

Full Length Research Paper

Altitude-related changes in activities of carbon metabolism enzymes and secondary plant products- menthoforon an active pharmaceutical constituents yield in peppermint (*Mentha piperita* L. Var. Kukarail)

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Accepted 15 September, 2011

Activities of some enzymes related to carbon metabolism were studied in different ecotypes of peppermint (*Mentha piperita* L. Var. Kukarail), growing at 950 and 1,250 m above mean sea level. Activities of peroxidase and superoxide dismutase (SOD) and geranyl pyro phosphate (GPP) are significantly higher in higher altitude cultivated cultivars. The GPP which converts to geranyl geranyl pyro phosphate (GGPP) comprises of two subunits, the smaller one and the large subunits. Electrophoretic bands of PCR showed the gene specific primers. In higher altitude, the smaller and large subunits of the key enzyme are present where as in the plain cultivated plants, the larger subunits are absent and the only smaller subunits are more conspicuously synthesized. The plain cultivated plants at 0.05 ppm Zn showed the higher carbon dioxide exchange rate ($0.65 \mu\text{g}(\text{CO}_2)\text{m}^{-2}\text{s}^{-1}$) and sacride formation $0.470 \mu\text{g}(\text{CH}_2\text{O})\text{m}^{-2}\text{s}^{-1}$). The higher altitude peppermints plants at 0.1 Zn mg/L grown plants, showed higher total amounts of monoterpene oil(s) (0.67%) and the increased was 26% over control, with higher menthol, menthyl actate and of medicinal uses menthofuron contents in comparison to plain cultivations. xide-dismutase (SOD) and peroxidise iso-enzymes with altitude were studied, where as activities of SOD did not show a significant difference with change in altitude. RFPD of the two altitude grown cultivars showed the different lines of conspicuous lane bandings. The key enzymes are from geranyl pyro phosphate (GPP).

Key words: Peroxidase, superoxide dismutase, peroxidise iso-enzymes, gernal geranyl pyrophosphate, phosphoenolpyruvate carboxylase; ribulose-1,5-bisphosphate, carboxylase/oxygenase.

INTRODUCTION

Peppermint (*Mentha piperita* L) was mostly affected by the leaf blight disease of *Alternaria alternata*. Therefore, a protocol has been established by tissue culture techniques, to get an improved and resistant variety of *Mentha piperita* L. var. Kukarail. Further, an adaptation to environment refers to the capacity of organisms or cells to alter their phenotype in response to changes. Plants display enormous plasticity to survive under changing environmental variables with altitude (Clements et al., 1950; Billings et al., 1961; Tranquillini, 1964). This plasticity in variety is according to altitude cropping response to environmental variables, This may involve

the changes at anatomical, morphological, physiological and biochemical levels to enable plants to combat 'harsh' climatic conditions at high altitude and maintain a reasonably efficient carbon harvesting system to compensate for the relatively short growing period (Tranquillini 1964, Larcher, 1995; Purohit, 2003). Plasticity in each of these responses could be of special significance under specific environment. Morphological plasticity is related to high competition in productive environments, whereas species acclimate through physiological plasticity in unproductive environments (Cordell et al., 1998). Mountain plants evolved in response to their particular altitude environment differ in physiological response to the respective ecotypes from lowland areas (Billings et al., 1961; Hiesey et al., 1971;

