# Natural crystalline fibers of (E)-(R)-4-thujanol: green kilogram production from a selected wild thyme. *X*-ray and NMR characterization of a spiral structure

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## ARTICLE INFO

## ABSTRACT

(E)-(R)-4-Thujanol present in thyme essential oils is an important aromatic ingredient for food, cosmetic and pharmaceutical applications. Until now, its extraction was challenging due to its low content in classical aromatic plants such as thyme or marjoram. Although this molecule is synthetically available, there is no production on an industrial scale because of very time-consuming and expensive chemical processes. We report here for the first time a new eco-responsible method to produce (E)-(R)-4-thujanol crystals on a kilogram scale. This new milestone is based on 2 fundamental research gaps: i) the selection of a wild thyme (*Thymus vulgaris*) for its high content of (E)-(R)-4-thujanol and its careful harvesting in the hills of French Provence and ii) a steam distillation producing an organic aromatic oil which aggregates spontaneously due to a favorable amphipathic partition at the air-water interface. Gas chromatography revealed a composition made of (E)-(R)-4-thujanol (76%), thymol (5%), *β*-myrcene (2,8%) and other minor monoterpenes. The white aggregate submitted to a cycle of sublimation/crystallization, led to accumulating translucent fibers made of (E)-(R)-4-thujanol (98%). *X*-ray diffraction unambiguously demonstrated that crystals of (E)-(R)-4-thujanol form a trimer (monoclinic asymmetric ($C_2$)) unit composed of three independent (E)-(R)-4-thujanol molecules. The trimer is further organized into a chiral P-type supramolecular helix of trimers through a network of intermolecular hydrogen bonds. This study is based on the cheap, easy and eco-responsible kilogram production of *(E)-(R)-4-thujanol* crystals may open up new opportunities for the flavor, fragrance and pharmaceutical industries. Fibers of (E)-(R)-4-thujanol may also have applications as bio-active fibrous assemblies.

## 1. Introduction

The food and perfume industries make extensive use of plant aromatic compounds under the form of essential oils or other natural extracts (cold pressing, maceration, absolute), for the preservation, flavoring and perfuming purposes (Burdock, 2010). For instance, all isomeric forms of *4*-thujanol are fragrance ingredients used in cosmetics (fine fragrances, shampoos, etc.) and household products (detergents) (Bhatia et al., 2008). However, as the abundance of *4*-thujanol in natural extracts is usually very low, its worldwide use is estimated less than 0.1 tons per year (of pure equivalent compound) (Bhatia et al., 2008). Despite this limiting bio availability, the active (E)-(R)-4-thujanol (Fig. 1) isomer has been tested in various fields of interests. Among them, (E)-(R)-4-thujanol has been reported to present genotoxic properties in human peripheral blood lymphocytes, without cytotoxic and/or cytostatic effects (Kocaman et al., 2011). (E)-(R)-4-thujanol is recognized in forestry science as a repellent against bark beetle (Blažytė-Čereškienė et al., 2016; Schiebe et al., 2019). Interestingly (E)-(R)-4-thujanol has also been identified in the aromas of the sunflowers as stimulating the antennae of worker bees (Thiery et al., 1990).
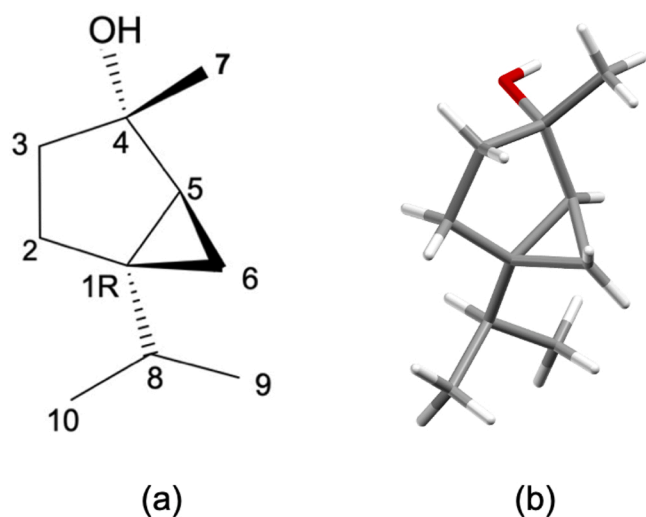
**Fig. 1.** Structure of (*E*)-(*R*)-*4*-thujanol. (a) Molecular structure representation in Cram (dash-wedge) mode, with carbon numbering. (b) Capped stick representation of the X-ray structure (this work).

These studies demonstrate the wide use that could be made of (*E*)-(*R*)-*4*-thujanol.

