) was identified as acetyl isocupressic acid[12] by ^{1}H-NMR and MS analyses: colorless oil, ^{1}H-NMR (CD_3OD): δ 0.63 (s, 3H, 20-Me), 1.22 (s, 3H, 18-Me), 1.71 (s, 3H, 16-Me), 2.07 (s, 3H, OAc), 4.52 (s, 1H, 17-H), 4.59 (d, 2H, J = 7.3Hz, 15-H), 4.86 (s, 1H, 17-H), 5.31 (t, 1H, J = 7.3Hz, 14-H); DI-MS: m/z (%) 362 (M^+; 1), 284 (49), 269 (100), 259 (1), 69 (11).

Abietinol: abieta-7,13-dien-18-ol (**10**)

The fraction Fr.24 (897.7mg) was separated from Fr.241 to Fr.245 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 95:5 v/v; flow rate 2.5ml/min). Fr.242 (267.2mg) was separated from Fr.2421 to Fr.2422 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 8:2 v/v; flow rate 2.5ml/min). Fr.2422 (152.0mg) was identified as abietinol[15] by ^{1}H-NMR and MS analyses: colorless oil, ^{1}H-NMR ($CDCl_3$): δ 0.84 (s, 3H, 20-Me), 0.88 (s, 3H, 19-Me), 1.01 (d, 3H, J = 7.0Hz, *i*-Pr.), 1.02 (d, 3H, J = 7.0Hz, *i*-Pr.), 2.22 (sept, 1H, J = 7.0Hz, 15-H), 3.13 (d, 1H, J = 11.0Hz, 18-H), 3.36 (d, 1H, J = 11.0Hz, 18-H), 5.39 (br.s, 1H, 7-H), 5.77 (s, 1H, 14-H); DI-MS: m/z (%) 288 (M^+; 100), 273 (8), 257 (23), 245 (13), 69 (35); IR ν_{max} (KBr) cm^{-1}: 3422, 1459, 1384, 1041.

Acetyl abietinol: 18-acetoxyabieta-7,13-diene (**11**)

The fraction Fr.144 (121.7mg) was identified as acetyl abietinol[16] by ^{1}H-NMR, MS, and elemental analyses. Fr.244 (99.4mg) was also separated from Fr.2441 to Fr.2442 by preparative HPLC (ODS-80Ts column; eluent MeOH; flow rate 2.5ml/min). Fr.2442 (35.0mg) was also identified as acetyl abietinol: colorless oil, ^{1}H-NMR ($CDCl_3$): δ 0.83 (s, 3H, 20-Me), 0.94 (s, 3H, 19-Me), 1.01 (d, 3H, J = 7.0Hz, *i*-Pr.), 1.02 (d, 3H, J = 7.0Hz, *i*-Pr.), 2.04 (s, 3H, OAc), 2.23 (sept, 1H, J = 7.0Hz, 15-H.), 3.67 (d, 1H, J = 11.0Hz, 18-H), 3.81 (d, 1H, J = 11.0Hz, 18-H), 5.41 (br.s, 1H, 7-H), 5.79 (s, 1H, 14-H); DI-MS: m/z (%) 330 (M^+; 100), 315 (4), 287 (4), 270 (81), 255 (90), 43 (95). Anal. calcd. for $C_{22}H_{34}O_2$: C, 79.95; H, 10.37%. Found: C, 79.41; H, 10.42%.

