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NOTE

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Induction of the biosynthesis of agatharesinol, a norlignan, in sapwood sticks of *Cryptomeria japonica* under humidity-regulated circumstances

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Abstract To establish an experimental approach for studying the biosynthetic pathway of agatharesinol, a norlignan, induction of the formation of agatharesinol in *Cryptomeria japonica* (Japanese cedar, sugi) was attempted. Wood sticks were prepared from the sapwood immediately after cutting a sugi tree down, and they were allowed to stand in desiccators in which the humidity was adjusted to 76% and 88%, and in a room. When the wood sticks were allowed to stand in 76% humidity and in a room, they dried rapidly and no formation of agatharesinol was demonstrated by gas chromatography-mass spectrometry. These results suggest that in the sapwood dried rapidly, the cells that were biologically active in the sapwood, probably ray parenchyma cells, die rapidly and completely before the biosyntheses of secondary metabolites including agatharesinol was wholly established. The wood sticks allowed to stand in 88% humidity, on the other hand, dried gradually compared with the sticks placed in 76% humidity and in a room, and agatharesinol was formed as one of the predominant ethyl acetate extractives. These results suggest that delaying the drying of the sapwood sticks can control the dying period to an appropriate length for the ray parenchyma cells, during which the biosynthesis of agatharesinol is induced.

Key words Biosynthesis · Norlignan · Agatharesinol · Sapwood · *Cryptomeria japonica*

Introduction

Norlignans are a class of natural secondary metabolites with diphenylpentane carbon skeletons (C_6-C_3-C_2-C_6), which are contained in many coniferous trees and some monocotyledonous plants. The biosynthetic pathway of norlignan is still open to further investigation, although the biosynthesis of *cis*-hinokiresinol in fungi-elicited suspension-cultured asparagus cells was studied recently by both feeding and enzymic experiments.[1,2] Concerning the biosynthesis of norlignan, Ohashi et al.[3–6] have demonstrated that the biosynthesis of agatharesinol, a heartwood norlignan of *Cryptomeria japonica* (Japanese cedar, sugi), was able to be induced in sugi sapwood. In their serial studies, they collected approximately 1-m-long stem logs immediately after cutting sugi trees down, and allowed the logs to stand in a room. Qualitative and quantitative changes of the chemical substances of the sapwood of the logs were periodically determined after cutting. Then, it was found that phenolic secondary metabolites including norlignan, namely agatharesinol, were newly formed and accumulated.

It has been shown that in many plant species formation of various secondary metabolites are induced by infection with fungi, wounding by insects, mechanical wounds, and so on. Although the direct trigger varies among the cases, formation of agatharesinol in sugi sapwood after cutting must be a biological action of the living cells in the sapwood, probably ray parenchyma cells, as they are dying. Ohashi et al.[4] also periodically determined the cytological changes of ray parenchma cells in the sapwood of the logs after cutting. Then, it was found that the nuclei of the ray parenchyma cells changed from elliptical to globular and the nuclei finally disappeared after cutting, and the cytological changes represented aging and dying of the parenchyma cells, which had been demonstrated by tracing the changes from sapwood to heartwood by Higuchi et al.[7] Furthermore, Ohashi et al.[6] administered water to sugi stem logs to prevent the logs drying after cutting, and in that study formation of the secondary metabolites including agatharesinol was suppressed. Then it was concluded that drying of the sapwood caused death of the ray parenchyma cells, and the dying parenchyma cells produced the secondary metabolites in the sugi stem logs that were allowed to stand in a room after cutting. In addition, the preliminary experiment demonstrated that in smaller samples, such as wood disks

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or wood blocks of sugi allowed to stand in a room, agatharesinol was not formed probably due to the dying of the ray parenchyma cells being too rapid for establishment of its biosynthesis. Therefore, regulation of dying of sugi sapwood ray parenchyma cells could lead to induction of the formation of agatharesinol. Using larger wood samples such as stem logs, however, the regulation is difficult in a laboratory, thus smaller samples are desirable. Furthermore, smaller samples are much more advantageous than larger ones for a feeding experiment that would be one of the available experimental approaches for studies on biosynthetic pathways.

