Bioconversion of (+)- and (-)-alpha-pinene to (+)- and (-)-verbenone by plant cell cultures of *Psychotria brachyceras* and *Rauvolfia sellowii*

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**Keywords:** alpha-pinene, biotransformation, verbenol, verbenone.

**Abbreviations:**  
2,4-D: 2,4-dichlorophenoxyacetic acid  
EtoAc: ethyl acetate  
GC: gas chromatography  
GC-MS: gas chromatography-mass spectrometry  
MS: Murashige and Skoog  
NMR: Nuclear Magnetic Resonance Spectroscopy

*Corresponding author*
experiments, but in much lower amounts and accompanied by several by-products, highlighting the usefulness of the biotransformation process.

The exploration of inexpensive and abundantly available terpenoids, widely distributed in nature and produced in bulk amounts, for the biotechnological production of value-added compounds using biotransformation approaches drives special interest for the production of natural flavors and fragrances due to their distinctive and pleasant odors, as well as taste notes. Moreover, several processes in chemical synthesis also use terpenoids due their particularly stereochemistry.

Alpha-Pinene is the major constituent of the turpentine oils from most conifers and a component of the wood and leaf oils obtained from leaves, bark, and wood of a wide variety of other plants. In Brazil, the pine resin tapping activity is increasingly important, producing resin mostly for export; 100,000.00 tons were produced in 2002, a market that moved some US$ 25 millions, providing over 12,000.00 direct jobs in the countryside (source: Brazilian Association of Resin Extraction http://www.aresb.com.br/estatisticas/index.htm in december 2006). In order to increase the commercial value of the turpentine oil, it would be of interest to convert alpha-pinene into more valuable compounds. Selective oxidation of alpha-pinene with some biocatalysts can yield value-added products, such as verbenone and verbenol, antiaggregation and aggregation pheromones, respectively, that are used in the control of southern pine beetle infestations, particularly of the genera *Tomicus* (Hylesininae), *Ips* e *Dendroctonus* (Scolytinae) (Huber and Borden, 2001; Lindgren and Miller, 2002; Diaz-Nuñez et al. 2006). (+)-verbenone is a particularly attractive starting material used in asymmetric synthesis, as chiral precursor to the preparation of the A-ring subunit of the antitumoral diterpene taxol® (Lajunen et al. 2000). (-)-verbenone is a major flavor constituent of strawberry, raspberry, dill, rosmarinus and spearmint flavor mixtures with high demand in the food industry (Ravid et al. 1997) and, more recently, has been used as starting material to prepare cyclobutyl GABA analogues (Mogliani et al. 2002) and cyclobutane carbocyclic nucleoside and oligopeptides (Rouge et al. 2003).

Over the past few decades a large number of biotransformations of alpha-pinene into verbenone has been reported using fungi: *Aspergillus niger* (Agrawal and Joseph, 2000; Divyashree et al. 2006), *Hormonema sp.* (van Dyk et al. 1998) and *Botrytis cinerea* (Farooq et al. 2002); bacteria: *Serratia marcescens* (Wright et al. 1986), *Pseudomonas spp.* (Divyashree et al. 2006) and *Nocardia sp.* (Perez et al. 1999); and plant cell suspension cultures: *Nicotiana tabacum*, *Cannabis sativa* (Hirata et al. 1994) and *Picea abies* (Lindmark-Henriksson et al. 2003; Vanek et al. 2005). However, selectivity coupled to improved yields is highly desirable to make industrial applications feasible. On the basis of these considerations, the objective of the present work was to find systems able to convert alpha-pinene (2,6,6-trimethyl-bicyclo[3.1.1]hept-2-ene) into verbenone (4,6,6-trimethyl-bicyclo(3.1.1)hept-3-en-2-one), using a biotransformation approach based on

### Table 1. Bioconversion of (-)-alpha-pinene (60 mg) by *Psychotria brachyceras* (30 g fresh weight). Numbers represent mean percentages ± standard deviation (four replications) of components starting with 100% alpha-pinene at the experimental onset.