However, (*E*)-(*R*)-*4*-thujanol is always a minor component in the natural extracts. Due to the complex mix of terpenoids in natural oils, with similar physical-chemical properties, large-scale purification of (*E*)-(*R*)-*4*-thujanol has been hampered. Furthermore, the chemical synthesis of (*E*)-(*R*)-*4*-thujanol is time-consuming and therefore costly (Baeckström et al., 1996). It is interesting to highlight that the chemical synthesis showed that (*E*)-(*R*)-*4*-thujanol exist as a white crystal needle (Baeckström et al., 1996). A more recent, potentially cheaper, alternative chemical synthesis exists, but it gives a mix of *4*-thujanol isomers 1R,1S, E, Z, and another by-product (Galopin, 2001). Therefore, only a few and expensive pure (*E*)-(*R*)-*4*-thujanol can be supplied for research experiments from manufacturers. An alternative supply of (*E*)-(*R*)-*4*-thujanol would be highly desirable to promote new studies.

Nowadays, companies tend to develop eco-responsible strategies to minimize their impact on the environment in order to achieve the future goal of a sustainable world (Blome et al., 2014). Therefore, the search for the production of (*E*)-(*R*)-*4*-thujanol must be done through environmentally friendly processes. This leads us to a renewed interest in plant breeding overexpressing (*E*)-(*R*)-*4*-thujanol with the aim of producing a crystalline form of (*E*)-(*R*)-*4*-thujanol. The relatively easy production of L-menthol crystals from *Mentha arvensis* extract inspired this method (Eccles, 1994).

The selection of a natural population of thyme (*Thymus vulgaris*, *Labiatae*) appears to be a relevant strategy to achieve the objective of natural production of (*E*)-(*R*)-*4*-thujanol enriched essential oil. Indeed, many thyme ecotypes produce a very diversified mixture of aromatic compounds. Some of them contain compounds such as thymol or linalool accounting for up to 85% of the aroma profile (Thompson et al., 2003). Geographical and ecological studies conducted several years ago in the Montpellier region of southern France, showed that wild thyme with a high (*E*)-(*R*)-*4*-thujanol profile grows on hills at altitudes of above 400 m with frost and cold, humid air in winter. The summer is also less dry and hot compared to sites closer to the sea where phenolic thyme (thymol and carvacrol) dominates (Thompson et al., 2003). It is worth mentioning that a chemotype analysis has proposed a biosynthetic pathway for these aromatic compounds present in thyme. This pathway is composed of a series of 5 loci under epistatic control (Vernet et al., 1986). More recently, wild thymes from the CNRS collection (Montpellier, France), producing (*E*)-(*R*)-*4*-thujanol were used to build a cDNA library (Krause et al., 2013). TPS6 and TPS7 cDNAs, which encode the

TPS6 and TPS7 sabinene hydrate synthases, were cloned and expressed in recombinant bacterial systems. However, and to our knowledge, no genetic manipulation using these cDNA, has been reported to increase (*E*)-(*R*)-*4*-thujanol in the yielding plant.

This study reports the search of a thyme in the garrigue of Provence (south of France) that naturally overexpresses (*E*)-(*R*)-*4*-thujanol. We selected a homogenous population with an apparently high level (up to 50% w:w) of (*E*)-(*R*)-*4*-thujanol in the essential oil. The individual plants were also extracted in the laboratory with glass elements (boiling flask and Graham condenser), in order to find out the variation of chemotypes within this thyme population. During this operation, we found that the (*E*)-(*R*)-*4*-thujanol in the aromatic vapor did not produce essential oil. Instead, an unexpectedly and abundant white aggregate appeared on the surface of the condensed water. From this aggregate, a pure crystal of (*E*)-(*R*)-*4*-thujanol was easily produced. X-ray diffraction revealed details of the crystal structure that showed supramolecular organization into helical entities. Our study demonstrated that a green and inexpensive alternative production of high purity (*E*)-(*R*)-*4*-thujanol is clearly feasible.

## 2. Material and methods

### 2.1. Plant materials

A selected population of wild thyme (*Thymus vulgaris*) was identified for its ability to produce (*E*)-(*R*)-*4*-thujanol in large quantities. About ten days after flowering, the plants were cut with a sickle. The seeds are collected in a canvas bag and used for sowing in the field in June. The young shoots appeared during the summer and were isolated in the nursery during the winter to promote their root development. The plants are transferred for growth on open ground at an altitude of 650 m on clay-limestone soil in the Alpes de Haute Provence department (France).

### 2.2. Chemicals, reagents, and gas

n-Heptane HPLC grade, ethanol 95% (v:v) laboratory reagent grade and methanol 99,9% (v:v) analytical reagent grade, were purchased from Fisher Scientific. Hydrogen and nitrogen gas were from Airs Products. Analytical standards (linalool, *alpha*-terpineol, thymol, terpinen-4-ol) were purchased form Sigma-Aldrich.

