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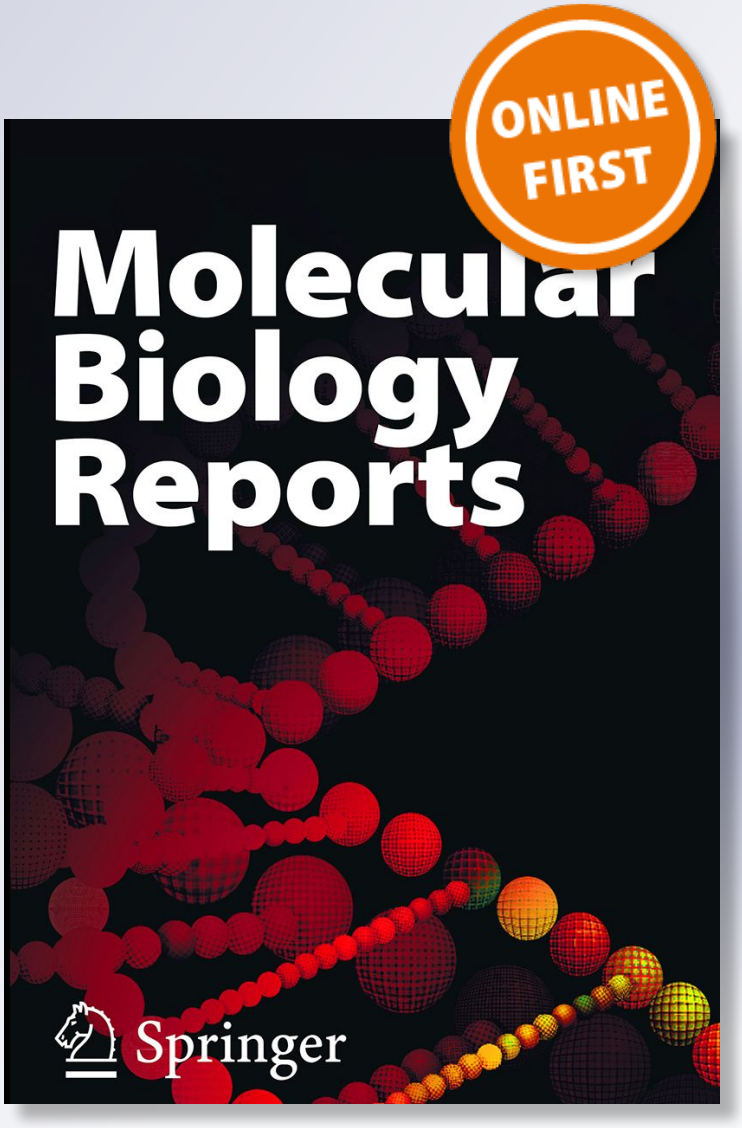
Molecular Biology Reports
An International Journal on Molecular and Cellular Biology

ISSN 0301-4851

Mol Biol Rep
DOI 10.1007/s11033-019-04678-x



Springer

Molecular Biology Reports
https://doi.org/10.1007/s11033-019-04678-x

ORIGINAL ARTICLE

Phenolic compounds, antioxidant capacity and antimutagenic activity in different growth stages of in vitro raised plants of *Origanum vulgare* L.

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Received: 14 December 2018 / Accepted: 6 February 2019