In the present article, to establish an experimental approach for studying the biosynthetic pathway of agatharesinol, induction of the formation of agatharesinol in a smaller wood sample was attempted. Sticks were prepared from the sapwood immediately after cutting a sugi tree down, and they were allowed to stand in desiccators in which high humidity was maintained. After standing for the predetermined period, the sapwood sticks were subjected to solvent extraction. The ethyl acetate extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) for qualitative and quantitative change of the extractives.

Experimental

Adjustment of humidity

The humidity of desiccators were adjusted to 76% and 88% by placing saturated barium chloride solution (100 ml) and saturated sodium acetate solution (100 ml) in the desiccators (20 × 20 × 15 cm), respectively.

Preparation of sapwood sticks

A 36-year-old sugi tree was cut down in the University Forest (Inabu, Aichi Prefecture) of Nagoya University in late June 2003, and a stem log (23 cm diameter ×1 m long) was collected. Wood disks (5 cm thick) having 14-year sapwood were cut off the log. The sapwood was divided into three parts in the radial direction, and sapwood sticks (1 × 1 × 5 cm, 10 g) were prepared from the middle 4-year ring part. The sapwood sticks were allowed to stand in the humidity-regulated desiccator, or in a room. All these procedures were carried out immediately after cutting the tree, i.e., on the cutting day.

Extraction

Monitoring the moisture content of the sapwood sticks, the sticks were cut into small pieces after the standing in the desiccators or the room. Then, they were thoroughly extracted with methanol (MeOH), and the MeOH extracts were fractionated with *n*-hexane and ethyl acetate (EtOAc), successively.

GC-MS of ethyl acetate extract

A part of the EtOAc extract was dissolved in pyridine, and an aliquot of the solution was submitted to trimethylsilylation (TMSi) using *N*,*O*-bis (trimethylsilyl)-trifluoroacetamide (Wako). The TMSi derivative was analyzed by GC-MS on a Shimadzu GCMS QP 2010 model equipped with DB-1 capillary column (30 m × 0.32 mm i.d., film thickness 1 μm) using helium gas as the mobile phase. The oven temperature was programmed to increase from 150°C to 280°C at 7°C/min, and the ionization energy was 70 eV.

Results and discussion

Change of the moisture content of the sapwood sticks

Ohashi et al.[3–6] allowed sugi stem logs to stand in a room after cutting trees down. In their studies, the sapwood was gradually dried and finally air-dried 70 days after cutting, and secondary metabolites including agatharesinol were newly formed in the sapwood in the drying profile.[3–6] No formation of the secondary metabolites was found in the stem logs administered with water, in which dying of the sapwood ray parenchyma cells should be suppressed.[6] In addition, no formation of the secondary metabolites was found in the smaller sapwood samples that were dried rapidly, in which dying of the ray parenchyma cells occurs quickly (data not shown). The biosynthesis of agatharesinol in sugi sapwood should occur in the period of the ray parenchyma cells dying. Therefore, it was necessary to prepare a circumstance in which the ray parenchyma cells of smaller wood sticks die and produce secondary metabolites. One possible approach is to delay the drying of the wood sticks to secure a dying period of appropriate length for the ray parenchyma cells, during which the biosyntheses of secondary metabolites including agatharesinol is induced. This is because there is no doubt that drying of the sapwood samples significantly influences the dying of the ray parenchyma cells. To prevent the rapid drying of the wood sticks we place sapwood sticks in high-humidity conditions, and drying of the sapwood sticks was first examined by determining their moisture content.

Figure 1 shows the changes of the moisture content of the sapwood sticks allowed to stand in the humidity-regulated desiccators, and in a room. When sapwood sticks were placed in a room and in a desiccator at 76% humidity, the wood sticks were almost air-dried within 3 days and 6 days, respectively. When sapwood sticks were placed in a desiccator at 88% humidity, they dried gradually; the moisture content decreased below 150%, 100%, and 50% after 11 days, 14 days, and 17 days, respectively. Nobuchi and Harada[8] studied the relation between moisture content and death of the ray parenchyma cells in sugi wood immediately after cutting. They determined the moisture content and the percentage of dead cells in every annual ring, and it was found that the moisture content of the sapwood was

