Enhanced rosmarinic acid production in cultured plants of two species of *Mentha*

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In the present investigation an attempt has been made to enhance rosmarinic acid level in plants, grown in vitro, of 2 species of *Mentha* in presence of 2 precursors in the nutrient media during culture. For in vitro culture establishment and shoot bud multiplication, MS basal media were used supplemented with different concentrations and combinations of different growth regulator like NAA (α-napthaleneacetic acid), BAP (6-benzylaminopurine). The medium containing NAA (0.25 mg/L) and BAP (2.5 mg/L) gave the highest potentiality of shoot formation (average 58.0 numbers of shoots) per explant for *Mentha piperita* L. and the medium containing BAP (2.0 mg/L) gave the highest potentiality of shoot (average 19.2 numbers of shoots) formation per explant for *Mentha arvensis* L. The complete plants were regenerated in above mentioned media after 8 weeks of subculture. For in vitro enhancement of rosmarinic acid production, the 2 precursors tyrosine (Tyr) and phenylalanine (Phe) were added in the nutrient media at different levels (0.5 mg/L to 15.0 mg/L). Tyrosine was found to be very effective for augmenting rosmarinic acid content in *Mentha piperita* L. It nearly increased the production up to 1.77 times. In case of *Mentha arvensis* L., phenylalanine significantly affected the production of rosmarinic acid and the production was nearly 2.03 times more than the control. No significant increase in biomass was observed after addition of these precursors indicating that the added amino acids acting as precursors for rosmarinic acid synthesis were readily utilized in producing rosmarinic acid without promoting growth. Total protein profile also revealed the presence of a specific band in polyacrylamide gel electrophoresis.

**Keywords:** *Mentha*, Micropropagation, Phenylalanine, Rosmarinic acid, Tyrosine

Genus *Mentha* belongs to the family Lamiaceae with approximately 25 species. Medicinal importance of *Mentha* is well known due to the presence of rosmarinic acid, the second most common ester of caffeic acid in the plant kingdom\(^1\), used as antioxidant as well as a potent and effective HIV-1 integrase and reverse transcriptase inhibitor. High costs and high demand of this plant for manual labour disallow large scale propagation with green cuttings. As all species of this genus contain high amount of secondary metabolites, in vitro rapid propagation for production of improved clones is desirable for conservation and commercial exploitation. Plant tissue culture technology offers a means to produce economically important secondary metabolites at an enhanced level within controlled laboratory environments\(^2\). Rosmarinic acid was detected in shoot cultures of *Mentha piperita*\(^3\). There are reports of synthesizing rosmarinic acid using shoot segments of plants\(^4\).

Shoot culture of *Mentha spicata* showed increased secondary metabolite content\(^2\). Nodal shoot cultures were found to be very beneficial over cell suspension cultures in case of rosmarinic acid production\(^5,6\).

Application of elicitors to plant cell and organ cultures is very useful for enhancing the biotechnological productivity of valuable secondary metabolites in vitro\(^7,9\). Biosynthesis of rosmarinic acid has been demonstrated the involvement of phenylpropanoids pathway and the tyrosine-derived pathway\(^1,10,11\). Rosmarinic acid synthesized by shoot cultures of *Mentha arvensis* in presence of agents like phenylalanine and sucrose was first reported by Phatak and Heble\(^12\). Actively growing tissue converts >20% of exogenously supplied phenylalanine and tyrosine to the caffeoyl ester and this high rate of synthesis coincides with increased enzyme activity in *Coleus* cell culture\(^13\).

In the present study effect of precursors on rosmarinic acid production during micropropagation of 2 species of *Mentha* has been investigated.

**Materials and Methods**

Two species of *Mentha*, *Mentha piperita* L. and *Mentha arvensis* L. were collected from NBPGR,
New Delhi bearing strain No. IC: 54537 and IC: 54538. Field grown plants were maintained in the experimental garden, Department of Botany, University of Calcutta. Total phenol and rosmarinic acid content in 4 seasons (summer, monsoon, autumn and winter) were taken into account and an average was calculated and the average annual productivity of field grown plants was compared with the in vitro regenerates.