Abstract

Efficient micropropagation procedure was developed for *Origanum vulgare*, a high-value culinary herb, and the phytochemicals, phenolic content, antioxidant and antimutagenic activity of leaf and stem, derived from different growing stages were analyzed. The agar solidified Murashige and Skoog (MS) medium supplemented with a combination of 6-benzylaminopurine and α-naphthaleneacetic acid was optimized as best shoot-multiplication-medium. Shoots were rooted best on 1/2 strength MS medium supplemented with 50 µM indole-3-butyric acid (IBA). The plantlets were successfully acclimatized ex vitro in a soil, sand and farmyard manure mixture (2:1:1 v/v/v) with 100% survival rate in greenhouse. The total anthocyanin and total phenolic content were observed significantly higher in leaves of in vitro-raised plants. However, total tannin, flavonoid and antioxidant activity remained higher in leaves of mother plant maintained under ployhouse condition. All the plant extracts have shown significant antimutagenic activity except in vitro-growing plants. A total of 13 polyphenolic compounds were detected in different extracts using high performance liquid chromatography. Among these, catechin was detected maximum in in vitro-growing cultures and chlorogenic acid in leaves of mother plant. These findings will help the farmers, medicinal plant growers, and industries for mass multiplication and effective extraction of phytochemicals from *O. vulgare*.

Keywords Anthocyanin · Shoot elongation · Micropropagation · Polyphenolics · HPLC

Abbreviations

AAE	Ascorbic acid equivalent
ABTS	2,2-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid)
asl	Above mean sea level
BAP	6-Benzylaminopurine
CN	Cyanidin 3-glucoside
DPPH	2,2-Diphenyl-1-picryhydrazyl
DAD	Diode-array detection
GA_3	Gibberellic acid
GAE	Gallic acid equivalent
HPLC	High performance liquid chromatography
IBA	Indole-3-butyric acid
IL	In vitro-raised plant leaf
IS	In vitro-raised plant stem
IVG	In vitro-growing cultures
MPL	Mother plant leaf
MPS	Mother plant stem
MS	Murashige and Skoog
NAA	α-Naphthaleneacetic acid
PBS	Phosphate-buffered saline
PGRs	Plant growth regulators
TAE	Tannic acid equivalent
TBE	Tris borate ethylenediaminetetraacetic acid
QE	Quercetin equivalent
µM	Micro mole

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11033-019-04678-x) contains supplementary material, which is available to authorized users.

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Published online: 12 February 2019

Introduction

Origanum vulgare L. (Lamiaceae), commonly known as Van Tulsi or Himalayan majorana, is an aromatic perennial herb, native to the Mediterranean region [1, 2]. In India, it is the only reported species of genus *Origanum* and distributed across the sub-temperate to temperate Himalayan regions, from Kashmir to Sikkim, at elevations from 600 to 4000 m asl [3]. *O. vulgare* is an important culinary herb and among the most traded and consumed spice plants in world trade [4]. Besides, it is known to possess antioxidant, antimicrobial, insecticidal, antifungal and antiseptic properties [5–8]. It is a vital source of polyphenols and their biosynthetic precursors, such as anthocyanins, flavonoids, flavonols, phenolic acids, pro-anthocyanins, tannins etc. which are known to possess antioxidant, anticancer, antimutagenic, antitumor activity [9–12], and helps in maintaining the homeostasis of the body by scavenging reactive oxygen species (ROS) [13]. Perhaps due to these proven biological properties, *O. vulgare* is used to treat various ailments such as spasmodic condition, digestive disorders, menstrual problems, whooping, convulsive coughs etc. since ancient times [2], and enjoy wide industrial, pharmaceutical and traditional demand around the world [12].

To date, most of the consumed plant material of *O. vulgare* is collected from wild, which is hampering its availability in natural populations [14]. Further, very less is known about its cultivation and domestication [4]. Traditionally, *O. vulgare* is propagated through seeds and cuttings (vegetative propagation), however, poor germination rate, low seed viability, seed sterility, hampers its large-scale propagation through seeds and the repeated vegetative propagation resulted progressive yield loss in the progenies [15]. Plant tissue culture-based techniques have emerged as an alternative for mass-multiplication, conservation and in vitro secondary metabolite production of high-value plant species across the globe [16, 17]. Although, some in vitro propagation studies are available for genus *Origanum* and mainly focused on plant regeneration response from the meristematic tips [15], axillary buds [18], and seeds [19, 20]. However, screening of polyphenols, and pharmaceutical activities in different growing stages and plant parts of *O. vulgare* is not reported so far.