### 2.3. Water steam extraction

For large scale production (100 kg biomass), water steam extraction set-up was made in a stainless-steel biomass container, a cooling system (condenser), and an oil separator (Hüsnü Can Başer and Buchbauer, 2010). For small scale experiments, plant material (5 or 30 g) was placed in a balloon (500 ml or 2 L) with distilled water (50 or 300 ml), topped with a Kjeldahl bulb and a 75° elbow. The outlet of the elbow fits in a Graham condenser (L, 300 mm; internal diameter, 8 mm) in a vertical position above a 50 ml beaker. All pieces were in PYREX™. The Water in the flask was boiled using a Bunsen burner.

### 2.4. Crystal sublimation

A macro vacuum sublimate apparatus was provided by Rettberg GmbH. The apparatus is composed of 1) a recipient linked to a vacuum pump, 2) an internal trap and 3) a refrigerant to regulate trap's temperature. Crystal (50 mg) was placed in the container. A heat source was adjusted under the recipient. Vacuum was generated using an LBX V10 pump (18 L/min, −670 mmHg). To initiate sublimation, heat and vacuum were adjusted as indicated in the results section. Vapor phase was condensed in the cold trap, where the pure crystal fibers were collected with a forceps.

## 2.5. GC-FID analysis

Samples were injected into the GC for analysis on a 7890 A gas chromatograph (Agilent Technologies). The detection was carried out by flame ionization (FID). A J&W DB-WAX capillary column (60 m x 0,25 mm inner diameter) was employed. Inletting was done à 250 °C with oven programming from 50 °C (5 min hold) to 200 °C (2 °C/min) and from 200 °C (50 °C /min) to 230 °C, 5 min hold, under a constant flow of 0,8 ml of hydrogen/min. Peak were assigned according to the retention times of the pure compounds or to Kovats indices of the AFNOR NF T75 standard (French Association of Normalization) when pure compounds were not commercially available. The percentage is the ratio of peak areas, according to ISO 11024-1,2.

## 2.6. NMR

1D and 2D experiments were conducted on a Bruker Avance III HD nanobay spectrometer (Bruker) operating at 400 MHz for proton and at 100 MHz for carbon by mean of a 5 mm diameter smartprobe. A few mg of the white aggregate were dissolved in 500 μL of $CDCl_3$ and transferred into a 5 mm NMR tube. All NMR experiments were recorded at room temperature (ca. 20 °C). For 1D $^1H$ and $^1H$-decoupled-$^{13}C$ liquid state NMR experiments, acquisition parameters used were respectively of 10 μs for $^1H$ and $^{13}C$ $\pi/2$ hard pulses, 16 and 3464 scans, relaxation delay of 2 s and 5 s and spectral windows of 10 and 24 kHz. 2D homonuclear experiments ($^1H$–$^1H$ COSY, $^1H$–$^1H$ TOCSY and $^1H$–$^1H$ NOESY) were acquired with a spectral window of 5.6 kHz, a number of scans between 8 and 32, a relaxation delay of 1.5 s or 2 s, an acquisition time of 183 ms in the direct dimension and 23 ms in the indirect dimension, the same mixing time of 300 ms was used for TOCSY and NOESY experiments. To correlate $^1H$ and $^{13}C$ resonances, 2D heteronuclear experiments ($^1H$–$^{13}C$ HSQC, $^1H$–$^{13}C$ HMBC) were performed with a number of scans between 24 and 32, a proton spectral window of 5.6 kHz, a carbon-13 spectral window of 18 kHz and 22 kHz, a relaxation delay of 1.5 s and 2 s, an acquisition time of 183 ms in the direct dimension and 7 ms or 6 ms in the indirect dimension. For 2D HSQC, delays were optimized for 145 Hz coupling constant ($^1J_{CH}$) and for 2D HMBC, for long distance correlation, a coupling constant of 8 Hz was used.

All NMR spectra were processed using the TopSpin software (Bruker). A Lorentzian noise filtering respectively of 3 Hz and 0.3 Hz was applied prior to Fourier transformation for 1D carbon and proton spectra. $^1H$ and $^{13}C$ chemical shifts were calibrated against the $CHCl_3$ signals (7.26 ppm for residual $^1H$ and 77.16 ppm for $^{13}C$) (Fulmer et al., 2010).

## 2.7. X-ray diffraction

A tiny crystal (less than 20 μm in the largest dimension) of the white aggregate stored at 4 °C was selected and flash frozen at 145 K in a cryo-loop. Data were collected on a Rigaku© FRX rotating anode (3 kW) equipped with an AFC11 quarter-chi goniometer and a direct photon-counting Dectris© Eiger2 1 M detector. Using Olex2 (Sheldrick, 2015), the structure was solved with the SHELXT (Sheldrick, 2008) structure solution program using Intrinsic phasing and refined with the SHELXL (Dolomanov et al., 2009) refinement package using Least Squares minimization. H atoms were positioned geometrically and constrained depending on their environment. The H-atoms were refined in the riding-model approximation, with Uiso(H) = 1.2 Ueq (CH, CH2, CH3, OH). Data collection and refinement statistics are detailed in the cif file deposited in the CCDC database with the number 2144069.