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-alpha-pinene</td>
<td>1.0 ± 0.9</td>
<td>0.5 ± 0.4</td>
<td>0.7 ± 0.4</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>trans-pinocarveol</td>
<td>3.3 ± 0.6</td>
<td>2.1 ± 0.5</td>
<td>2.7 ± 0.6</td>
<td>1.9 ± 0.6</td>
<td>0.0</td>
<td>2.6 ± 1.5</td>
</tr>
<tr>
<td>trans-verbenol</td>
<td>73.7 ± 4.8</td>
<td>63.8 ± 1.3</td>
<td>59.1 ± 3.6</td>
<td>37.1 ± 6.8</td>
<td>15.7 ± 3.8</td>
<td>17.2 ± 2.2</td>
</tr>
<tr>
<td>myrtenol</td>
<td>9.5 ± 3.6</td>
<td>9.6 ± 0.7</td>
<td>8.3 ± 1.1</td>
<td>3.5 ± 1.3</td>
<td>1.8 ± 3.6</td>
<td>2.1 ± 2.0</td>
</tr>
<tr>
<td>(-)-verbenone</td>
<td>10.7 ± 1.9</td>
<td>19.5 ± 4.2</td>
<td>22.0 ± 3.6</td>
<td>48.4 ± 5.9</td>
<td>80.9 ± 2.9</td>
<td>76.3 ± 1.5</td>
</tr>
</tbody>
</table>
Bioconversion of (+)- and (-)-alpha-pinene to (+)- and (-)-verbenone by plant cell cultures of *P. brachyceras* Müll Arg. (Rubiaceae) used in this investigation were induced from young stem segments (developed under indoor conditions) of cuttings cultured in nutrient solution, as described by Gregianini et al. (2003). After surface sterilization using standard procedures, stem segments were cultured under darkness in MS (Murashige and Skoog) medium containing 3% w/v of sucrose, 1% w/v soluble polyvinylpolypyrrolidone (PVP), 0.75% w/v microbial grade agar, 10 mg/l of naphthaleneacetic acid (NAA, Sigma Chemical Co. St. Louis, USA) and 1 mg/l kinetin (KIN, Sigma Chemical Co. St. Louis, USA). Calli were developed and maintained in this medium with monthly subcultures at the Laboratory of Plant Physiology, UFRGS. A voucher of the plant, which (1S,5S)(-)-verbenone was purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA).

**Cell culture stocks**

The callus tissues of *P. brachyceras* Müll Arg. (Rubiaceae) used in this investigation were induced from young stem segments (developed under indoor conditions) of cuttings cultured in nutrient solution, as described by Gregianini et al. (2003). After surface sterilization using standard procedures, stem segments were cultured under darkness in MS (Murashige and Skoog) medium containing 3% w/v of sucrose, 1% w/v soluble polyvinylpolypyrrolidone (PVP), 0.75% w/v microbial grade agar, 10 mg/l of naphthaleneacetic acid (NAA, Sigma Chemical Co. St. Louis, USA) and 1 mg/l kinetin (KIN, Sigma Chemical Co. St. Louis, USA). Calli were developed and maintained in this medium with monthly subcultures at the Laboratory of Plant Physiology, UFRGS. A voucher of the plant, which

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**Table 2. Bioconversion of (-)-alpha-pinene (60 mg) by *Rauvolfia sellowii* (30 g fresh weight).** Numbers represent mean percentages ± standard deviation (four replications) of components starting with 100% alpha-pinene at the experimental onset.

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-alpha-pinene</td>
<td>11.9 ± 1.3</td>
<td>6.1 ± 1.4</td>
<td>2.0 ± 2.6</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>trans-pinocarveol</td>
<td>10.4 ± 4.4</td>
<td>6.4 ± 2.4</td>
<td>6.2 ± 3.1</td>
<td>11.0 ± 1.4</td>
<td>10.5 ± 0.6</td>
<td>13.5 ± 2.4</td>
</tr>
<tr>
<td>trans-verbenol</td>
<td>50.6 ± 7.0</td>
<td>56.9 ± 4.3</td>
<td>46.4 ± 3.5</td>
<td>41.4 ± 2.5</td>
<td>40.2 ± 5.1</td>
<td>38.4 ± 0.8</td>
</tr>
<tr>
<td>trans-pinanone</td>
<td>0.0</td>
<td>0.7 ± 1.6</td>
<td>0.5 ± 1.2</td>
<td>5.6 ± 1.3</td>
<td>7.5 ± 1.7</td>
<td>8.6 ± 1.8</td>
</tr>
<tr>
<td>myrtenol</td>
<td>5.3 ± 0.9</td>
<td>4.2 ± 1.9</td>
<td>4.3 ± 2.4</td>
<td>6.5 ± 0.6</td>
<td>7.0 ± 0.4</td>
<td>6.4 ± 1.2</td>
</tr>
<tr>
<td>(-)-verbenone</td>
<td>16.7 ± 1.1</td>
<td>24.6 ± 3.0</td>
<td>36.6 ± 6.9</td>
<td>37.6 ± 1.8</td>
<td>33.9 ± 4.3</td>
<td>28.9 ± 3.4</td>
</tr>
</tbody>
</table>
was harvested at Morro Santana (campus of UFRGS, Porto Alegre, RS, Brazil), is deposited in the University Herbarium (ICN Sobral and Kerber 7899). Cell suspension cultures of *R. sellowii* Müll Arg. (Apocynaceae) were originally developed by Rech et al. (1998) and maintained on B5 (Gamborg) medium containing 1 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D, Sigma Chemical Co. St. Louis, USA) and 4% w/v sucrose. Suspension cultures were maintained in this medium, being transferred onto fresh medium fortnightly.