*In vitro culture*—Apical and nodal portions with axillary buds of 2-3 cm length from field grown plants were taken as explants. Explants were first washed in aqueous liquid detergent solution (Tween 20, SRL; 2 drops in 100 mL) for 10 to 12 min at room temperature and rinsed thrice with distilled water. These were then treated with an aqueous 0.1% (w/v) HgCl₂ solution for surface sterilization and finally washed repeatedly in sterile distilled water under aseptic condition. Media used for *in vitro* culture was Murashige and Skoog (MS) modified basal medium supplemented with 3% (w/v) sucrose, 0.05 mg/L (w/v) ascorbic acid, 0.1 mg/L (w/v) glutamine, 0.25% (w/v) Gelrite® and different combinations of growth regulators. Different growth regulators, NAA: α-naphthaleneacetic acid and BAP: 6-benzylaminopurine were added in different combinations and concentrations in MS basal media. The pH of the medium was adjusted to 5.7 with either 1N sodium hydroxide (NaOH) or 1N hydrochloric acid (HCl) for each set. In the preparation of solid media, 0.25% Gelrite® (w/v) was used. Prepared media were taken in culture tubes (150 × 25 mL), 20 mL liquid media was poured in each culture tube. The culture vessels containing media were autoclaved at 121 ºC for 15 min.

Explants were inoculated in sterile nutrient media contained in culture vessel under aseptic condition in a laminar air flow chamber. All the cultures were maintained in a culture room at 24±1 ºC, under 16 h photoperiod which was provided by cool white fluorescent tubes. This gave a light intensity of 48 µmol m⁻² s⁻¹ photosynthetic photon fluxes. Cultures were maintained by sub-culturing the material after every 4 weeks under aseptic condition.

Shoots collected from plants regenerated in *vitro* in a medium with best response were considered as control. Total phenol and rosmarinic acid contents were analyzed from control tissues and compared with *in vivo* plants as well as precursor treated plants. Two biosynthetic precursors responsible for rosmarinic acid synthesis, phenylalanine and tyrosine were used at a wide range of concentrations as additives during culture. Stock solutions of each of these were prepared in distilled water and added at different concentrations to the culture media (Table 1). A wide range of concentrations were chosen (0.5-15.0 mg/L) for addition in culture media. All the shoots were maintained till the increase in fresh weight become stabilized. *In vitro* regenerates of both the species were carried out by normal propionic orcein staining method. Plantlets were transferred to pre-acclimatization chambers (PAC) consisted of 2 halves of polystyrene culture vessels (Magenta boxes) joined together with a connector ring. Three holes of equal sizes, placed at an equal distance, were made on top of the upper half of the PAC to permit free air exchange. After filling with a 1:1 mixture of Soilrite™ and sand, water and ¼ MS solution were sprayed on plants periodically. The upper half of the PAC was removed after 3 weeks. PACs were kept in BOD at 26±1 ºC and 16 h photoperiod. After 3 weeks of acclimatization all the plants were shifted to the field. 

*Extraction of total phenolics including rosmarinic acid*—*In vivo* plant materials of 2 species of *Mentha* were first collected from the field condition during the 4 seasons (summer, monsoon, autumn and winter) and washed thoroughly to remove the surface contaminants. 10 g of fresh tissue (root and shoot separately) was taken and then 100 mL of HPLC grade methanol (Spectrochem®) was added. The conical flask was then fitted with a reflux condenser and was placed on a heater with a magnetic stirrer at 60 ºC and extracted for 4 hours. The extract was then filtered and this filtrate was taken as the sample for further analysis.

<table>
<thead>
<tr>
<th>Precursors</th>
<th>Concentration (mg/L)</th>
<th>Mentha piperita</th>
<th>Mentha arvensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>0.5</td>
<td>T₁</td>
<td>T₁₁</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>T₂</td>
<td>T₂₂</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>T₃</td>
<td>T₃₃</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>T₄</td>
<td>T₄₄</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.5</td>
<td>P₁</td>
<td>P₁₁</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>P₂</td>
<td>P₂₂</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>P₃</td>
<td>P₃₃</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>P₄</td>
<td>P₄₄</td>
</tr>
</tbody>
</table>
until the tissue became colourless\textsuperscript{1,18}. After filtration the filtrate was evaporated to dryness. The crude was further dissolved in a HPLC grade methanol and filtered under suction using a nylon membrane filter with a 0.45 \mu m pore size\textsuperscript{19}. This was kept aside until further use in qualitative and quantitative estimation of rosmarinic acid. Extraction from \textit{in vitro} regenerates was also carried out by the same method.