## 3. Results

### 3.1. Selection of wild thyme species overproducing (E)-(R)-4-thujanol

Our goal was to isolate a wild thyme with a high level of (E)-(R)-4-

thujanol. As mentioned by previous reports, we searched in the garrigue around 400 m altitude along the north face of the hills where frost occurs in winter (Thompson et al., 2003), in the Provencal landscape (south-east of France). We also used our olfactory memory to smell diverse areas of wild thyme in situ. Indeed, the chemotypes of phenol, linalool or (E)-(R)-4-thujanol (even in low abundance) have very different scents. A mat of thyme on an area that met geographic and olfactory criteria, as well as a red earth floor, caught our attention. A 100 kg sickle cut of this thyme was steam extracted in a stainless-steel distillery. Essential oil was collected using an oil separator. Gas Chromatography (GC) analysis showed the presence of 50% (w:w) of (E)-(R)-4-thujanol compared to the other monoterpenes. The following year, we conducted another cutting in the same area with similar analysis results. Seeds (20,000) were collected and sown. The young plants appeared during spring of 2021. Several young shoots were extracted individually with boiling water in a glass balloon (500 ml) and the vapor stream went through a Graham condenser. For the extraction with analytical purposes, not more than 5 g of fresh plants were used (such small quantities of plants can therefore not generate essential oil). Instead, we obtained condensed water that can be directly injected onto a GC equipped with a polar capillary. Results showed a very regular amount of (E)-(R)-4-thujanol in condensed water: 52 ± 4% on 15 individual plants of the selected thyme population.

### 3.2. Easy production of (E)-(R)-4-thujanol in solid state

Very interestingly, we noticed very small white aggregates on the surface of condensed water from each plant-to-plant distillation. All condensed water samples were pooled, filtered on paper and washed with distilled water. The aggregate could only be dissolved with 95% ethanol or methanol using a vortex mixer. A GC analysis revealed the presence of 76% (w:w) of (E)-(R)-4-thujanol, 5% (w:w) thymol and diverse minor monoterpenes (Table 1, Fig. 2A.). To check reproducibility, 13 water steam distillations of fresh thyme (30 g each) were performed, in all cases, shapeless white aggregates were always produced with a 2.2 ± 0.2% yield (w:w), (Fig. 2A, insert). The white aggregate was observed with a microscope and suggested a crystal structure (needles) at a 50 μm resolution (not shown).

### 3.3. Production of pure crystal fibers of (E)-(R)-4-thujanol

Due to the presence of crystals in the white aggregate and its enrichment to only 76% (E)-(R)-4-thujanol, an additional enrichment step by sublimation/condensation was achieved using a Rettberg apparatus (description in Materials and Methods). Physical parameters were adjusted as follows: − 670 mmHg, 50 °C bath to initiate sublimation and a refrigerated trap (6 °C) to promote crystal nucleation. The refrigerated trap accumulated disordered spools of translucent fibers

**Table 1**

Thyme solid aggregate composition from GC analyses. Compound assignments of peaks in the GC chromatogram (Fig. 2A) were performed according to Materials and Methods section.

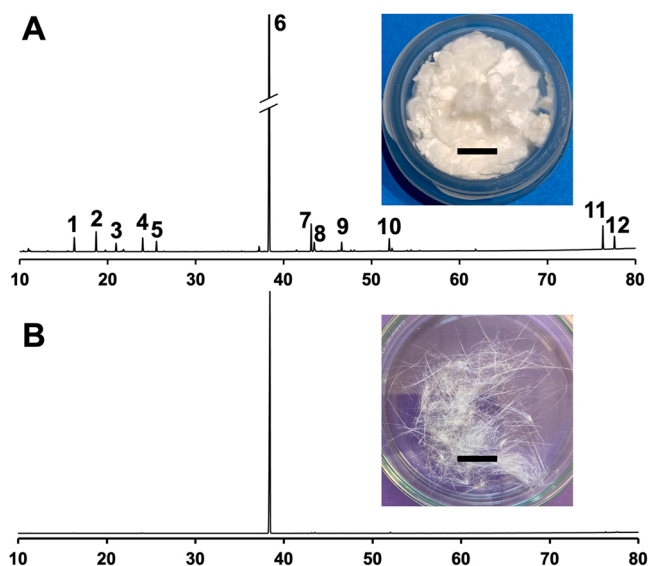| Compounds | % (w:w) ± 0.1 |
|---|---|
| 1. Sabinene | 2.2 |
| 2. β-Myrcene | 2.8 |
| 3. Limonene | 1.2 |
| 4. γ-terpinene | 1.9 |
| 5. p-Cymene[1] | 1.5 |
| 6. (E)-(R)-4-thujanol[1] | 76.2 |
| 7. Linalol | 3.5 |
| 8. (Z)-(R)-4-thujanol | 1.5 |
| 9. β-Caryophyllene | 1.2 |
| 10. α-Terpineol | 1.7 |
| 11. Thymol[1] | 5.0 |
| 12. Carvacrol[1] | 1.8 |

[1] Molecular structure found by NMR (this study).