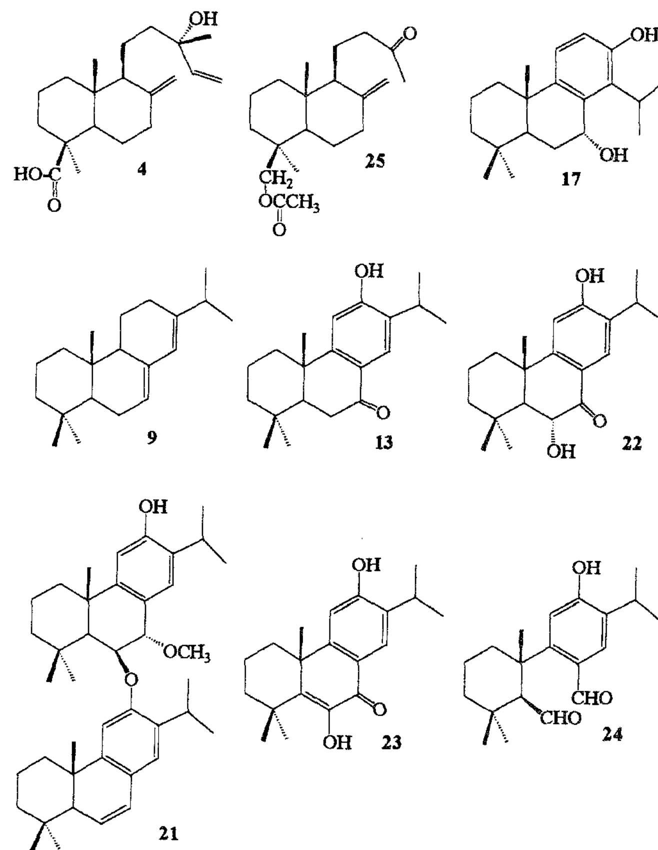

Fig. 3. Structures of terpenes from related reports[10,11] (Hanari et al., unpublished observations)

Ferruginol (**12**)

The fraction Fr.133 (159.8 mg) was separated from Fr.1331 to Fr.1333 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 85:15 v/v; flow rate 2.5 ml/min). Fr.1332 (42.2 mg) was identified as ferruginol[10–12] by ^{1}H-NMR and MS analyses. Fr.234 (104.0 mg) was also separated from Fr.2341 to Fr.2343 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 85:15 v/v; flow rate 2.5 ml/min). Fr.2342 (23.6 mg) was also identified as ferruginol: colorless oil, ^{1}H-NMR ($CDCl_3$): δ 0.91 (s, 3H, 20-Me), 0.93 (s, 3H, 19-Me), 1.17 (s, 3H, 18-Me), 1.22 (d, 3H, J = 6.9 Hz, *i*-Pr.), 1.24 (d, 3H, J = 6.9 Hz, *i*-Pr.), 3.11 (sept, 1H, J = 6.9 Hz, 15-H), 6.63 (s, 1H, 11-H), 6.83 (s, 1H, 14-H); DI-MS: *m/z* (%) 286 (M^+; 100), 271 (81), 243 (5), 69 (45).

Dehydroabietane: abieta-8,11,13-triene (**14**)

The fraction Fr.14 (429.0 mg) was separated from Fr.141 to Fr.145 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 9:1 v/v; flow rate 2.5 ml/min). Fr.145 (44.8 mg) was identified as dehydroabietane[17,18] by ^{1}H-NMR and MS analyses. Fr.245 (73.6 mg) was also identified as dehydroabietane: colorless oil, ^{1}H-NMR ($CDCl_3$): δ 0.90 (s, 3H, 20-Me), 0.94 (s, 3H, 19-Me), 1.18 (s, 3H, 18-Me), 1.22 (d, 6H, J = 7.0 Hz, *i*-Pr.), 2.87 (sept, 1H, J = 7.0 Hz, 15-H), 6.88 (s, 1H, 14-H), 6.98 (d, 1H, J = 8.0 Hz, 12-H), 7.19 (d, 1H, J = 8.0 Hz, 11-H); DI-MS: *m/z* (%) 270 (M^+; 23), 255 (100), 227 (6), 69 (33); IR ν_{max} (KBr) cm^{-1}: 1459, 889, 822.