**Biotransformations**

Biotransformations of (1S,5R)-(-)-alpha-pinene and (1R,5S)-(+)-alpha-pinene were carried out by *Psychotria brachyceras* and *Rauvolfia sellowii* cell suspension cultures. Before each experiment, ca. 30 g of cells or callus tissue were transferred to a 250 ml conical flask containing 30 ml of freshly prepared SH medium (Schenk and Hildebrandt, 1972) containing 1 mg/l of 2,4-D and 3% w/v sucrose. Cells were then grown for 1 week at 25 ± 2ºC, under diffuse light (3 µmol. s⁻¹.m⁻²), on a rotary shaker (100 rpm). After this time, 1.0 ml of a methanolic solution (60 mg/ml) of substrate, without prior sterilization, was added to the cell suspensions, and the cultures were returned to the shaker for 15 days. Controls were prepared by the addition of 1 ml of a methanolic solution (60 mg/ml) of alpha-pinene to 30 ml of medium, and, in other flasks, ca. 30.0 g cells and 30 ml medium. Experiments (each with four replicates per sampling time) were independently repeated three times with similar results.

**Extraction and analysis**

For optimization of extraction procedure, portions of the incubation mixture were pipetted out and extracted with different polarity solvents, such as hexane, chloroform and ethyl acetate (EtOAc), in order to establish the best solvent to extraction procedure. A reminiscent strong emulsion could be observed by the use of EtOAc, even after...

Gas Chromatography analyses were performed using a Shimadzu GC-17A chromatograph equipped with a fused silica capillary column (30 m x 0.25 mm x 0.25 mm, coated with DB-5). Injector and detector temperatures were set at 220ºC and 250ºC, respectively; the oven temperature was programmed from 60-230ºC at 3ºC/min. All the samples...
were analyzed by GC-MS in the same apparatus and chromatographic conditions as described above, using a quadrupole MS system (QP 5000) operating at 70 eV. The percentage composition of unreacted substrate and the amount of products were obtained from electronic integration measurements using flame ionization detection, without taking into account relative response factors. Compounds identification was based on a comparison of retention indexes (determined relatively to the retention times of a series of n-alkanes) and mass spectra with those of authentic standard purchased from Sigma-Aldrich and literature data (van Dyk et al. 1998; Adams, 2001). The retention indexes obtained were 945 to alpha-pinene, 1132 to (-)-alpha-pinene, 10.071 min to (-)-alpha-pinene, 10.338 min to (+)-alpha-pinene, 28.033 min to (+)-verbenone. In the confirmatory studies, 27.858 min to (-)-verbenone, and 23.3 min to (+)-alpha-pinene. Retention times obtained were 10.071 min to (-)-alpha-pinene, 10.338 min to (+)-alpha-pinene, 28.033 min to (+)-verbenone. The results showed that under the evaluated conditions, 