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Mächler et al., 1977; Körner and Diemer, 1987; Cordell et al., 1998; Hovenden and Schimanski, 2000; Hovenden and Schoor, 2003; Kumar et al., 2005, 2006; Vats and Kumar, 2006). However, there is little information on altitude related changes in enzymatic activities related to carbon metabolism except for enzymes such as peroxidase, superoxide-dismutase (SOD) and peroxidase iso-enzymes and geranyl geranyl pyrophosphate (GGPP), geranyl geranyl pyrophosphate (GGPP), comprises of two subunits, the smaller one and the large subunits. Further apart from the monoterpenes biosynthesis, the primary carbon metabolism, that is, the ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBPCO) plays an important role in carbon sequestration and carbon capturing for carbon balancing. The higher RuBPCO activity was reported in high altitude ecotypes of *Selinum vaginatum* (Pandey et al., 1984). Lower activation state of RuBPCO but higher total carboxylase activity in barley, pea, and wheat at high elevation suggested responsiveness of the enzyme to low partial pressures of CO₂ at high altitude (Kumar et al., 2004). While low temperature could stimulate RuBPCO activity in C4 plant *Atriplex* (Osmond et al., 1982), neither high altitude nor chilling could enhance RuBPCO capacity in C4 plants *Bouteloua gracilis* and *Muhlenbergia montanum* (Pittermann and Sage, 2000, 2001). Increased phosphoenolpyruvate carboxylase (PEPC) activity was reported in C3 species *Glycine soja* with increase in elevation from about 500 to 3650 m, though the implication of this fact was not discussed (Pandey and Purohit, 1980). Earlier, we reported that crop plants like barley and wheat when grown at high altitude significantly increased carboxylase and oxygenase activities of RuBPCO and activities of PEPC, aspartate aminotransferase (AspAT), and glutamine synthetase (GS) as compared to those grown at low altitude (Kumar et al., 2006). The objective of the present study was to find the response of some carbon capturing enzymes for secondary plant products production in altitudinal ecotypes of wild species, using *Rumex nepalensis* as the target plant that has its natural distribution spread along a wide altitudinal gradient in Himalaya (Bahar, 2002). Keeping in view the facts that a study has been made to raise the disease free and efficient genotype of *M. piperita* L. Var. Kukarail at high altitude at Purera and in plains to see the carbon sequestration.

MATERIALS AND METHODS

Plant

Plant tips (5 to 6 inches) with of efficient and disease free *M. piperita* L. Var. Kukarail genotype were obtained from the tissue culture techniques as described previously (Saxena et al., 2008) in CIMAP, Lucknow, India. Uniform cuttings were initially planted in 10000 cm³ earthen pots filled with purified silica sand (Agarwala and Sharma, 1961), for the development of roots. After 15 days, rooted cuttings were transferred to 2500 cm³ pots. The salts used

in nutrient solution culture were purified for Zn (Hewitt, 1952). Hoagland and Arnon's (1952) nutrient solution was used in the experiment except Fe which was supplied as Fe-EDTA. Three pots each of Zn treatments ranging from 0.0 to 1.0 µg Zn ml⁻¹ were maintained in controlled glass house condition at ambient temperature (30±5°C) and irradiance (between 800 and 1000 µmol m⁻²s⁻¹). The nutrient solution in each treatment was added at alternate days. With onset of deficiency and toxicity (after 20 days) growth observation and detailed physiological and biochemical data with growth attributes were performed.

CO₂ exchange rate (P_N)

CO₂ exchange rate was measured using a computerized portable photosynthesis system (Srivastava and Misra, 1991) (Model LiCOR 6000, LiCOR, USA).

Chlorophyll (Chl) content

A known mass of leaf tissue (3rd leaf) was extracted with 80% acetone and observance was recorded on spectrophotometer (Pye Unicam PU8610, USA) for determination of Chl a and b according to Arnon (1949), and carotenoids were calculated as described by Deming-Adams (1992).

Growth attributes and Zn analysis

Leaf fresh and shoot dry mass and area (area meter LICOR Li-3000) were recorded. For tissue elemental analysis 1g. dried leaf samples were digested with 1 N HCl at 60°C for 24 h. Aliquot samples of the clear digest were diluted with water (10 cm³) and analyzed for Zn by atomic absorption spectrophotometer (Pye Unicam SP 2800) (Misra and Sharma, 1991).

For antioxidant reactive peroxidase enzyme assays of peroxidase, iso-enzymes of peroxidase, SOD and GGPP enzymes, frozen leaf samples were ground with a mortar and pestle in extraction buffer containing 5 ml 0.1 M phosphate buffer (pH 6.8), as described previously (Shanon et al., 1960) and peroxidase iso-enzymes by polyacrylamide gel electrophoresis (PAGE). Leaf samples of *Mentha piperita* were collected for enzymatic studies from three different altitudinal locations, including Purera (1,250 m, 32°17'41"N, 7°10'76"E), and of plains (950 m, 32°20'47"N, 77°13'17"E). Fully developed young leaves were harvested between 09:00 and 10:00 h on a clear sunny day and stored in liquid nitrogen for further use. All the assays were performed in the Institute's laboratory at Lucknow. The range for photosynthetic photon flux density varied from 1500 to 1700 and 2200 to 2500 µmol m⁻² s⁻¹ for Purera, and Plains, respectively. Mean monthly day temperatures during the month of data recording at these localities were 9.2±2.2 and 22.2±3.2°C, respectively. Further, using 2 g of fresh chopped leaves at 3rd position, were homogenized with 5 ml of 0.1 M phosphate buffer (pH 6.8). Each treatment was replicated 3 times and assayed on SDS page electrophoresis. Superoxide dismutase (SOD) activity was assayed by the method of Henery et al. (1976). Geranyl pyrophosphate synthetase (GPP) assayed was as described previously (Misra and Sharma, 1991). RFPD through PCR and cDNA analysis were performed as described by Saxena et al. (2008).