The present study focuses on (i) development of effective in vitro propagation protocol for *O. vulgare*, (ii) comparative evaluation of phytochemicals, antioxidant and anti-mutagenic activity in different plant parts (leaf and stem) of *O. vulgare* across different growth stages and wild plants, (iii) screening of antioxidant-polyphenolics in different growth stages using HPLC.

Materials and methods

Plant material

Plants of *O. vulgare* L. were collected during the month of September 2011 from the wild population at Mukteshwer, Uttarakhand, West Himalaya, India (latitude 79°37′18″N, longitude 29°26′60″E; altitude 2186 m asl) and established in the greenhouse (under 24 ± 5 °C atmospheric temperature and 70–80% relative humidity). Nodal explants were taken from wild plant established in greenhouse (mother plant) as starting material for in vitro propagation of *O. vulgare*.

In vitro micropropagation

Nodal segments were excised from the mother plant and washed thoroughly under tap water using a few drops of Tween 20 for 30 min. Thereafter, nodal segments were subjected to 0.50% w/v systematic fungicide solution (Bavistin, BASF India Ltd.) for 15 min and surface sterilized with freshly prepared 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5 min under laminar air flow cabinet (Vista Biotech, India). Each treatment was followed by five time washing with sterile ultrapure water (Rions: 0.2 µm capsule filter, Labpure series). After proper disinfection, these nodal explants were inoculated on MS medium supplemented with 3% (w/v) sucrose, and different concentrations of 6-benzylaminopurine (BAP; 1.0–8.0 µM) alone for shoot induction and/or in combination with α-naphthaleneacetic acid (NAA; 0.10–0.50 µM), Gibberellic acid (GA_3; 0.25 µM) for shoot multiplication and elongation, respectively. Nodal explants inoculated onto MS medium without supplementation of plant growth regulators (PGRs) were served as control. All in vitro cultures were maintained in a growth room at 25 ± 2 °C under a 16/8 h light/dark photoperiod with 42 µM m^{-2} s^{-1} illumination provided by cool white fluorescent tubes (Philips 40 W). The MS medium was solidified with 0.8% (w/v) agar and pH of the medium was adjusted to 5.8 with 1N NaOH before autoclaving at 121 °C for 15 min. Based on a comparative assessment of the influence of these PGR levels on shoot induction, proliferation and elongation, growth parameters were monitored up to 6-week. The regenerated shoots (3 to 5 cm long) from actively growing cultures were excised and subjected to two-step rooting procedure described by Pandey and Tamta [21]. Briefly, excised microshoots were subjected to solid MS medium supplemented with 50 µM IBA for 24 h and then rooted individually on a hormone-free 1/2-strength MS basal medium. Rooted plants were carefully taken out from

culture flasks and washed in ultrapure water to remove the traces of medium. These rooted plants were transferred to hardening pots containing a mixture of soil, sand and farmyard manure in a ratio of 2:1:1 (v/v/v) and covered by transparent polybags and initially hardened inside the growth room for 4-week. The potting mixture was moistened twice a week with 1/2 strength MS without sucrose. After 4-week, the semi-hardened plants were transferred to greenhouse on a normal day/night condition at the 24 ± 5 °C temperature and 60–70% relative humidity. The survival rate of plantlets was recorded after 6-week of transfer in the greenhouse.

Phytochemical analysis

Extract preparation

Leaves and stem samples were collected from (i) mother plant maintained in the greenhouse, (ii) well acclimatized 1 year old in vitro-raised plants, and (iii) in vitro-growing cultures of *O. vulgare*. These were washed properly with ultrapure water to remove the traces of dust/MS medium and then dried at room temperature. The dried samples were grounded to make a fine texture using mortar and pestle. For extraction, 1 g of the powder was mixed into 10 ml of 80% methanol (v/v) and homogenized under Ultrasonicator (Toshiba India) for 5 min. The homogenized mixture was kept at 60 °C in a water bath for 1 h. This was stored in tightly capped bottles for 24 h at room temperature and then filtered by Whatman filter paper no 1. The filtrate (extract) was stored in glass vials at − 20 °C prior to analysis.