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-alpha-pinene</td>
<td>25.9 ± 8.3</td>
<td>3.7 ± 2.2</td>
<td>3.2 ± 1.8</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>trans-pinocarveol</td>
<td>7.3 ± 0.5</td>
<td>8.5 ± 2.8</td>
<td>7.0 ± 3.2</td>
<td>7.5 ± 5.5</td>
<td>7.8 ± 2.9</td>
<td>12.2 ± 1.9</td>
</tr>
<tr>
<td>trans-verbenol</td>
<td>54.5 ± 6.9</td>
<td>68.5 ± 3.0</td>
<td>61.7 ± 4.3</td>
<td>59.1 ± 4.4</td>
<td>48.0 ± 2.0</td>
<td>39.1 ± 1.5</td>
</tr>
<tr>
<td>myrtenol</td>
<td>2.3 ± 0.7</td>
<td>3.2 ± 1.7</td>
<td>4.6 ± 1.9</td>
<td>3.8 ± 2.5</td>
<td>2.2 ± 0.8</td>
<td>2.9 ± 0.8</td>
</tr>
<tr>
<td>unidentified*</td>
<td>2.2 ± 1.2</td>
<td>4.7 ± 3.1</td>
<td>4.8 ± 2.7</td>
<td>3.1 ± 4.2</td>
<td>3.4 ± 1.1</td>
<td>4.3 ± 3.0</td>
</tr>
<tr>
<td>(+)-verbenone</td>
<td>7.4 ± 1.2</td>
<td>10.4 ± 2.0</td>
<td>17.9 ± 3.2</td>
<td>24.0 ± 2.1</td>
<td>32.2 ± 0.7</td>
<td>31.9 ± 3.1</td>
</tr>
</tbody>
</table>

*m/z = 95(100); 41(47.1); 93(25.2); 43(21.5); 55(17.4); 79(14.1); 67(13.9); 91(13.7); 121(13.4); 105(12.5); 53(11.6); 77(10.7); 81(7.8); 110(7.8); 139(6.5); 136(4.6); 154(2.8).

RESULTS AND DISCUSSION

Biotransformations of (-)-alpha-pinene and (+)-alpha-pinene were carried out in order to achieve (-)- and (+)-verbenone formation, by the use of *Psychotria brachyceras* and *Rauvolfia sellowii* cell suspension cultures. The cultures were selected due to our interest in explore the potential of native plant cell suspension cultures. The stereochemistry of stereogenic center were evaluated by chiral GC coinjection with commercial samples purchased from Sigma-Aldrich and confirmed by [α] values from purified verbenones. The results showed that under the evaluated conditions, *P. brachyceras* was able to modify only the (-)-enantiomer, whereas *R. sellowii* was effective towards both enantiomers of alpha-pinene with similar profile. When the (+)-alpha-pinene were added to the *P. brachyceras* suspension cultures, the substrate was completely consumed and no further products could be formed by other biocatalysts (Hirata et al. 1994; van Dyk et al. 1998).
Table 4. Degradation of (-)-alpha-pinene (1.0 ml of a methanolic solution of 50 mg/ml) in reaction medium (50 ml). Similar profiles were obtained with 50 ml of the following types of solvents evaluated separately: distilled water, Milli-Q® water, phosphate buffer, MS medium prepared with Milli-Q® water, or MS medium prepared with distilled water. Numbers represent mean percentages ± standard deviation (four replications) of components starting with 100% alpha-pinene at the experimental onset.

<table>
<thead>
<tr>
<th>Component</th>
<th>3 hrs</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-alpha-pinene</td>
<td>2.2 ± 1.3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>campholenal</td>
<td>7.2 ± 2.5</td>
<td>3.2 ± 0.9</td>
<td>2.0 ± 0.9</td>
<td>1.5 ± 0.6</td>
<td>0.8 ± 0.2</td>
<td>1.6 ± 0.4</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>trans-pinocarveol</td>
<td>10.9 ± 3.7</td>
<td>6.5 ± 2.7</td>
<td>5.6 ± 2.3</td>
<td>4.4 ± 0.8</td>
<td>3.1 ± 1.7</td>
<td>3.0 ± 1.4</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>cis-verbenol</td>
<td>2.1 ± 0.5</td>
<td>2.3 ± 1.3</td>
<td>3.1 ± 0.8</td>
<td>2.7 ± 1.0</td>
<td>2.0 ± 1.4</td>
<td>2.9 ± 0.4</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>trans-verbenol</td>
<td>62.5 ± 3.5</td>
<td>64.6 ± 6.0</td>
<td>67.5 ± 2.9</td>
<td>67.6 ± 2.4</td>
<td>64.6 ± 2.4</td>
<td>59.4 ± 1.6</td>
<td>60.0 ± 2.5</td>
</tr>
<tr>
<td>myrtenol</td>
<td>6.7 ± 2.5</td>
<td>6.1 ± 1.2</td>
<td>6.6 ± 0.5</td>
<td>6.5 ± 0.6</td>
<td>6.0 ± 0.5</td>
<td>6.5 ± 0.5</td>
<td>6.9 ± 0.4</td>
</tr>
<tr>
<td>(-)-verbenone</td>
<td>7.6 ± 0.8</td>
<td>8.1 ± 1.3</td>
<td>10.9 ± 0.8</td>
<td>12.9 ± 0.7</td>
<td>17.6 ± 2.2</td>
<td>19.6 ± 1.3</td>
<td>23.8 ± 0.3</td>
</tr>
<tr>
<td>myrtanol</td>
<td>2.3 ± 1.5</td>
<td>1.7 ± 0.9</td>
<td>2.1 ± 0.3</td>
<td>2.2 ± 0.4</td>
<td>2.7 ± 0.6</td>
<td>2.6 ± 0.2</td>
<td>2.7 ± 0.3</td>
</tr>
</tbody>
</table>