Estimation of essential monoterpene oil(s)

Geranium oil estimation was done by steam distillation of 100 g freshly plucked leaves in a Clevenger's apparatus (Clevenger,



DISEASE FREE PLANTS PRODUCED THROUGH PLANT TISSUE CULTURE



Figure 1. Disease free plants produced through plant tissue culture technique in the culture tubes.

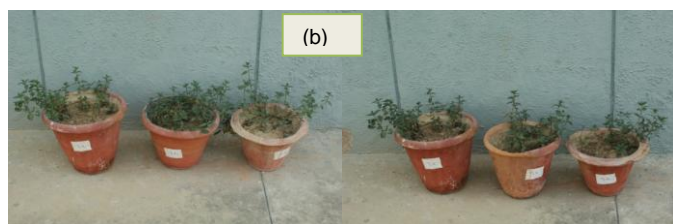


Figure 2. The plants with full growth in the controlled conditions at glass house. (a) At hills cultivation; (b) At plains cultivation.

1928). The oil constituents mainly geraniol, citronellol and other associated oil contents were determined by gas liquid chromatography (Perkin –Elemer model 3920 B). The stainless steel column was packed with 10% carbowax (20 mesh) on

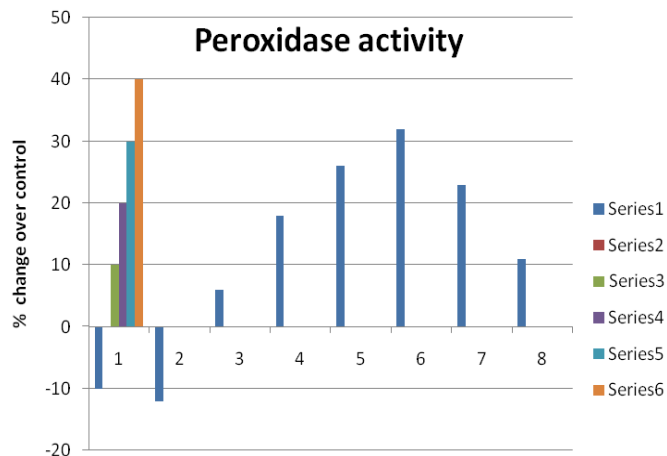


Figure 3. Effect of peroxidase activity in peppermint cultivated at higher altitude- Purera and plains: Series#1 to 4 at hills, and series#5 to 8 are at plains cultivation.

chromosorb WNAW. Injector and detector temperature was maintained at 200°C. The flow of H_2 was 0.47 cm S^{-1} data processing for area % was done on a Hewlett Packard integrator model HP-33%.

Statistical analysis

The results were statistically analyzed for the least significant differences (LSD) using the layout of a complete randomized design (CRD). Further, the results were analyzed for the correlation coefficient to determine the relationship among the characters studied, using the relationship $Y = a + b x$.

RESULTS AND DISCUSSION

Disease free somaclonal variants of *Mentha piperita* Var. Kukarail were obtained from tissue culture techniques *in-vitro* from agar medium at controlled condition (Figure 1), and further planted in plains and at higher altitudes of CIMAP Resource Center at Purera.

Peroxidase, isoenzymes of peroxidases, increased with increase in altitude and was higher by 60%, at Purera, than in plants grown at Plains (Figure 1). Similar trend was shown by and an antioxidant enzyme - SoD and activities of GPP (Figure 2). Activity also showed similar trend with almost double activity c-DNA at Purera as compared to plains (Figure 3). Three out of four probable enzymes of antioxidants activities in monoterpene synthesis during primary plant products and secondary plant products- the monoterpenes metabolism, including Peroxidase, iso-enzymes of peroxidases (Figure 5) and SOD exhibited lower activities at plains, compared to Purera (Figure 2). The fourth probable enzyme of GPP of involved in Carbon metabolism monoterpene metabolism, showed higher activity by 43% at Purera, compared to plains (Figure 3).