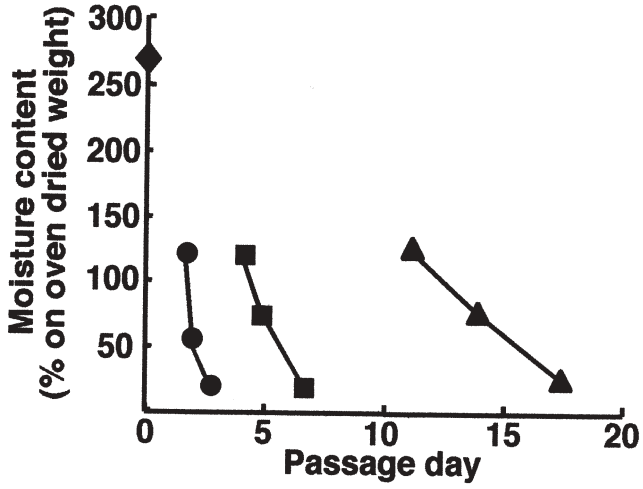

Fig. 1. Changes in the moisture content of sugi sapwood sticks allowed to stand in a room (*circles*), in a desiccator at 76% humidity (*squares*), and in a desiccator at 88% humidity (*triangles*). The moisture content immediately after cutting is indicated by the *diamond* on the y-axis

more than 200% where all ray parenchyma cells were alive. In the transition zone, the moisture content decreased with the increase in the number of dead cells; the outermost annual ring of the transition zone with 150% moisture content had more than 90% living cells, and the innermost ring with approximately 100% moisture content had approximately 50% living cells.[8] In the gradual drying profile, prepared by placing the sapwood sticks in the desiccator at 88% humidity, the dying period of the ray parenchyma cells of the sapwood sticks must be prolonged, and this is likely to be essential for induction and formation of the secondary metabolites.

Detection and identification of compounds newly formed in the sapwood sticks

Figure 2 shows the GC profiles of the EtOAc extracts from the sapwood sticks allowed to dry in a room (b), at 76% humidity (c), and 88% humidity (d). All these sapwood sticks were subjected to solvent extraction when their moisture content decreased below 100%. The GC profile of the EtOAc extracts from the sapwood extracted on the cutting day is shown as Fig. 2a. Peaks that were not present in the GC profile of the cutting day sample (Fig. 2a) were found in those of the sapwood sticks placed in a room (Fig. 2b) and in the humidity-regulated desiccators. These results indicate that the qualitative changes of the EtOAc extractives of the sapwood sticks occurred in the drying period.

Ohashi et al. previously identified EtOAc extractives that were predominantly formed and accumulated in sugi sapwood after cutting as agatharesinol, catechin, and others. In the sapwood sticks allowed to dry in a room (Fig. 2b) and in 76% humidity (Fig. 2c), agatharesinol was not detected. However, one compound (referred to as compound **2**) was identified as catechin by GC-MS by comparing the

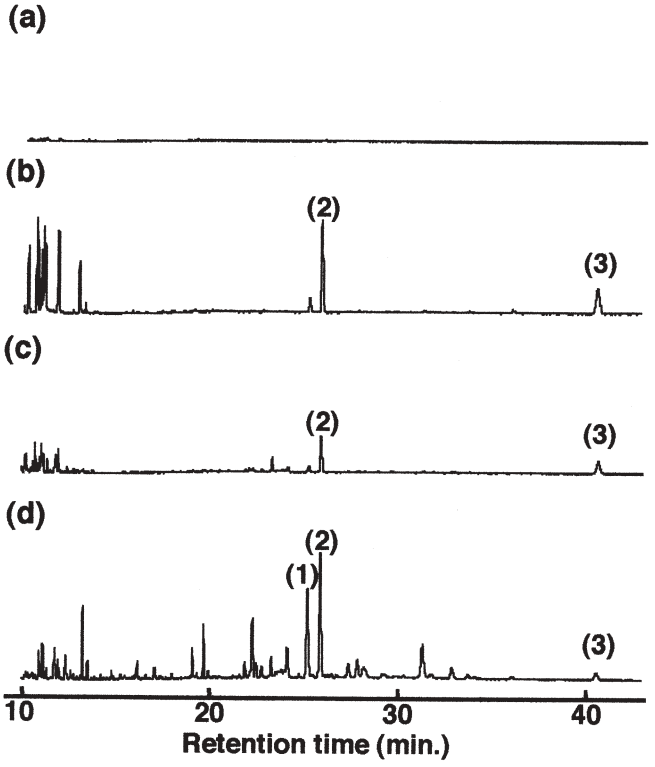

Fig. 2. Gas chromatograms of the EtOAc extracts of sapwood of the cutting day (**a**), and sapwood sticks allowed to stand in a room (**b**), in a desiccator at 76% humidity (**c**), and in a desiccator at 88% humidity (**d**). All sapwood sticks were subjected to solvent extraction when their moisture content decreased below 100%