Dehydroabietinol: abieta-8,11,13-trien-18-ol (**15**)

The fraction Fr.2333 (21.0 mg) was identified as dehydroabietinol[19] by ^{1}H-NMR and MS analyses: colorless oil, ^{1}H-NMR ($CDCl_3$): δ 0.89 (s, 3H, 20-Me), 1.21 (s, 3H, 19-Me), 1.22 (d, 6H, J = 7.0 Hz, *i*-Pr.), 2.83 (sept, 1H, J = 7.0 Hz, 15-H), 3.23 (d, 1H, J = 11.0 Hz, 18-H), 3.47 (d, 1H, J = 11.0 Hz, 18-H), 6.88 (s, 1H, 14-H), 6.98 (d, 1H, J = 8.2 Hz, 12-H), 7.18 (d, 1H, J = 8.2 Hz, 11-H); DI-MS: *m/z* (%) 286 (M^+; 74), 271 (100), 253 (90), 69 (15); IR ν_{max} (KBr) cm^{-1}: 3421, 1043, 824.

Totarol (**16**)

The fraction Fr.1343 (143.3 mg) was identified as totarol[20] by ^{1}H-NMR and MS analyses. Fr.2353 (27.2 mg) was also identified as totarol: colorless oil, ^{1}H-NMR ($CDCl_3$): δ 0.90 (s, 3H, 20-Me), 0.93 (s, 3H, 19-Me), 1.17 (s, 3H, 18-Me), 1.33 (d, 3H, J = 7.3 Hz, *i*-Pr.), 1.35 (d, 3H, J = 7.3 Hz, *i*-Pr.), 3.28 (sept, 1H, J = 7.3 Hz, 15-H), 6.52 (d, 1H, J = 8.2 Hz, 12-H), 6.98 (d, 1H, J = 8.2 Hz, 11-H); DI-MS: *m/z* (%) 286 (M^+; 48), 271 (100), 243 (5), 69 (22); IR ν_{max} (KBr) cm^{-1}: 3449, 1273, 903, 804.

7α-Methoxytotarol (**18**)

The fraction Fr.225 (252.5 mg) was separated from Fr.2251 to Fr.2253 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 8:2 v/v; flow rate 2.5 ml/min). Fr.2253 (136.7 mg) was separated from Fr.22531 to Fr.22533 by preparative HPLC (ODS-80Ts column; eluent CH_3CN-H_2O 7:3 v/v; flow rate 2.5 ml/min). Fr.22532 (19.4 mg) was identified as 7α-methoxytotarol by ^{1}H-NMR and MS analyses: colorless oil, ^{1}H-NMR (CD_3OD): δ 1.06 (s, 3H, 20-Me), 1.08 (s, 3H, 19-Me), 1.23 (s, 3H, 18-Me), 1.46 (d, 3H, J = 7.0 Hz, *i*-Pr.), 1.48 (d, 3H, J = 7.0 Hz, *i*-Pr.), 3.16 (sept, 1H, J = 7.0 Hz, 15-H), 3.51 (s, 3H, OMe), 4.55 (s, 1H, 7b-H), 6.75 (d, 1H, J = 8.6 Hz, 12-H), 7.05 (d, 1H, J = 8.6 Hz, 11-H); ^{13}C-NMR (CD_3OD): δ 21.4 (2-CH_2), 21.8 (16-Me), 22.0 (17-Me), 22.9 (19-Me), 24.2 (6-CH_2), 25.7 (20-Me), 30.4 (15-CH), 34.5 (18-Me), 34.6 (4-C), 40.2 (10-C), 41.3 (1-CH_2), 43.4 (3-CH_2), 46.2 (5-CH), 56.2 (OMe), 77.4 (7-CH), 118.8 (12-CH), 124.8 (11-CH), 134.5 (14-C), 135.4 (8-C), 143.6 (9-C), 156.3 (13-C); high resolution (HR)-MS: *m/z* 316.2409 (M^+: $C_{21}H_{32}O_2$ requires 316.2402); DI-MS: *m/z* (%) 316 (M^+; 17), 284 (62), 269 (100), 227 (45), 157 (45); IR ν_{max} (KBr) cm^{-1}: 3449, 1273, 903, 804.