GPP activity, which is associated with the capacity of

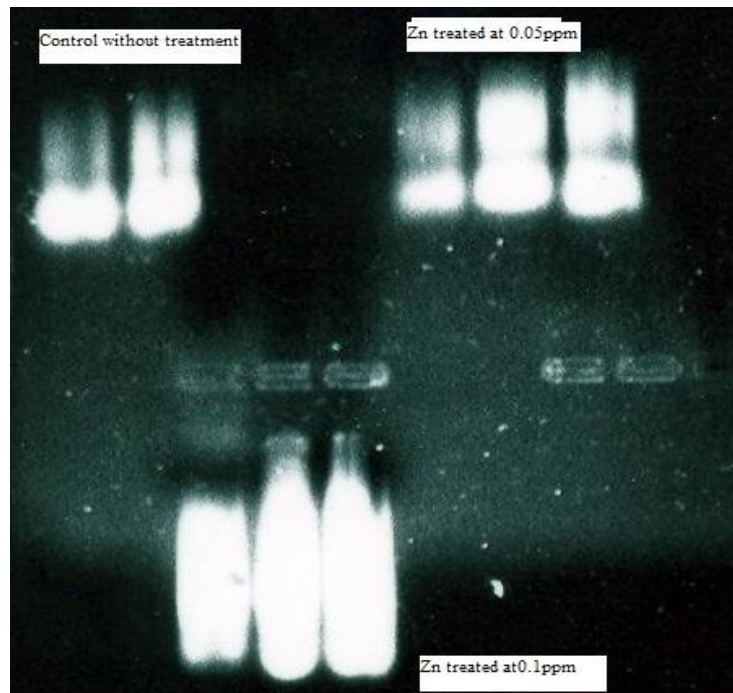


Figure 4. RNA isolated from leaves and showing the bands in electrophoresis.

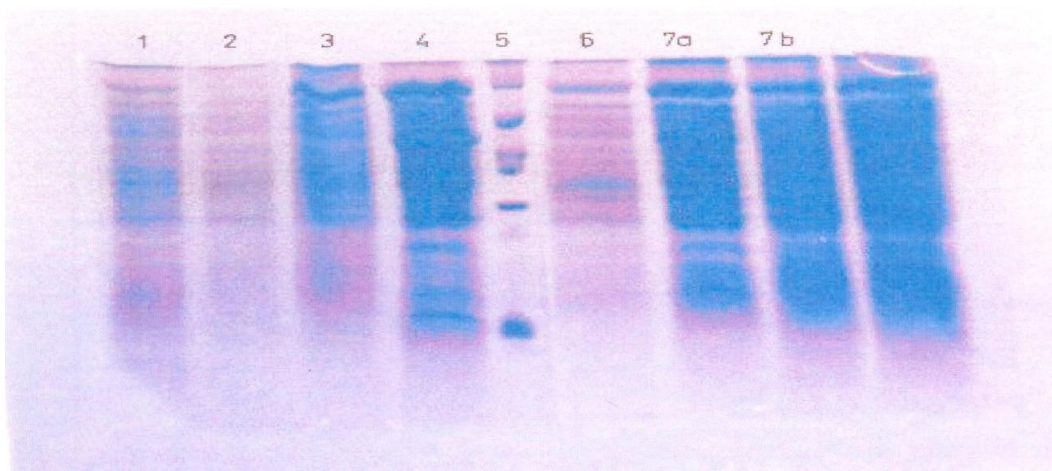


Figure 5. Native polyacrylamide gel electrophoresis (PAGE): Zn treatment 0.00 to 1.0 U μ g/ml. Showed the peroxidase isoenzyme band profiles in Peppermints cultivation at plains (#1 to 4) and at higher altitudes of Purera (#5 to 7b and unnumbered the last band).

carbon fixation, may increase with altitude (Chabot et al., 1972). Temperature drops consistently with altitude and may impart a major impetus to shape leaf's photosynthetic response at high elevation. Response of GPP to temperature is largely explained by the function of its activating enzyme, GPP activase which has a low temperature optimum (Robinson and Portis, 1989; Crafts-Brandner et al., 1997). RuBPCO activase is instrumental

in maintaining high GPP activity at low temperatures (Percy, 1977). GPP was transformed into GGPP through GPP synthase enzyme. GPP synthase is composed of two subunits, one larger subunits and another of smaller subunits. Here in our studies the plains cultivated peppermint is composed of only smaller subunits which showed the lesser activity of GPP of plain crops (Figure 4). The same trend was also obtained in