\textbf{Quantitative determination of total phenolics following spectrophotometry—}Total phenol compounds from the extracts were determined and expressed as milligrams of gallic acid equivalents per gram of fresh weight (mgGAEE/gFW)\textsuperscript{12,20}. 0.1 mL of stock solution (100 mg/mL) was dissolved in 7.9 mL of double distilled water. Folin ciocalteau’s phenol reagent\textsuperscript{21} (0.5 mL) was added to the sample. Between 30 sec and 8 min, 1.5 mL of 20\% sodium carbonate (w/v) was added and allowed to stand at 18–20 °C for 2 h. Dark blue colour was developed and the absorbance was measured by spectrophotometer at 765 nm. Double distilled water was used as blank sample. The content of total phenol compounds was determined using a standard curve prepared with gallic acid.

\textbf{Qualitative determination of rosmarinic acid following thinlayer chromatography—}Presence of rosmarinic acid in methanolic extract was detected using thin layer chromatography\textsuperscript{22} (TLC). Concentrated extract was loaded on to a 10 \times 3 cm TLC plate, which was cut off from a 20 \times 20 cm, 0.25 mm thick; Silica gel 60 F 254 analytical TLC plate (Merck Co. Ltd., Darmstadt, Germany) by glass capillary tubes of 20 \mu L capacity and the plate was thoroughly air-dried. The solvent mobile phase was prepared using a mixture of chloroform: methanol: water (67.5: 0.69; v/v/v) and poured in a TLC jar. The solvent was evaporated to dryness. The crude was further dissolved in a 0.45 \mu m pore size\textsuperscript{19} filter under suction using a nylon membrane filter with a 0.45 \mu m pore size\textsuperscript{19}. This was kept aside until further use in qualitative and quantitative estimation of rosmarinic acid. Extraction from \textit{in vitro} regenerates was also carried out by the same method.

\textbf{Liquid chromatography} separation was achieved by using a C18 column, (150 \times 4.6 mm, Hypersil) with a particle size 5.0 \mu m and the temperature was set at 30 °C. The flow rate was 0.5 mL/min. The mobile phase for chromatographic analysis was with water: acetonitrile (83:17 v/v). Ultraviolet detection was set at 330 nm\textsuperscript{24}.

\textit{Extraction and estimation of total protein—}Tissue (2 g) was weighed on a fresh weight basis and mixed with 1 mL of protein extraction buffer (PEB) [Composition for 100 mL; 0.1M TRIS, 0.25M sucrose, 1.0\% PVP, 0.1\% ascorbic acid, 0.1\% cysteine HCl, 1.0 mM EDTA, 0.4 mM MgCl\textsubscript{2}, pH was adjusted to 6.8] and pulverized in a pre-chilled mortar and pestle. The crushed material was taken in 1.5 mL Eppendorf tubes and centrifuged at 12,000 rpm for 30 min at 4 °C in a cold centrifuge. The supernatant was collected and re-centrifuged again at 20,000 rpm for 10 min at 4 °C. The supernatants (protein suspended in buffer) were decanted carefully and stored at –20 °C for further analysis.

\textbf{Quantitative estimation of protein—}This was done following the method of Bradford\textsuperscript{25}. The quantity of total protein was determined and expressed as milligram of protein per gram of fresh weight (mg/g FW). Extracted sample (30 \mu L) was mixed with 270 \mu L of 0.15 M NaCl solution. Laboratory made Bradford reagent (3 mL) was added to it and incubated at 37 °C for 10-12 min. Absorbance of the developed dark blue colour was measured by spectrophotometer at 595 nm. Deionised water was used as a blank sample. The content of total protein was determined using a standard curve prepared with bovine serum albumin.