retention time and mass fragmentation pattern with those of an authentic sample (Fig. 3). A compound (referred to as compound **3**) of which the retention time and mass fragmentation pattern were identical to those of pinoresinol was also found. Although matairesinol has been isolated from sugi needle,[9] lignans had not been detected in sugi xylem until the recent report of identification, formation, and accumulation of pinoresinol together with matairesinol in sugi sapwood after cutting. The details of this recent finding will be published in another report.[10]

In the sapwood sticks allowed to stand at 88% humidity (Fig. 2d), one of the predominant compounds (compound **1**) was identified as agatharesinol by GC-MS by comparing with the retention time and mass fragmentation pattern with those of an authentic sample (Fig. 4). Catechin was also detected as a predominant component in this sample by GC-MS.

Periodic changes of the content of agatharesinol in the sapwood sticks

Figure 5 shows the content of agatharesinol in the sapwood sticks allowed to stand in a room and in the humidity-regulated desiccators. The sapwood sticks were subjected to solvent extraction on the days when their moisture content decreased below 150%, 100%, and 50%.

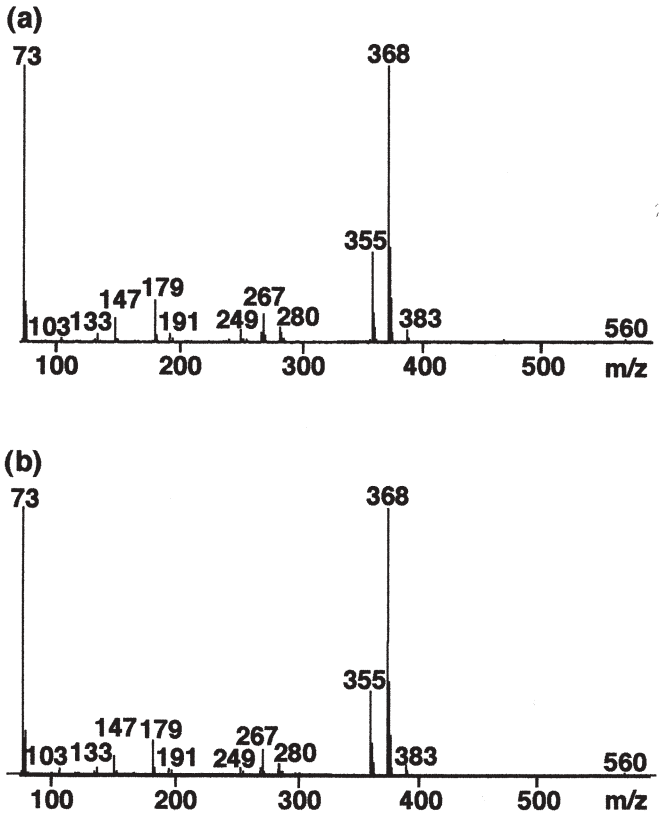

Fig. 3. Mass spectra of trimethylsilyl (TMS) derivatives of **a** catechin (authentic sample) and **b** compound **2**