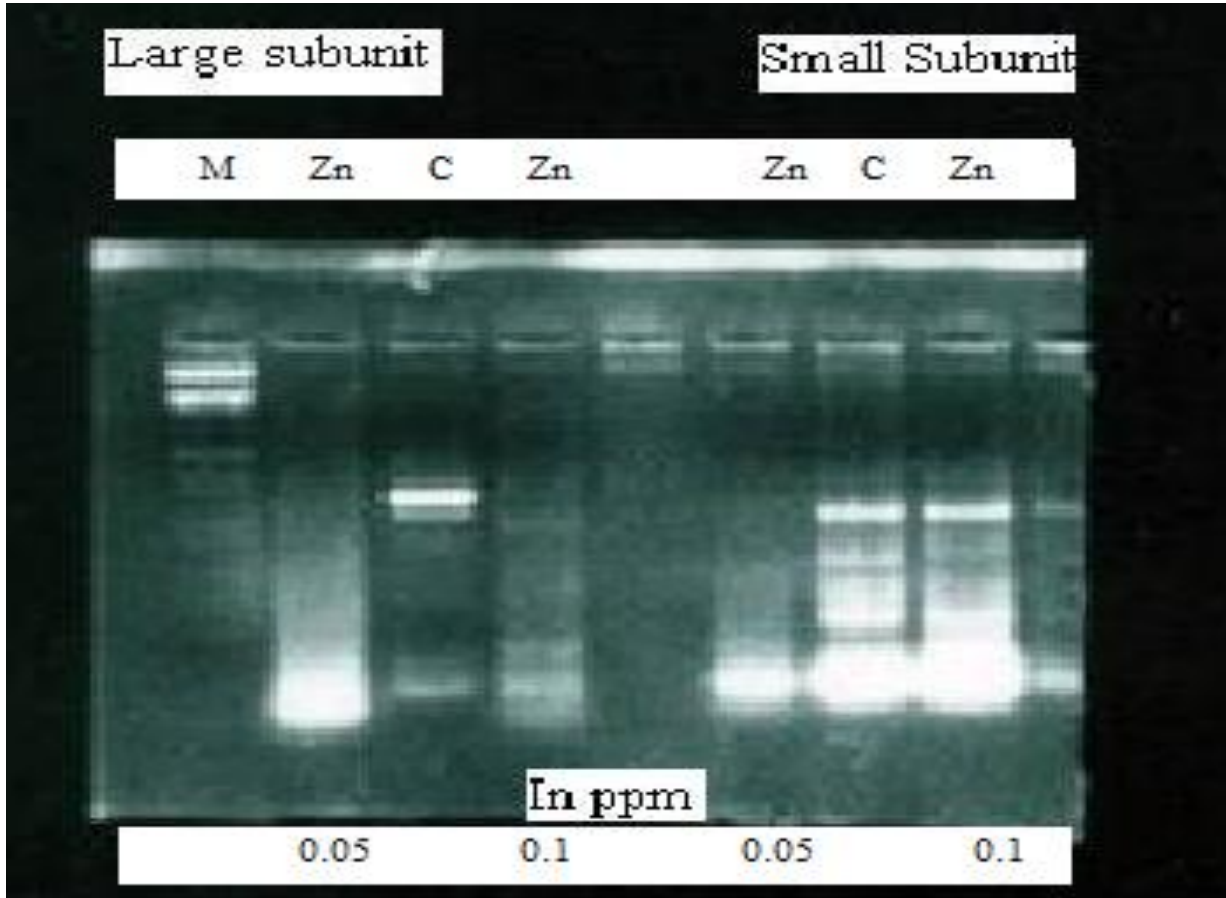


Figure 6. Electrophoretic bands after PCR with gene specific primer.

cDNA analysis with RFPD lesser bands than the cultivations of higher altitudes of *Purera* (Figure 6). Again the total photosynthesis, saccharides formation, and total oil production was 2 folds much higher than the plain cultivations (Table 1). The same production of menthofuron was obtained in the higher cultivation of peppermints. Further, CD values indicated significant differences at $p < 0.05$ and $p < 0.01$. Saccharides formation with photosynthesis and total oil formation with menthofuron production are also a good measure of capacity and efficiency of leaf photosynthesis under high irradiance (Walters, 2005). Significant correlations were also obtained in between the photosynthesis and total oil production (0.974, $p < 0.05$). In between saccharides formation and total oils with menthofuron (0.912 and $0.946 < p = 0.05$, respectively). Further, leaves acclimated to high irradiance are less susceptible to photoinhibition or photodamage, and the advantage arises largely due to increased electron transport, increased capacity to assimilate, or capacity to dissipate energy through various mechanisms including enhanced photorespiration (Streb et al., 1998; Savitch et al., 2000). High irradiance in combination with low temperature have the potential to induce chronic photoinhibition of photosystem 2 (Allen