\textbf{Qualitative estimation of protein—}This was done by Sodium dodecyl sulphate polyacrylamide gel electrophoresis [SDS PAGE]\textsuperscript{26}. The gel consisted of Acrylamide, Bisacrylamide, SDS and a Tris-Cl buffer with adjusted pH. Ammonium persulfate and TEMED (Tetramethylethylenediamine) were added when the gel was ready to be polymerized. First the separating gel (15\%) was poured and allowed to polymerize. After electrophoresis, gel was immersed in stain (Composed of 0.25 g Coomassie brilliant blue in 100 mL 5:4:1 mixture of methanol, water and acetic acid) for overnight and destained with the solution of 5:4:1 mixture of methanol, water and acetic acid. Image was taken under a visible light illuminator and analysed in a computer with software PhotocaptMw. Electrophoregrams were evaluated on the basis of band mobility and relative intensity. Protein profile of

\begin{align*}
\text{R}_{f} &= \frac{\text{Length of the spot from the starting point of the run (cm)}}{\text{Total length of run (cm)}}
\end{align*}

\textbf{High performance liquid chromatography (HPLC) analysis of rosmarinic acid for quantification—}High Performance Liquid Chromatography (HPLC) (Shimadzu, SPD-10A UV-Vis detector, LC-10 AD
each sample showed its own electrophoresis pattern with subunits of varied molecular weight. Differences were observed in both presence and absence of a particular band.

Statistical analysis—Data were analyzed statistically following one-way Anova assay to determine the least significant difference among the treatments. Five replications of each set of experiment were taken for study and an average was calculated.

Results

Apical buds were most suitable explant sources for multiplication of both peppermint and field mint. The bud break was observed mostly between 8-11 days after culture in all cases. All the cultures were maintained up to 15 weeks. For peppermint various combinations of different cytokinins on shoot bud multiplication were used with one auxin NAA (0.25 mg/L). The media containing 0.25 mg/L NAA and 2.5 mg/L BAP (media E<sub>2</sub>) produced highest number of shoot buds (58.0 ± 0.82 shoot buds per explant) (Fig. 1). BAP showed the highest potentiality for shoot bud multiplication than the other 2 cytokinins tested. For field mint also the medium containing BAP (2.0 mg/L) (L<sub>2</sub>) gave the highest potentiality of shoot (average 19.2 shoots per explant) formation per explant (data not shown). Root formation started after 15 days of culture. Presence of auxin only gave a better response in root production. After complete regeneration they were transferred to the pre acclimation chamber (PAC). The plants were maintained there up to 3 weeks. The upper half of the PAC was removed after 3 weeks and kept in outer environment. It was seen that plants survived with a good growth. Somatic chromosome analysis of regenerated plants of both the species showed chromosome number stability with 2n=36 in <i>M. piperita</i> and 2n= 36 in <i>M. arvensis</i>.

Seasonal variation in total phenol and rosmarinic acid content was observed for <i>in vivo</i> plants of 2 species in <i>Mentha</i>. A detailed account was given in Table 2. Total phenol content was found to be higher in shoots than roots in every season indicating the fact that phenols are mainly accumulated in shoots. The data were analyzed statistically following one-way Anova assay to determine the least significant difference among the treatments. Five replications of each set of experiment were taken for study and an average was calculated.

Table 2—Spectrophotometric analysis of total phenol content during the seasons
[Values are mean ± SE of 5 replicates]

<table>
<thead>
<tr>
<th>Plant material</th>
<th>Phenol content (shoot) (mg GAE / gFW)</th>
<th>Phenol content (root) (mg GAE/gFW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Summer</td>
<td>Monsoon</td>
</tr>
<tr>
<td>&lt;i&gt;Mentha&lt;/i&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;i&gt;piperita&lt;/i&gt;</td>
<td>5.29±0.23</td>
<td>4.12±0.03</td>
</tr>
<tr>
<td>&lt;i&gt;Mentha&lt;/i&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;i&gt;arvensis&lt;/i&gt;</td>
<td>2.19±0.03</td>
<td>1.68±0.03</td>
</tr>
</tbody>
</table>

One way ANOVA; **<i>P</i> ≤ 0.05
average phenol content in shoots was found to be 2.98 times higher than in root in case of peppermint and 3 times higher from root for field mint. Average rosmarinic acid content of *Mentha piperita* shoots was 2.18 times more than shoots of *Mentha arvensis*.