Estimation of total phenolic content

Total phenolic content (TPC) was determined by Folin–Ciocalteu's colorimetric method [22]. The quantification of TPC was done on the basis of the standard curve of gallic acid and results were expressed in mg gallic acid equivalents (GAE)/g of dry weight.

Estimation of total tannin content

Total tannin content (TTC) was measured by Folin's Dennis method described by Nwinuka et al. [23]. The quantification of TTC was done on the basis of the standard curve of tannic acid and results were expressed in mg tannic acid equivalent (TAE)/g of dry weight.

Estimation of total flavonoid content

Total flavonoid content (TFC) in the methanolic extract was determined by aluminium chloride colorimetric method [24]. The quantification of TFC was done on the basis of the standard curve of quercetin and results were expressed in mg quercetin equivalent (QE)/g of dry weight.

Estimation of total anthocyanin content

Total anthocyanin content (TAC) was measured by pH differential AOAC method [25]. The TAC was expressed as milligram cyanidin-3-glucoside equivalent per 100 g dry weight (mg CGE/100 g dw) of the sample and quantified with the following formulae:

$$TAC\ (mg\ CGE/100g\ dw) = \left(\frac{\Delta A \times MW \times df \times 100}{\varepsilon}\right) l$$

where, ΔA = [(A520–A700 nm) pH 1.0 − (A520–A700 nm) pH 4.5]; molecular weight (449.2 g/mol of cyanidin-3-glucoside); df = dilution factor; l = path length in cm; ε = 26,900 M extinction coefficient in L mol^{-1} cm^{-1} for cyanidin-3-glucoside.

Antioxidant activity

DPPH radical-scavenging assay

The free radical scavenging activity was tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals scavenging assay [26]. Ascorbic acid was used as a standard and results were expressed in millimole (mM) ascorbic acid equivalent (AAE)/g of dry weight.

ABTS radical-scavenging assay

The 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging assay was performed using an improved ABTS method [27], using ascorbic acid as a standard. The radical scavenging activity of the extracts was expressed as mM ascorbic acid equivalent (AAE)/g dry weight.

DNA damage protection assay (antimutagenic activity)

Conversion of the supercoiled form of plasmid DNA to the open-circular and/or linear forms were analysed as an indicator of DNA damage. For this, pBR322 plasmid DNA was photolyzed via UV radiation in the presence of H_2O_2 as described by Pandey et al. [28], with minor modifications. Different extracts of *O. vulgare* were tested for their antimutagenic potential, along with a standard antioxidant (ascorbic acid). Reaction mixtures (15 µL) containing 180 ng of pBR322 plasmid DNA, 7.5% H_2O_2, plant extract (1 mg/mL) and/or ascorbic acid (1 mg/mL) while one tube was kept as control (C) without plant extract and ascorbic

acid. The reaction was carried out at room temperature and UV radiation was provided from 40 cm distance for 30 min. After 30 min reaction tubes were placed in − 20 °C for 20 min to stop the reaction. Moreover, 180 ng of pBR322 plasmid DNA mixed in 1 × PBS buffer (pH 7.4) was placed in a separate tube for non-irradiated control (P). To visualize results, electrophoresis was performed on 1.0% agarose gel in 0.5 × TBE buffer at 45 V for 2 h. After proper separation, the gel was photographed under gel documentation system (Uvitech, Cambridge, UK) and band density was determined using the software (Uvipro Platinum 1.1). The following formulae were used for calculating the percentage of supercoiled pBR322 plasmid DNA (S %), relative supercoiled pBR322 plasmid DNA (RS %) and relative ascorbic acid prevention of supercoiled DNA (RAS %):

$$S\% = \left(\frac{Band\ density\ of\ (s)}{Band\ density\ of\ (s+l+oc)}\right)100$$

$$RS\% = \left(\frac{S\%\ of\ test\ samples}{S\%\ \ of\ control}\right)100$$

$$RAS\% = \left(\frac{S\%\ of\ test\ \ samples}{S\%\ \ of\ \ ascorbic\ \ acid}\right)100$$

where s: supercoiled, l: linear and oc: open circular forms of pBR322 plasmid DNA.