and Ort, 2001), and have exclusive significance in high altitude locations where plants are mostly exposed to this combination of stresses. Photorespiration is a useful strategy at high elevation for barley and wheat, which showed increased peroxidase and Sod with iso-enzymes of peroxidase with altitude (Kumar et al., 2006). Similar response of enhanced activity of RuBPCO in *R. nepalensis* suggests its possible role to protect against photooxidative damage. We found in *R. nepalensis* an increase in activity of yet another carboxylating enzyme, at high altitude. This is in contrast to, decrease in PEPC activity with altitude in C3 plant *Fagopyrum esculentum* (Pandey and Purohit, 1980) and *Salsola australis* (Pyankov et al., 1997), suggesting that where it has been increased. Here, it may be related to GPP, as an altered carbon metabolism for optimal photosynthetic performance at high altitude. GPP probably could play an important role in capturing environmental or photorespired CO_2 at high altitude in C3 plants (Kumar et al., 2006). The higher GPP activity at higher altitude suggested the role, whereby it supported the formation of higher saccharides and total oil formations with menthol and menthofuron, of total monoterpenes oil contents. These findings strongly be a causative effect of a

Table 1. Effect of *M. piperita* cultivation on growth parameters.

Growth attribute	Harvesting						LSD At 5%	LSD At 1%
	Plane side			Hill side				
	1 st Harvest	2 nd Harvest	3 rd Harvest	4 th Harvest	5 th Harvest	6 th Harvest		
Plant height (cm)	57.0	58.0	61.0*	63.4**	64.1**	59.0	2.5	4.1
No. of branches	9	10*	13**	10*	10*	8	1.1	3.2
Fresh mass (g plant ⁻¹)	218.8	238.6*	224.8	282.5**	215.5**	196.2	11.1	16.3
Dry mass (g plant ⁻¹)	14.11	16.33*	16.81*	19.36**	18.46**	15.85	2.10	3.30
Leaf area (cm ²)	8.2	12.1*	25.2**	40.3**	37.2**	11.2	3.5	6.2
Chl <i>a</i> (g kg ⁻¹ (FM))	0.68	0.79*	0.94**	1.48**	1.01**	0.82*	0.11	0.15
Chl <i>b</i> (g kg ⁻¹ (FM))	0.50	0.56	0.61*	0.79**	0.40	0.29	0.08	0.12
Chl <i>a/b</i>	1.36	1.41	1.54	1.87	2.53	2.83	-	-
<i>P_N</i> (μg(CO ₂) m ⁻² s ⁻¹)	0.15	0.19*	0.75**	0.82**	0.71**	0.42**	0.03	0.06
Saccharides (μg (CH ₂ O) m ⁻² s ⁻¹)	0.102	0.129	0.510	0.558	0.483	0.286	-	-
Oil %	0.35	0.36	0.47*	0.56**	0.46	0.47	0.02	0.04
Menthone % of total oil	21	27**	27**		25**	38**	37**	0.01
Menthol % of total oil	0.59	59	67**		67**	69**	69**	0.01
Menthofuron % of total oil	5	10**	9.**		19**	18**	17**	0.04
Fe (mg kg ⁻¹)	98	112	142**		537**	419**	312**	21
Mn (mg kg ⁻¹)	26	37**	41**		98**	62**	53**	9
Zn (mg kg ⁻¹)	12	19*	34**		64**	41**	36**	7
Cu (mg kg ⁻¹)	7	9	11**		12**	7	5	3

Chl, Chlorophyll; *P_N*, net photosynthetic rate; oil amounts in % of total oil. * and **, Values are significant at P=0.05 and P=0.01 levels, respectively.

possible source. Further, these enzymatic alterations could provide adaptive advantage to plant in order to conserve carbon at high elevation. The high total oil contents, menthofuron at higher altitude cropping of peppermints at Purera leads to value addition. This higher menthofurn enrich plants at higher altitude than the plains, leads to control of dangerous smoking and as a good reliever to smoker if added into the candies. Further, it is highly available in candies of states, which have menthofuron as an additive in candies.

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