The entire precursor treated clones showed a decrease in shoot bud multiplication than the control for the 2 species. In case of peppermint the fresh weights of control plants were found to be 0.26 g after 6 weeks of culture and 2.5 g after 12 weeks of culture. Lower concentration of tyrosine added clones (0.5-5.0 mg/L) showed an increase in biomass with respect to leaf size and texture. Total phenol content increased (10.04 - 10.94 mg GAE/gFW) with the addition of higher concentration of tyrosine (10.0–15.0 mg/L) after 6 weeks of culture. Biomass growth also increased with decreased phenolic content after repeated sub-cultures (Fig. 2). Phenyllalanine showed an increase in fresh tissue weight or biomass than tyrosine (up to 5.56 g after 10 weeks of subculture). Increasing concentration of phenylalanine promoted tissue growth than augmentation of total phenol production (Table 3). Among the 2 precursors tested rosmarinic acid content was highest in 15.0 mg/L tyrosine added clones (Table 4). After repeated subculture rosmarinic acid content decreased.

In case of field mint the fresh weights of control plants were found to be 0.34 g after 6 weeks of culture and 0.66 g after 12 weeks of culture. For *Mentha arvensis* fresh tissue weight was maximum in tyrosine added clones (up to 2.44 g after 10 weeks of subculture), which was reflected in shoot length, leaf size and fleshy plant texture. Phenol content decreased with increased tissue growth (Table 3). Between the 2 precursors tested phenylalanine was more efficient in total phenol production than tyrosine. Total phenol content augmented up to $5.31 \pm 0.56$ mg GAE/gFW after addition of 10 mg/L phenylalanine. It is nearly 2.25 times greater than the control plants. Higher concentration of phenylalanine did not favor increment in total phenol content (Fig. 3).

It was then revealed that tyrosine was beneficial for rosmarinic acid production in *M. piperita* and it increased the production up to 77% that of *in vivo* plants after 6 to 7 weeks of culture. In *M. arvensis* phenylalanine increased the productivity up to

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**Table 3**—Spectrophotometric analysis of total phenol content in tyrosine treated *in vitro* propagated shoots of *Mentha arvensis* and in phenylalanine treated *in vitro* propagated shoots of *Mentha piperita*.

* [Values are mean ± SE of 5 replicates]

<table>
<thead>
<tr>
<th>Media</th>
<th>45th day</th>
<th>60th day</th>
<th>75th day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh weight (g)</td>
<td>Phenol content* (mg GAE/gFW)</td>
<td>Fresh weight (g)</td>
</tr>
<tr>
<td>Control</td>
<td>0.34</td>
<td>2.36±0.44</td>
<td>0.45</td>
</tr>
<tr>
<td>T11</td>
<td>0.44</td>
<td>3.09±0.98</td>
<td>0.69</td>
</tr>
<tr>
<td>T22</td>
<td>0.31</td>
<td>3.28±0.36</td>
<td>0.79</td>
</tr>
<tr>
<td>T31</td>
<td>0.39</td>
<td>3.88±0.74</td>
<td>0.54</td>
</tr>
<tr>
<td>T44</td>
<td>1.60</td>
<td>4.07±0.01</td>
<td>1.84</td>
</tr>
<tr>
<td>F Value</td>
<td>56.309**</td>
<td></td>
<td>145.077**</td>
</tr>
<tr>
<td>Control</td>
<td>0.26</td>
<td>6.24±0.68</td>
<td>1.79</td>
</tr>
<tr>
<td>P1</td>
<td>0.31</td>
<td>8.41±0.98</td>
<td>2.12</td>
</tr>
<tr>
<td>P2</td>
<td>0.30</td>
<td>8.02±1.08</td>
<td>1.92</td>
</tr>
<tr>
<td>P3</td>
<td>0.40</td>
<td>8.22±0.02</td>
<td>2.09</td>
</tr>
<tr>
<td>P4</td>
<td>0.61</td>
<td>8.16±0.44</td>
<td>3.30</td>
</tr>
<tr>
<td>F Value</td>
<td>210.252**</td>
<td></td>
<td>778.735**</td>
</tr>
</tbody>
</table>

Control : without any additives; One way ANOVA; **P ≤ 0.05
2.03 times from *in vivo* plants (Table 4). Repeated subculture was not found to be beneficial for rosmarinic acid production in both these species.