Phenolic profile analysis

Phenolic profiles were evaluated using a high performance liquid chromatography (HPLC) system equipped with diode array detector (DAD-MZOA) and two LC-10ATvp HPLC pumps (Shimadzu LC-10AT, Shimadzu, Japan), based on the method [27, 29]. The quantity of each phenolic compound was calculated by peak areas and standard curves of corresponding standards and results were expressed as mg/g dry weight of the sample. All the standards were purchased from Sigma-Aldrich, Steinheim, USA.

Chemicals

All the micro and macronutrients, vitamins and iron-source of Murashige and Skoog [30] (MS) medium were purchased from the HiMedia, Laboratories Pvt. Limited, Mumbai, India. Plant growth regulators, HPLC standards, caffeic acid, catechin, chlorogenic acid, ellagic acid, ferulic acid, gallic acid, *m*-coumaric acid, p-coumaric acid, phloridzin, quercetin, rutin, *trans*-cinnamic acid, vanillic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid ascorbic acid, catechin, cyanidin 3-glucoside, gallic acid, quercetin and 2,2-Diphenyl-1-picryhydrazyl (DPPH) were purchased from Sigma-Aldrich, (St. Louis, Missouri, United States). Aluminium chloride, acetic acid, ferric chloride, hydrochloric acid, potassium acetate, potassium chloride, sodium acetate, sodium carbonate and potassium persulphate from Sisco Research Laboratories Pvt. Ltd. Mumbai, India. Ethanol, methanol 2,2-Azinobis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and 2,4,6-tri-2-pyridyl-1,3,5-triazin (TPTZ) from Merck KGaA, Darmstadt, Germany and pBR322 vector from Promega, Madison, WI, USA. All the chemicals purchased were of analytical and HPLC grade.

Statistical analysis

All the experiments were set up in a completely randomized design and performed in triplicate. The data were subjected to one-way analysis of variance (ANOVA) [31]. Significant differences ($P < 0.05$) between mean values were detected using Duncan's multiple range test. All the statistical analysis were done using SPSS statistical package for Windows (Version 20; SPSS Inc., Chicago, USA) statistical software package.

Result and discussion

In vitro propagation

Culture establishment and shoot induction

The physiological status of explant plays a significant role in the establishment of cultures, thus the mother plants were established in the greenhouse as a fresh source of the explant. The shoot emergence was observed after 2 week of culture (Fig. 1a); BAP (4.0 µM) showed significantly ($P < 0.05$) higher responses in terms of shoot induction (91.67%) and maximum shoot length (2.29 ± 0.03 cm). Increased concentration of BAP showed a deleterious effect on shoot induction (Table S1). Similarly, all the explants cultured on MS basal medium (control) turned brown within 15 days of culture without initiating shoots. This may be due to the insufficient endogenous level of hormones to sustain the growth of these explants in the basal medium. Similar results have been reported from the plants of Lamiaceae, *Ocimum sanctum* [32] and other such as, *Quercus serrata* [21], *Canscora decussate* [33], *Couroupita guianensis* [34]. After induction of shoots from lateral buds, these were excised and cultured in shoot multiplication medium, *i.e.* MS medium supplemented with optimized BAP concentrations (4.0 µM) along with different concentrations of 1-naphthaleneacetic acid (NAA, 0.10–0.50 µM).