**Fig. 2.** Crystal of (*E*)-(*R*)-4-thujanol. A, Crude crystal was obtained using steam distillation of fresh thyme. GC analysis of the crude crystal solubilized in n-heptane. Peak assignments are indicated Table 1. Insert: picture of crude crystal (white aggregate). B, Pure crystal fibers were produced by sublimation/condensation of crude crystal. GC analysis of the fibers solubilized in n-heptane. Peak of (*E*)-(*R*)-4-thujanol (98,2%). Insert: picture of pure crystal fiber. Black scale bar: 1 cm. Chromatogram time in minute.

that can be collected with tweezers (Fig. 2B, insert). GC analysis of the fibers dissolved in n-heptane, revealed that the purity of the fibers was around 98% (w:w) of (*E*)-(*R*)-4-thujanol (Fig. 2B). The purification yield from the white aggregate to the isolated fibers of pure of (*E*)-(*R*)-4-thujanol was near 80% (w/w).

### 3.4. NMR analysis of (E)-(R)-4-thujanol

The solubilized white aggregate (76% of (*E*)-(*R*)-4-thujanol) was submitted to various liquid-state NMR experiments (see methods and Supplementary material). The major constituent, (*E*)-(*R*)-4-thujanol, was identified and the full molecular structure easily determined with the help of 2D $^1$H and $^{13}$C NMR. The Table 2 collects the $^1$H and $^{13}$C chemical shifts, which agree with those reported from chemical synthesis. However, we report here the molecular structure assignment that was not made earlier or made in different solvents (Baeckström et al.,

**Table 2**
Assignment of $^1$H and $^{13}$C and chemical shifts to molecular structure of (*E*)-(R)-4-thujanol in CDCl$_3$.

| | $^1$H $\delta$ (ppm); nH[a] | $^{13}$C $\delta$ (ppm) |
|---|---|---|
| C1 | – | 34.80 |
| C2 | 1.59 and 1.84; 2H | 26.04 |
| C3 | 1.28 and 1.52; 2H | 36.76 |
| C4 | – | 80.76 |
| C5 | 1.05; 1H | 34.53 |
| C6 | 0.39 and 0.21; 2H | 13.48 |
| C7 | 1.29; 3H; s | 25.11 |
| C8 | 1.42; 1H; septuplet; 6.8Hz | 32.32 |
| C9[b] | 0.97 or 0.88; 3H; d; 6.7 Hz | 20.18 or 20.08 |
| C10[b] | 0.88 or 0.97; 3H; d; 6.7 Hz | 20.08 or 20.18 |

[a] Chemical shift referenced to CDCl$_3$; number of protons, multiplicity (s: singlet, d: doublet, t: triplet, …); $^3$J$_{HH}$ coupling constant
[b] Assignment could be reversed. Accuracy 0.01 ppm. Right hand side: molecular structure representation in dash-wedge mode with carbon numbering.
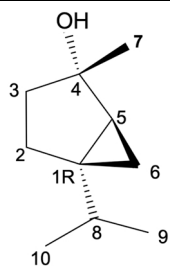
1996; Galopin, 2001; Bohlmann and Zeisberg, 1975) The molecular structure of other minor components (thymol, carvacrol and p-cymene) was also achieved (S1–5 Fig.).

### 3.5. Molecular and supramolecular structure

A small needle of the crystal was mounted on the *X*-ray goniometer and subsequent experiments were performed. The system diffracts in a monoclinic system C$_2$. Diffraction spots were collected on the low noise pixel hybrid detector and analyzed using appropriate software (see Materials and Methods, and S6–7 Fig.). There are 12 molecules in a compact cell connected via a helical hydrogen bonding network (cyan, Fig. 3) involving trimers of independent molecules.

The asymmetric unit is indeed composed of three independent molecules (in blue, green and red, Fig. 3) linked together by intermolecular hydrogen bonds to form the repeating unit of the supramolecular assembly (Fig. 4a). The Fig. 3 displays the end result along the 3 crystallographic axes. Interestingly, a supramolecular helix with a pitch of 5.495 Å (Fig. 4b) is clearly seen along the c-axis. As C2 is a chiral space group, this leads to a unique R isomer and hence to a supramolecular helix of P-chirality. Diffraction experiments were also conducted on the whole powder (S6 Fig.) and showed coincidence of powder diffraction peaks (broader) with single crystal peaks. This clearly indicates that the white aggregate is essentially made of crystals of (*E*)-(*R*)-4-thujanol. The other minor molecules are not in the crystalline state. This prompted us to further purify the withe aggregate by sublimation and obtain pure crystalline fibers.