Quantitative analysis of total protein content of control shoot tissue and tissue from precursor treated clones reveal minor variation. Regenerated plants showed higher protein content than field grown plants. In case of *M. piperita* highest protein content was observed in T1 (Tyrosine 10.0 mg/L) and T4 (Tyrosine 15.0 mg/L) shoot tissue and they showed 1.88 times increased content than field grown plants and only 15% increased content than control (Fig. 4). In case of *M. arvensis*, on the other hand, highest protein content was observed in P33 (Phenylalanine 10.0 mg/L) shoot tissue (Fig. 4). These plants showed 31.3% increased content than field grown plants and only 8.2% increased content than control.

PAGE analysis of all precursor treated clones was compared with control. Higher phenol and rosmarinic acid producing clones were taken into account. For peppermint, control (C) and lower concentrations of tyrosine added shoot tissue (T2) produced fourteen main protein bands with the molecular weight ranging from 13.930 KD to 111KD. Higher concentration of tyrosine added shoot tissue (T3 and T4) showed one extra band of 29.0 KD (Fig. 5A). The phenylalanine treated clones of peppermint P1, P2, P3 and control clones showed fourteen main protein bands. No major difference in band with respect to molecular weight was observed in all the 3 clones with control.

In case of PAGE analysis of tyrosine treated clones of field mint, all clones (T22, T33 and T44) revealed twenty main protein bands with molecular weight ranging from 16.782 KD to 107.213 KD which is similar to that of control. Control and lower concentration of phenylalanine added clones (P11 and P22) revealed twenty main protein bands with molecular weight ranging from 16.782 KD to 107.213 KD. Higher concentration of phenylalanine added clones (P33) revealed one extra specific band with the molecular weight of 41.826 KD which is absent from control (Fig. 5B).

**Discussion**

The present investigation on multiplication of 2 species of *Mentha, Mentha piperita* L. and *Mentha arvensis* L. revealed that the cytokinin BAP is more efficient in shoot bud multiplication than other cytokinins. Effectiveness of BAP in shoot bud multiplication was also earlier reported in *Mentha* by different authors and in other plant species. The requirement of such different levels of cytokinins may be correlated with the difference in its

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**Table 4**—Rosmarinic acid contents of *in vivo* and *in vitro* shoots in 2 species of *Mentha* after 6 to 7 weeks of culture

<table>
<thead>
<tr>
<th></th>
<th><em>Mentha piperita</em></th>
<th><em>Mentha arvensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosmarinic acid content* (mg/gFW)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td>7.00±0.08</td>
<td>3.2±0.32</td>
</tr>
<tr>
<td>Control</td>
<td>7.18±0.28</td>
<td>3.5±0.98</td>
</tr>
<tr>
<td>T1</td>
<td>9.60±0.34</td>
<td>4.7±1.38</td>
</tr>
<tr>
<td>T2</td>
<td>9.53±0.78</td>
<td>4.9±0.88</td>
</tr>
<tr>
<td>T3</td>
<td>10.10±1.22</td>
<td>5.3±0.56</td>
</tr>
<tr>
<td>T4</td>
<td>12.41±0.54</td>
<td>5.2±0.04</td>
</tr>
<tr>
<td>F Value</td>
<td>656.893**</td>
<td>1773.522**</td>
</tr>
<tr>
<td>P1</td>
<td>9.10±1.88</td>
<td>4.8±0.78</td>
</tr>
<tr>
<td>P2</td>
<td>10.20±0.64</td>
<td>5.1±0.32</td>
</tr>
<tr>
<td>P3</td>
<td>10.40±0.68</td>
<td>6.5±0.50</td>
</tr>
<tr>
<td>P4</td>
<td>7.80±1.24</td>
<td>4.3±0.76</td>
</tr>
<tr>
<td>F Value</td>
<td>371.298**</td>
<td>4259.345**</td>
</tr>
</tbody>
</table>

Control: without any additives; One way ANOVA; **P ≤ 0.05**

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Fig. 3—Total phenol content and biomass growth in phenylalanine treated clones of *M. arvensis* [Values are Mean ± SE of 5 replicates].