The lower concentration of NAA with different cytokinins has been proven the most efficient in various in vitro propagation studies including genus *Origanum*, viz. *O. vulgare* x *applii* [15], *O. acutidens* [20], and others

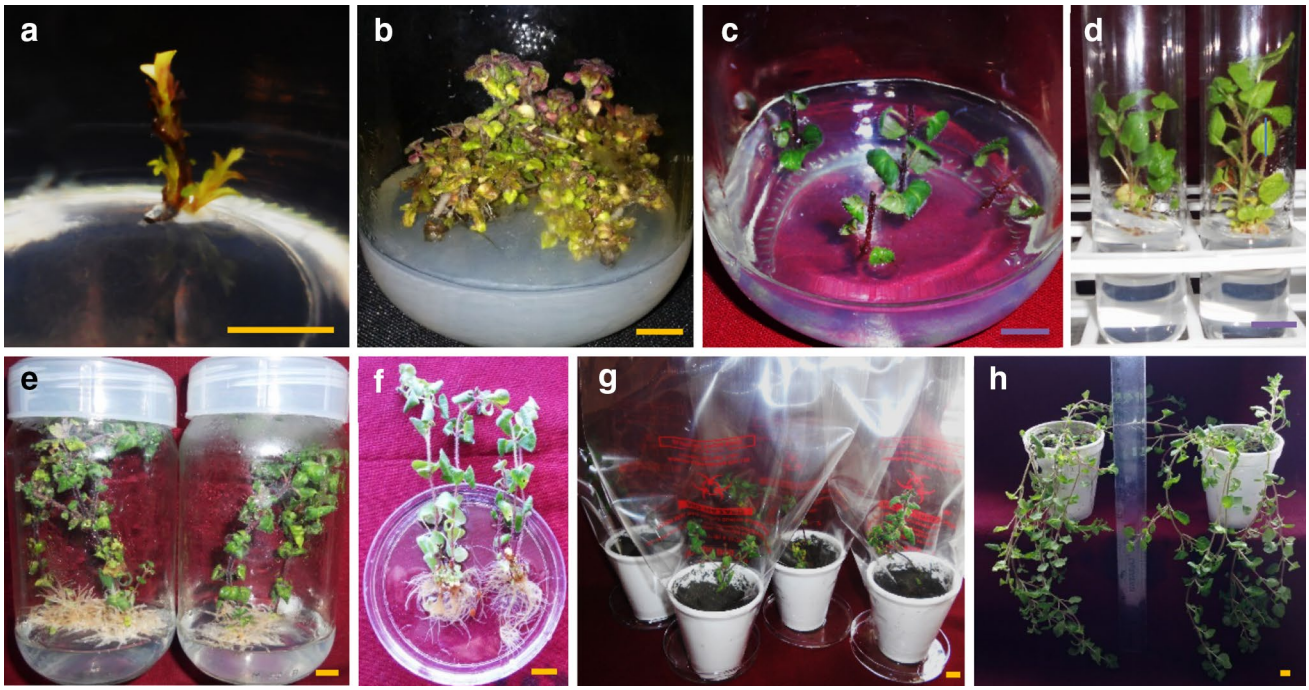

Fig. 1 In vitro propagation of *O. vulgare*. **a** Shoot induction in nodal explant (15 days) in MS medium supplemented with 4 µL BAP; **b** shoot multiplication in MS medium supplemented with BAP + NAA (4.0 + 0.25 µL) (60 days); **c**, **d** in vitro shoot elongation in MS medium with BAP + GA_3(4.0 + 0.25 µL) (30 days); **e** microshoots rooted on MS medium supplemented with BAP + GA_3 (4.0 + 0.25 µM); **f** well rooted shoots (30 days) prior to acclimatization; **g** potted plants inside culture room for gradual acclimatization; **h** well acclimatized plants showing promising growth after 60 days of acclimatization