### 3.6. In field production of (E)-(R)-4-thujanol

To further demonstrate the use of this selected wild thyme as an industrial crop and a source of large-scale production of (*E*)-(*R*)-4-thujanol, a first field of seedlings was initiated in November 2021 (Fig. 5). On June 2022, thyme was harvested with sickles (180 kg), and was immediately steam distilled. As with the previous results, the distillate spontaneously produced the crude crystal of (*E*)-(*R*)-4-thujanol (3.2 kg).

### 4. Discussion

This report presents several key results: 1) the natural selection of a thyme variety overexpressing (*E*)-(*R*)-4-thujanol, 2) an eco-responsible method to produce crystal fibers of (*E*)-(*R*)-4-thujanol (overview Fig. 6), 3) the X-ray resolution of the crystal edifice in spiral structure. These 3 aspects will be briefly discussed with possible implications for pharmaceutical, food and perfume industries.

### 4.1. Natural selection of thyme varieties overexpressing (E)-(R)-4-thujanol

The first step was to collect in the mountains of Provence (400 m hills facing north) a variety of thyme that contained a high percentage of (*E*)-(*R*)-4-thujanol. This is not a novelty, as previous studies had already reported the influence of geoclimatic conditions on (*E*)-(*R*)-4-thujanol enrichment (Thompson et al., 2003). The conditions of altitude, exposure and soil are apparently very favorable for enrichment, compared to the wild thyme species near the Mediterranean Sea. The novelty here is that seeds collected from this thyme population, grown in a similar ecological environment, generated young shoots and adult plants that retain a high level of (*E*)-(*R*)-4-thujanol production. Indeed, 180 kg of fresh thyme were collected in June 2022 and 3.2 kg of crude (*E*)-(*R*)-4-thujanol crystals were produced (Fig. 5).

### 4.2. Green and pure crystal fibers of (E)-(R)-4-thujanol

We used conventional methods of extraction of essential oil, i.e., steam extraction and cold condensation to produce (*E*)-(*R*)-4-thujanol
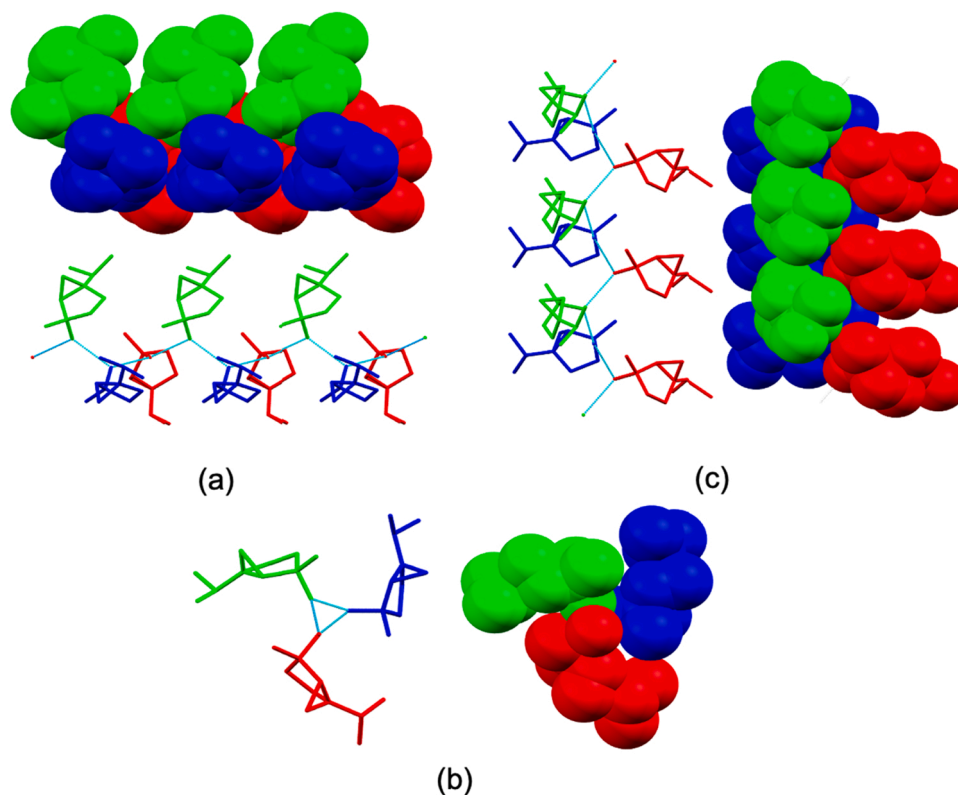
**Fig. 3.** Crystal structure of (*E*)-(*R*)-*4*-thujanol. Monoclinic C$_2$ system with a= 20,375(3) Å, b= 6,2495(8) Å and c= 24,636(3) Å. Hydrogen bonding network and space filling representation of the supramolecular assembly (Helix of P-chirality) along the crystallographic a-axis (a), b-axis (b) and c-axis (c).
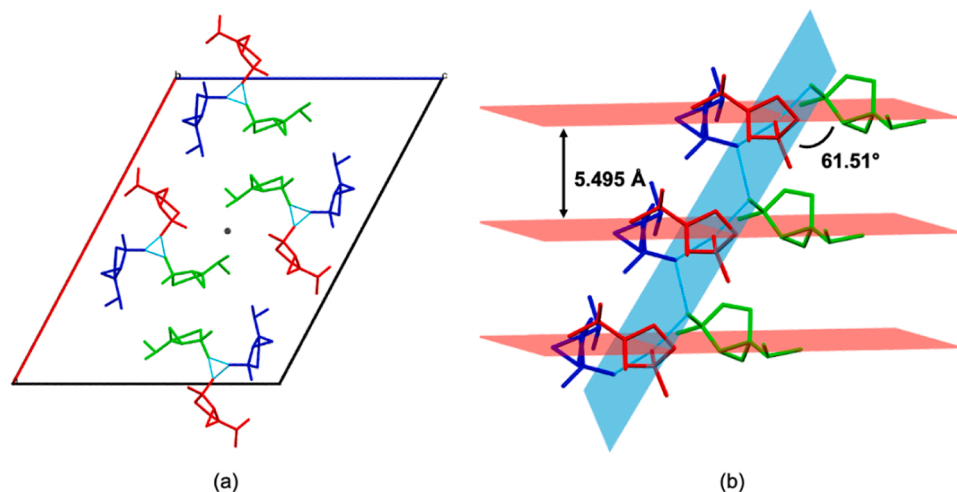