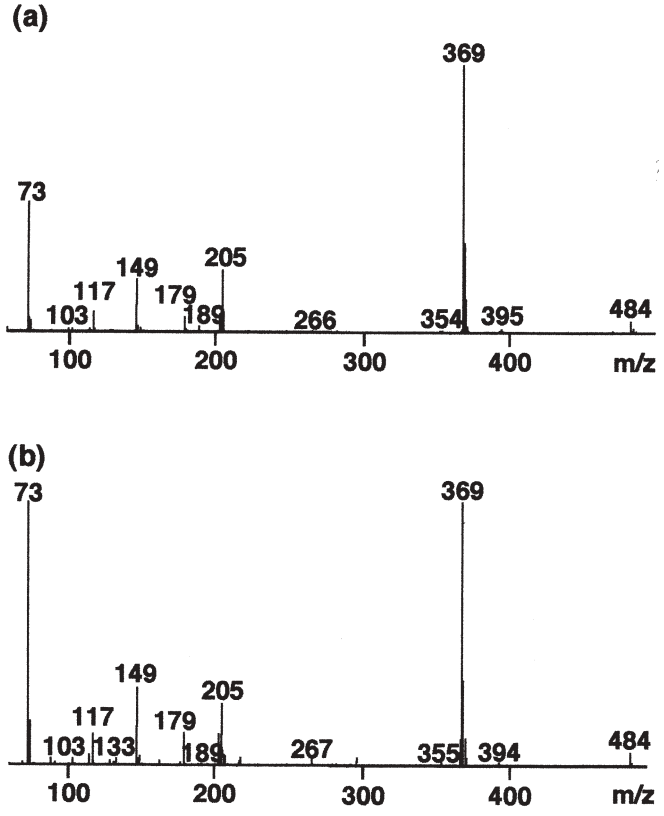

Fig. 4. Mass spectra of TMS derivatives of **a** agatharesinol (authentic sample) and **b** compound **1**

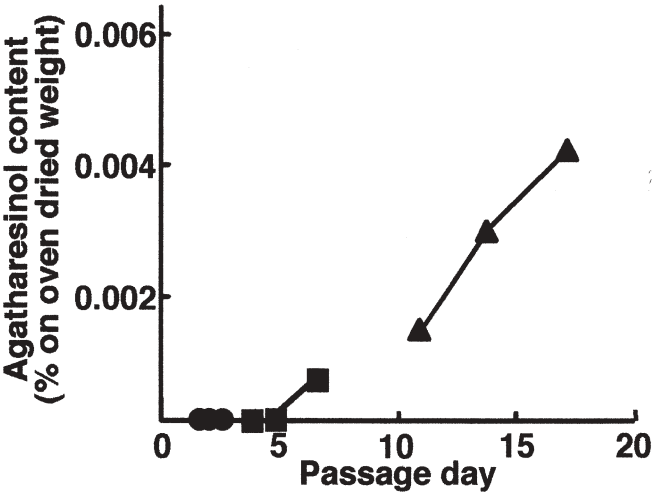

Fig. 5. Changes in the content of agatharesinol in the sugi sapwood sticks allowed to stand in a room (*circles*), in a desiccator at 76% humidity (*squares*), and in a desiccator at 88% humidity (*triangles*). The sapwood sticks were subjected to solvent extraction when their moisture contents decreased below 150%, 100%, and 50%

Agatharesinol was not detected in the sapwood sticks that were extracted on the cutting day, and it was not found in the sapwood sticks allowed to stand in a room. On the other hand, agatharesinol was detected in the sapwood sticks allowed to stand in the humidity-regulated desiccators. In the case of standing in 88% humidity, especially, a large amount of agatharesinol was formed and accumulated in the sapwood sticks. After 11 days, agatharesinol was detected in slight amounts, and then its content increased to a maximum after 17 days.

To date, the factors that directly induce the biosynthesis of agatharesinol in the smaller wood sticks are not fully known, although drying of the wood, fungi infection, or mechanical wounding are possible. Ohashi et al.[4] concluded in their serial studies using sugi stem logs that drying of the sapwood caused death of the ray parenchyma cells and the dying cells produced the secondary metabolites including agatharesinol. This was because no formation of agatharesinol was found in the sugi stem logs that were administered with water to prevent the logs from drying after cutting. As demonstrated in this study and in the previous preliminary studies, agatharesinol was not formed in the sapwood samples that were dried too rapidly. This must be due to the fact that the sapwood ray parenchyma cells should die rapidly and completely before the biosynthesis of agatharesinol can be wholly established. Consequently, for induction of the biosynthesis of agatharesinol in sugi sapwood sticks it was necessary to control the dying period to an appropriate length for the ray parenchyma cells, during which biosynthesis of agatharesinol was induced. Prolongation of the period was achieved by standing the wood sticks in high-humidity conditions. In the prolonged period, the dying ray parenchyma cells in the sapwood produced agatharesinol until they lost production ability due to their complete death.

Conclusions

For the induction of biosynthesis of agatharesinol in sugi sapwood sticks, it was necessary to regulate the dying of the ray parenchyma cells of the sapwood. Delaying the drying of the sapwood sticks in high-humidity conditions was able to control the dying period to an appropriate length for the ray parenchyma cells, during which biosynthesis of agatharesinol was induced.

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