Sandaracopimaric acid (**19**)

The fraction Fr.1333 (70.2 mg) was identified as sandaracopimaric acid[12] by ^{1}H-NMR and MS analyses. Fr.2343 (66.3 mg) was also identified as sandaracopimaric acid: colorless needles, ^{1}H-NMR (DMSO-d_6): δ 0.80 (s, 3H, 20-Me), 1.01 (s, 3H, 17-Me), 1.11 (s, 3H, 19-Me), 2.17 (s, 1H, 9-H), 4.86 (d, 1H, J = 10.9 Hz, 16-H), 4.88 (d, 1H, J = 17.5 Hz, 16-H), 5.19 (s, 1H, 14-H), 5.73 (dd, 1H, J = 10.9 and 17.5 Hz, 15-H), 11.99 (br.s, 1H, 18-COOH); DI-MS: *m/z* (%) 302 (M^+; 33), 287 (47), 257 (12), 121 (100).

Nezukone (**20**)

The fraction Fr.112 (55.3 mg) was separated from Fr.1121 to Fr.1124 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 75:25 v/v; flow rate 2.5 ml/min). Fr.1122 (10.9 mg) was identified as nezukone[21] by ^{1}H-NMR and MS analyses. Fr.1123 (19.3 mg) was also separated from Fr.11231 to Fr.11235 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 65:35 v/v; flow rate 2.5 ml/min). Fr.11232 (0.4 mg) was also identified as nezukone: colorless oil, ^{1}H-NMR (CD_3OD): δ 1.26 (d, 6H, J = 7.0 Hz, *i*-Pr.), 2.85 (sept, 1H, J = 7.0 Hz), 6.99–7.14 (m, 2H), 7.25–7.33 (m, 2H); DI-MS: *m/z* (%) 148 (M^+; 77), 120 (17), 105 (100), 77 (61).

Results and discussion

HPLC analysis of the *n*-hexane and ethyl acetate extracts of bark-glued resin obtained from the resinous stem canker of T. dolabrata var. hondae

The *n*-hexane and ethyl acetate extracts were obtained from the bark-glued resin of the resinous stem canker of *T. dolabrata* var. *hondae*, respectively. 14 diterpenes were isolated from the *n*-hexane extracts (Hanari et al., unpublished observations). The ethyl acetate extracts contained characteristic compounds, which may be present as minor con-

stituents in the n-hexane extracts. The isolation scheme for the ethyl acetate extracts is shown in Fig. 1. Identified compounds are shown by boldface numbers.

A monoterpene and 15 diterpenes were isolated from the ethyl acetate extracts of the bark-glued resin of the resinous stem canker of *T. dolabrata* var. *hondae* and were identified by NMR and MS analyses (Fig. 2). Among them, monoterpene **20** and **4** diterpenes **5**, **8**, **11**, **18** were characteristic compounds in the ethyl acetate extracts because they were not isolated from the n-hexane extracts (Hanari et al., unpublished observations). These terpenes were first isolated from *T. dolabrata* var. *hondae* and *T. dolabrata*. Diterpene **18** was a new compound. The molecular formula $C_{21}H_{32}O_2$ was determined on the basis of the HR-MS spectrum data. The ^{1}H-NMR spectrum contained one isopropyl, three quaternary methyl, one methoxy, and two aromatic signals; these spectrum data are similar to those of 7α-hydroxytotarol (Hanari et al., unpublished observations).[22] The ^{13}C-NMR spectrum showed the existence of 21 carbons in diterpene **18**, which is also similar to 7α-hydroxytotarol (Hanari et al., unpublished observations) except for two chemical shift values (C-6: δ 24.2ppm; C-7: δ 77.4ppm) and a methoxy signal (δ 56.2ppm). Therefore, diterpene **18** was assigned to 7α-methoxytotarol, of which the correlated spectroscopy (COSY) spectrum supported this structure.