**Fig. 4.** View of the (*E*)-(*R*)-*4*-thujanol crystal packing. (a) Crystal packing along the b axis showing the 12 molecules in the cell. The helical hydrogen bonding network is represented in cyan and the C2 center depicted in black. The asymmetric unit is composed of three independent molecules (in blue, green and red) linked together by hydrogen bonds to form the repeating unit of the supramolecular assembly. (b) representation of three axial planes defined by the centroids of the molecules in the repeating unit (in red) and the median plane of the hydrogen bonding network along one spiral unit (cyan) showing an interplane distance of 5.5 A and an angle of 61°.

rich oils (Fig. 6B). The novelty here lies in the liquid to solid phase transition of (*E*)-(*R*)-*4*-thujanol due to the presence of water on the surface (Fig. 6B). From this solid state a cycle of sublimation/crystallization was performed to produce translucent fibers of pure (*E*)-(*R*)-*4*-thujanol (Figs. 2B, Fig. 6 C). The phenomenon of natural and spontaneous liquid/solid phase transition of (*E*)-(*R*)-*4*-thujanol from essential oil at 25 °C and the crystallization of pure natural (*E*)-(*R*)-*4*-thujanol using solvents-free methods have never been reported. In addition, the description of crystal fibers made of an aromatic monoterpene is without precedent in terpenoids biochemistry. Only one other monoterpene alcohol exists in crystal form: natural L-menthol. Steam extraction of *Mentha arvensis* produces an essential oil containing up to 90% menthol and crystal nucleation occurs at temperatures up to 4 °C depending on the menthol content in the oil (Eccles, 1994). However, the crystals are not stable and rapidly sublime at room temperature (Bombicz et al., 1999). In contrast, the pure crystal of (*E*)-(*R*)-*4*-thujanol is perfectly stable with a melting point of 60 °C (Baeckström et al., 1996).

### 4.3. Spiral crystal structure of (E)-(R)-4-thujanol

The crystal edifice of (*E*)-(*R*)-*4*-thujanol is a supramolecular spiral structure with three molecules forming an asymmetric monoclinic unit. Hydrogen bonds form the backbone along the b-axis direction of the asymmetric unit (Fig. 3). This hydrogen bonding network is very similar to that of the L-menthol crystal (Bombicz et al., 1999) (S7 Fig.). While the latter appears as a transparent prism (Bombicz et al., 1999), in
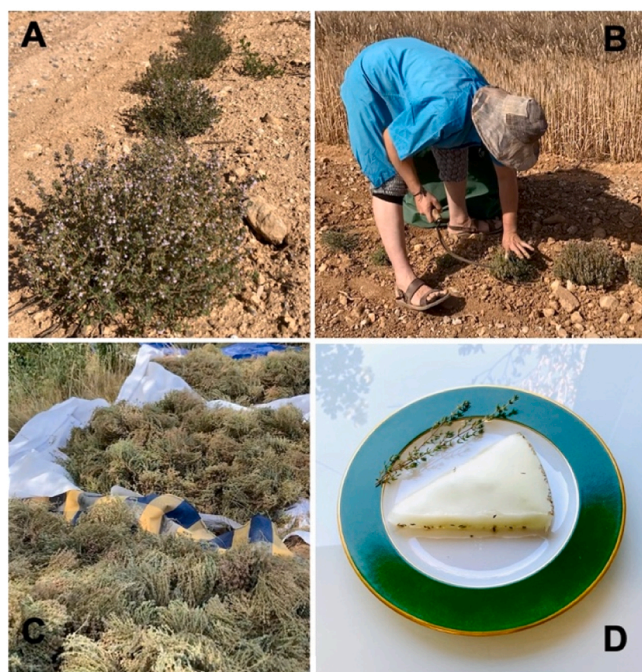
**Fig. 5. In field production of (*E*)-(*R*)-4-thujanol. A.** Selected wild-type thyme in field. **B.** Manual sickle cutting. **C.** harvested thyme (180 Kg). **D.** Crude crystal of (*E*)-(R)– 4-thujanol from water steam distillation (3,2 Kg).