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Fig. 4—Total protein content of different clones of *M. piperita* and *M. arvensis* [Values are Mean ± SE of 5 replicates].
endogenous level in different populations and species studied, that may also be linked to its habitats. Also, the genotypic differences might have been responsible for the difference in responses in different populations and species\textsuperscript{34,35}. Chromosome analysis revealed chromosome number stability in the regenerates. This may indicate the efficacy of BAP and the culture conditions in maintaining the genome stability of the regenerates. Such chromosome number stability in the regenerates has also been observed using BAP as the only cytokinin in culture medium\textsuperscript{34}.

The present investigation also dealt with the analysis of total phenolics and rosmarinic acid in 2 species of Mentha, after addition of 2 precursor molecules namely tyrosine and phenylalanine. By providing tissue culture approaches, a shoot based clonal line was tested for experimental purpose. There are scanty reports on shoot based clonal lines in Mentha piperita\textsuperscript{3,12} and in Mentha arvensis\textsuperscript{3,12}. It was also reported that mint shoots grown in solid agar medium was found to contain more amount of secondary metabolites than the shoots grown in liquid medium\textsuperscript{2}.

Application of precursors to the nutrient medium had increased the synthesis of phenolic content as well as rosmarinic acid in culture. An increment in secondary metabolite contents had also been observed in culture regenerated plants of different species grown in presence of precursor chemicals or elicitors\textsuperscript{8,9}. Both the precursors did not reveal similar responses for 2 species of Mentha for enhancement of rosmarinic acid. Among the 2 precursors tested phenylalanine was found to be useful for M. arvensis\textsuperscript{12}. Application of excess amino acid exogenously resulted in an increase in tissue growth. The reason might be because of the supplies of excess carbon level, which can be used for growth and phenol accumulation\textsuperscript{36,37}. Enhancement of secondary metabolite like total phenol and rosmarinic acid might be due to stimulation of key enzyme Phenylalanine Ammonia Lyase (PAL) activity which is the gateway of shikimic acid pathway. Previous researches supported the view that addition of precursor amino acids in the culture medium resulted in stimulation of some compounds which were intermediates in or at the beginning of a secondary metabolic biosynthetic route\textsuperscript{38}. Previous studies also demonstrated that actively growing tissue converted more than 20% of exogenously supplied phenylalanine and tyrosine to the caffeoyl ester and this high rate of synthesis coincided with an increase in Phenylalanine Ammonia Lyase specific activity which in turn produced rosmarinic acid\textsuperscript{13}.

In the present study addition of amino acid in culture media simultaneously promoted biomass growth and rosmarinic acid synthesis. A positive correlation was observed in polyphenol synthesis and
rosmarinic acid production in this study and similar results were also observed in elicitor treated sweet basil\textsuperscript{39}.

The total protein contents in different precursor and elicitor added clones along with control and field grown plants were quantified and evaluated following Bradford’s method. The analysis revealed differences in total protein contents especially with the field grown plants. The plant tissue of in vivo origin revealed the least amount of protein. The difference was also observed in one dimensional SDS – PAGE profile of the buffer soluble total protein. Number of bands increased in cultured plants than the field grown plants and similar finding was also observed by previous workers\textsuperscript{35}. A differential gene expression might be responsible for such increment during in vitro plant growth and development\textsuperscript{40,41}.

Enhanced phenol and rosmarinic acid producing clones produced a detectable band in PAGE. The additives might induce the higher activity of key enzymes of the phenylpropanoid biosynthetic pathway. High activity of these enzymes in precursor fed culture of different plant species was observed by previous workers\textsuperscript{13,42,43}. The enzyme Phenylalanine Ammonia Lyase (PAL) might have been stimulated by the addition of precursors and elicitors which resulted in increased protein synthesis\textsuperscript{44}.

Conclusion

In the present investigation a simple and rapid in vitro propagation protocol has been established. By utilizing specific amino acids, the present study has also revealed an enhanced and continuous production of rosmarinic acid which can be utilized for pharmaceutical exploitation.

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