Quantities of extracts from the resinous stem cankers of C. obtusa and T. dolabrata var. hondae

Table 1 shows the yields of the extracts from the resinous stem cankers and the healthy barks of *C. obtusa*[11,12] and *T. dolabrata* var. *hondae* (Hanari et al., unpublished observa-

Table 1. Yields of extracts from the healthy barks and from the bark of the resinous stem cankers in *Chamaecyparis obtusa* and *Thujopsis dolabrata* var. *hondae* (based on 100g dry weight)

Sample	Extracts
Bark of the resinous stem canker of *C. obtusa*	40.9
Healthy bark of *C. obtusa*	2.62
Bark of the resinous stem canker of *T. dolabrata* var. *hondae*	27.8
Healthy bark of *T. dolabrata* var. *hondae*	5.61

Each extraction was total of the n-hexane and ethyl acetate extracts

Table 2. Terpenes identified in the healthy barks and in the bark of the resinous stem cankers of *Chamaecyparis obtusa* and *Thujopsis dolabrata* var. *hondae*

Compound	*C. obtusa*		*T. dolabrata* var. *hondae*	
	Healthy bark	Resinous stem canker	Healthy bark	Resinous stem canker
1 (L)	○ (++)	○ (+++)*	○	○ (+)**
2 (L)	× (−)	○ (++)*	×	○ (+)**
3 (L)	× (−)	○ (+)*	○	○ (++)**
4 (L)	× (−)	○ (+)*	×	○ (+)**
5 (L)	× (−)	○ (+)*	×	○ (++)**
6 (L)	○ (++)	○ (+++)	×	○ (+++)
7 (L)	× (−)	○ (+)*	×	○ (+++)
8 (L)	× (−)	○ (+)*	×	○ (+)**
9 (A)	× (−)	× (−)	○	○ (+)**
10 (A)	× (−)	× (−)	×	○ (+++)**
11 (A)	× (−)	× (−)	×	○ (+)**
12 (A)	○ (+++)	○ (+++)	×	○ (+)
13 (A)	○ (+)	○ (+)	×	× (−)
14 (A)	× (−)	× (−)	○	○ (+)
15 (A)	× (−)	× (−)	×	○ (+)**
16 (T)	× (−)	× (−)	○	○ (+)
17 (T)	× (−)	× (−)	×	○ (+)
18 (T)	× (−)	× (−)	×	○ (+)**
19 (P)	× (−)	○ (+)	×	○ (+)**
20	× (−)	× (−)	×	○ (+)**
21 (A)	○ (+++)*	× (−)	×	× (−)
22 (A)	○ (+)*	× (−)	×	× (−)
23 (A)	○ (+)*	× (−)	×	× (−)
24 (A)	○ (+)	× (−)	×	× (−)
25 (L)	× (−)	○ (+)*	×	× (−)
Methyl esters of higher fatty acids	× (−)	× (−)	○	× (−)

The boldface numbers mean the number of isolated terpenes
The structures of compounds refer to those in Figs. 2 and 3
Diterpene skeletons – (A), (L), (P), (T) – are the abietane, labdane, pimarane, and totarane types, respectively
Not detected and detected compounds are shown by crosses and circles, respectively
Compounds with an asterisk (*) were first isolated from *C. obtusa*. Compounds with two asterisks (**) were first isolated from *T. dolabrata* var. *hondae*
Plus (+), double plus (++), and triple plus (+++) denote that the yields are <2%, 2%–4%, and >4%, respectively, based on the n-hexane and ethyl acetate extracts weight; the healthy bark of *C. obtusa* is based on the n-hexane extracts weight

Table 3. Yields of labdane-type diterpenes in the bark of the resinous stem cankers of *Chamaecyparis obtusa* and *Thujopsis dolabrata* var. *hondae* (based on 100 g of extracts weight)

Compound	Resinous stem canker (%)	
	C. obtusa	*T. dolabrata* var. *hondae*
1 (L)	5.20	0.67
2 (L)	2.90	1.70
3 (L)	0.73	3.30
4 (L)	0.53	0.37
5 (L)	0.88	3.00
6 (L)	4.00	6.10
7 (L)	0.09	5.70
8 (L)	0.29	0.26
25 (L)	0.11	–
Total yield of labdane diterpenes	14.73	21.10

tions), respectively. In the resinous stem cankers of *C. obtusa* and *T. dolabrata* var. *hondae*, the amount of extract was 15.6 and 4.96 times heavier than that of the healthy ones, respectively. The extracts of both trees greatly increased with the increasing presence of the resinous stem canker.