**Fig. 6.** Extraction process of (*E*)-(*R*)-4-thujanol crystal from a selected wild thyme. A. Selected wild-type thyme. B. Water steam extraction of thyme biomass and aggregate formation at the surface of condensed water. C. Production of pure crystal of (*E*)-(*R*)-4-thujanol using one cycle of sublimation/ crystallization.

contrast, the (*E*)-(*R*)-*4*-thujanol shows translucent and fine fibers (Fig. 2B). The easy liquid to solid phase transition of (*E*)-(*R*)-*4*-thujanol in the presence of water probably comes from the fact that the oil-water partition coefficient of monomeric *4*-thujanol is very high (Log(P)= 3.2 (Bhatia et al., 2008)), i.e., very much in favor of the oil phase and the inner network of hydrogen bonds (Fig. 3) leading to a trimer, which should be even more hydrophobic. The presence of water will sequester the (*E*)-(*R*)-*4*-thujanol trimers away from the hydrophilic milieu and favor the supramolecular organization in helix leading to a fibrous crystal (Fig. 2B).

*4.4. Potential industrial applications*

Many monoterpene alcohols such as menthol, linalool, *alpha*-terpineol, citronellol or geraniol are important aromatic compounds used as flavor enhancers or in medicine or fine chemistry (Schwab et al., 2013). (*E*)-(*R*)-*4*-thujanol, which belongs to the same class of molecules, is still absent from the catalogs of cheap aromatic molecules, which prevents its use, despite its importance in food, cosmetic and pharmaceutical applications. The stable pure crystal of (*E*)-(*R*)-*4*-thujanol will probably open new fields of investigation in biomedical, agricultural research or flavoring applications. Its fiber structure may open new application opportunities in bio-active fibrous assemblies.

We would like to emphasize that our approach is very simple in the production of very high-quality natural products and allows for new research steps. We are offering the community a new, environmentally responsible method for producing (*E*)-(*R*)-*4*-thujanol crystals on a kilo-gram scale. This new milestone is based on two very simple yet fundamental steps: i) careful natural selection of a wild thyme (*Thymus vulgaris*) for its high (*E*)-(*R*)-*4*-thujanol content and ii) taking advantage of amphipathic interactions in a steam distillation to produce crystals at the air-water interface. We believe that this very simple approach can be
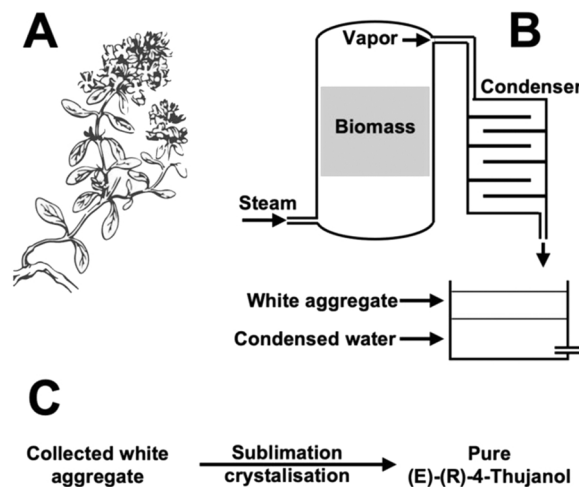
of great interest for industrial scale-up.

**CRediT authorship contribution statement**

**Denis Rontein:** Conceptualization, funding acquisition, investigation, methodology, resource, supervision, writing original draft, review and editing. **Erick J. Dufourc:** Funding acquisition, methodology, project administration, resources, supervision, writing original draft, review and editing. **Estelle Morvan:** Formal analysis, methodology, validation. **Brice Kauffmann:** Formal analysis, methodology, validation. **Axelle Grélard:** Formal analysis, methodology, validation. **Antoine Loquet**: Formal analysis, methodology, validation. **Anne-Julie Dixon:** Resources, methodology, funding acquisition.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Data availability**

Data will be made available on request.

**Appendix A. Supporting information**

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2022.115451.

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