The time courses analysis of bioconversion of alpha-pinene by P. brachyceras and R. sellowii are shown in Table 1 to Table 3. Values correspond to the overall mean concentrations ± standard deviations (since individual means did not differ between themselves) obtained in three independent experiments carried out in quadruplicates. P. brachyceras afforded the best results, achieving 80.9% conversion (relative integrated area GC-MS) of (-)-alpha-pinene to (-)-verbenone after a 10-day-incubation (Table 1). R. sellowii was less efficient for the production of (-)-verbenone (37.6% conversion in 7-day-incubation - Table 2), when compared with P. brachyceras, but showed the ability to convert (+)-alpha-pinene, with (+)-verbenone peaking at 32.2% conversion on day 10 (Table 3).

Control experiments, in which both enantiomers of alpha-pinene were added to culture medium, as well as suspension cultures not supplemented with substrate, were carried out for all incubation periods. In control flasks containing only cells and medium, no monoterpene metabolites could be detected, whereas in the control flasks supplemented with substrate, but devoid of biocatalyst cells, a quick conversion to a variety of autoxidation products was observed (Table 4). The autoxidation products were characterized by proceeding with a wider range of compounds. For instance, the formation of verbenone has also been found among the autoxidation products of alpha-pinenes, nevertheless, this amount was much smaller than the amount of verbenone produced from the same substrates by the cell suspension cultures, under the same conditions and time (Figure 2 and Figure 3). Thus, in the presence of biocatalyst, a greater extent of this ketone was produced, being the bioconversion increased by almost 2 fold with R. sellowii and more than 4 fold with P. brachyceras. The results are in agreement to those reported by Lindmark-Henriksson et al. (2003), which had also been observed that verbenone, verbenol and sobrerol were found among the autoxidation products of alpha-pinene. The authors also report that alpha-pinene subjected to the formation of verbenone and sobrerol were found among the autoxidation products of alpha-pinene; the authors suggested that this lack of specificity could reflect a transformation via a radical mechanism with possible involvement of peroxidases in the reactions. This might also be the case of R. sellowii.

CONCLUDING REMARKS

R. sellowii and P. brachyceras were able to convert alpha-pinene into verbenone without changes in the stereogenic center of the molecules. The verbenone was also present among the autoxidation products, but in much lower
amounts under the same conditions and time, highlighting the usefulness of the biotransformation process. *P. brachyceras* work in a selective way, affording the flavonoid (-)-verbenone with high conversion rates. It is clearly interesting and could be considered as an alternative to direct and selective obtaining of (-)-verbenone in future scaling up processes. A different behavior was observed with *R. sellowii*, which was characterized by giving relatively lower production of (-)-verbenone than that found with *P. brachyceras*, with little or no enantioselectivity. However *R. sellowii* was able to convert the antipode (+)-alpha-pinene into (+)-verbenone, a particularly attractive starting material for asymmetric synthesis. The importance of these findings is heightened by the natural status of biocatalytic processes and the lack of synthetic methods with equivalent efficiencies in the production of optical pure verbenone. Natural verbenone is currently obtained by extraction from pine and eucalyptus sources with great demand in the food industry for use as the main component of several flavors (Ravid et al. 1997); market prices of verbenone are much higher than those of pinene, suggesting economic viability (Agrawal and Joseph, 2000).

REFERENCES