The GC-MS analysis data have demonstrated the *n*-hexane extracts of the resinous stem cankers and the healthy barks in *C. obtusa*[10] and *T. dolabrata* var. *hondae* (Hanari et al., unpublished observations). The GC-MS analysis of both trees showed that the ratios and compositions of compounds in the extracts vary between the resinous stem canker and the healthy bark. Large differences were seen between the resinous stem canker and the healthy trees. The reason for this may be that numerous traumatic resin canals were formed in secondary phloem, and much resin was released from these parts as a defensive reaction.

Common diterpenes isolated from the resinous stem cankers of *C. obtusa* and *T. dolabrata* var. *hondae*

12 diterpenes were isolated from the *n*-hexane and ethyl acetate extracts of the resinous stem canker of *C. obtusa*,[11,12] and 18 diterpenes were isolated from the *n*-hexane extracts of the resinous stem canker of *T. dolabrata* var. *hondae* (Hanari et al., unpublished observations). This study showed that its ethyl acetate extracts contained the same common terpenes. Many of the labdane-type diterpenes were particularly involved in the resin and bark of the resinous stem canker.[9,11,12] These diterpenes were characteristic compounds in the resinous stem canker[10–12] (Hanari et al., unpublished observations). The healthy bark lacks these compounds (Table 2). Compound **19** might also be a characteristic diterpene. Table 3 shows the yields of the labdane-type diterpenes from the resinous stem cankers of *C. obtusa* and *T. dolabrata* var. *hondae*, which were seen in large amounts. The yields of the labdane-type diterpenes were 14.73% and 21.1%, on the basis of 100 g extracts weight. In contrast, the yields of the abietane-, pimarane-, and totarane-type diterpenes from the resinous stem canker of *C. obtusa* were small: 6.26%, 0.95%, and 0%, respectively. Similarities were observed in the resinous stem canker of *T. dolabrata* var. *hondae*: 12.68% for the abietane yield, 1.79% for the pimarane yield, and 1.46% for the totarane yield. The labdane-type diterpenes, therefore, were main components in each extract of the resinous stem canker. Compared with the resinous stem canker and the healthy bark, the amount of the extracts increased considerably, and the composition of the characteristic compounds varied. Obviously, the labdane-type diterpenes increased in terms of both kind and yield with the resinous stem canker. Because the labdane-type diterpenes have been known as diterpene precursors,[23] an increase in these diterpenes suggests the occurrence of an irregular metabolic pathway. These results suggest that the labdane-type diterpenes are closely related to the resinous stem canker or the causal fungi of these diseases. Many diterpene acids were also contained in commonly isolated diterpenes; that is, trees released stickier resin to protect the scar.[24]

In future, we are going to study the relations between the isolated labdane-type diterpenes and the causal fungi, the influence of the growth location, and the differences of breed.

Acknowledgments The authors thank Dr. Y. Yada, Ishikawa Forest Experiment Station, for samples of the resinous stem canker of *T. dolabrata* var. *hondae*. The authors are grateful to the Chemical Analysis Center, University of Tsukuba, for NMR spectra and elemental analysis data.

References

1. Suto Y (1987) The resinous stem canker of *Chamaecyparis obtusa* (in Japanese). For Pests 36:117–122
2. Suzuki K, Kamitani T (1989) The resinous stem canker of Ate (*Thujopsis dolabrata* var. *hondae*) (in Japanese). For Pests 38:24–29
3. Suzuki K, Fukuda K, Kaji M, Kamitani T (1988) Mechanisms on the incidence of "Rooshi" pitch canker of *Chamaecyparis obtusa* and *Thujopsis dolabrata* var. *Hondai* (in Japanese). Bull Tokyo Univ For 80:1–23
4. Kuroda K, Suzuki K (1985) Anatomical studies on "Rooshi" resinous canker of hinoki (*Chamaecyparis obtusa*). J Jpn For Res 67:63–66
5. Suto Y (1998) Traumatic resin-canal formation caused by inoculation with *Cistella japonica* in secondary phloem of *Chamaecyparis obtusa*. J For Res 3:99–102
6. Fukutomi M, Yada Y, Esaki K, Miura S (1998) Scanning electron microscopic studies on resinous canker of *Thujopsis dolabrata* Siebbold et Zuccarini var. *hondae* (in Japanese). Bull Ishikawa For Exp Station, pp 21–33
7. Suto Y (1992) A new species of *Cistella* (Discomycetes) inhabiting bark of *Chamaecyparis obtusa* and *Cryptomeria japonica*, and its cultural characters. Trans Mycol Soc Jpn 33:433–442
8. Suto Y (1997) Etiology of the resinous stem canker of *Chamaecyparis obtusa*: *Cistella japonica* as the causal agent. J For Res 2:59–65
9. Ohashi H, Imamura H (1972) Resin exudates of the diseased Japanese cypress (*Chamaecyparis obtusa* Endl.). I. Mokuzai Gakkaishi 18:409–413
10. Hanari N, Yamamoto H, Ooi T, Kusumi T, Kuroda K (2001) A new diterpene dimer from the bark of *Chamaecyparis obtusa*. J Wood Sci 47:36–40
11. Yamamoto H, Asano N, Sawano C, Sone T, Gasha T, Ono Y (1997) Diterpenes isolated from the resin of the resinous stem canker of Japanese cypress, *Chamaecyparis obtusa*. Mokuzai Gakkaishi 43:558–565

12. Hanari N, Ono Y, Ooi T, Yamamoto H (1998) Diterpenes extracted by a polar solvent from the bark of the resinous stem canker of *Chamaecyparis obtusa* (in Japanese). Mokuzai Gakkaishi 44:342–350
13. Arndt F (1966) Organic syntheses, vol 2. John Wiley, New York, pp 165–167, pp 461–464
14. Shimizu M, Tsuji H, Shogawa H, Fukumura H, Tanaami S, Hayashi T, Arisawa M, Morita N (1988) Anti-inflammatory constituents of topically applied crude drugs. II. Constituents and anti-inflammatory effect of *Cryptomeria japonica* D. Don. Chem Pharmaceutical Bull 36:3967–3973
15. Cambie RC, Rutledge PS, Ryan GR, Strange GA, Woodgate PD (1990) Conversion of abietic acid into analogues of ambergris odorants. Aust J Chem 43:867–881
16. Caputo R, Mangoni L, Monaco P (1974) Araucariaceae. III. Two new labdane norditerpene diols from *Araucaria excelsa*. Gazz Chim Ital 104:491–498
17. Tahara A, Shimagaki M, Ohara S, Tanaka T, Nakata T (1975) Diterpenoids. XXXVI. Synthesis of *d*-kaurene and *d*-phyllocladene from *l*-abietic acid. Chem Pharmaceutical Bull 23:2329–2338
18. Kitadani M, Yoshikoshi A, Kitahara Y, Campello JP, McChesney JD, Watts DJ, Wenkert E (1970) Natural ar-abietatriene. Chem Pharmaceutical Bull 18:402–405
19. Yatagai M, Takahashi T (1981) Hydroborations of abietic and levopimaric acids. Mokuzai Gakkaishi 27:649–653
20. Kuo YH, Wu TR, Cheng MC, Wang Y (1990) Five new compounds from the heartwood of *Juniperus formosana* Hayata. Chem Pharmaceutical Bull 38:3195–3201
21. Hirose Y, Tomita B, Nakatsuka T (1966) Terpenoids. XI. The structure of nezukone. Tetrahedron Lett 47:5875–5879
22. Kuo YH, Cheng MC, Wu TR (1984) The structure of suginal from the wood of *Juniperus formosana* Hayata. J Chin Chem Soc 31:417–419
23. Devon TK, Scott AI (1972) Handbook of naturally occurring compounds, vol 2. Academic, San Diego, p 186
24. Phillips MA, Croteau RB (1999) Resin-based defenses in conifers. Trends Plant Sci 4:184–190