Berberis chitria [35], *Berberis aristata* [36], *Jeffersonia dubia* [16]. Similarly, the positive effect of BAP and NAA was observed during the present study in maximizing the number of shoots (Fig. 1b). Results of shoot multiplication responses are depicted in Table S1. Although, no significant ($P < 0.05$) difference was observed in shoot induction frequency among different PGRs combinations; however, a significant ($P < 0.05$) difference was observed in remaining parameters such as the number of shoots, shoot length and the length of longest shoot (Table S1). Highest shoot induction frequency (100%), with 27.50 ± 2.05 shoots per explant and an average shoot length of 3.47 ± 0.15 cm was observed in MS medium supplemented with 4.0 µM BAP and 0.25 µM NAA (Table S1). To increase the average shoot length, multiple shoots obtained after 60 days of culture in shoot multiplication medium were transferred into elongation medium (Fig. 1c, d). In elongation medium NAA (0.25 µM) was replaced with GA_3 (0.25 µM). Replacement of NAA with GA_3 has shown positive response and significantly ($P < 0.05$) enhanced average shoot length (6.57 ± 0.38 cm) and length of longest shoot (9.93 ± 0.83 cm), while the shoot multiplication rate was reduced to 8.33 ± 2.08 shoots per explant (Fig. 2). The stimulating effect of GA_3 on elongation of diminutive shoots, raised on BAP supplemented medium, has been

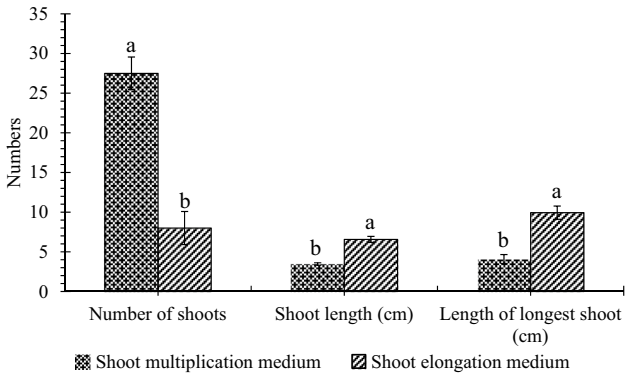

Fig. 2 Effect of (BAP + GA_3) on in vitro shoot elongation. *BAP* 6-benzylaminopurine; *GA_3* gibberellic acid. Vertical bars, representing mean ± SD, followed by same letters within a growth medium are not significantly different and separated by using Duncan's multiple range test (DMRT; $P < 0.05$)

reported in several other plant species [37]. The study of Arney and Mancinelli [38] indicates that the cell elongation effect of GA_3 is a derivative of the increased mitotic activity, possibly through an increased production of auxin in the apical and sub-apical meristematic regions. This might have reduced the shoot multiplication rate of *O. vulgare* in the elongation medium.

Root induction and acclimatization

Well-developed shoots (> 5 cm long) were subcultured on 1/2 strength MS medium containing indole-3-butyric acid (IBA) for rooting. A 100% rooting with 42.33 number of roots and 2.20 ± 0.53 cm average root length (LR) was observed in MS medium supplemented with IBA in a two-step rooting procedure. The root induction was observed in shoot multiplication medium (91.67%, 11.33 ± 1.15 and 1.67 ± 0.42 cm, percent rooting, number of roots and cm average root length, respectively) and shoot elongation medium (83.33%; 24.33 ± 9.29;3.37 ± 0.51 cm, percent rooting, number of roots and cm average root length, respectively). Root induction was observed in each treatment with significantly ($P < 0.05$) different responses. A comparative rooting response of shoots under different treatments is depicted in Table S2. Similar results were also observed in *Origanum acutidens* [20]. The two-step rooting procedure was successfully used in our earlier studies [21, 28, 35] for in vitro root induction. Further, it has been observed that shoots rooted in elongation medium have shown 100% survival rate, while shoots rooted in growth regulator-free MS medium did not survive (Fig. 1g, h). It is reported that both cytokinins and auxins can be produced in roots and shoots [39, 40], but their production is regulated by the location of the synthesizing cells in the plant and their developmental stage and environmental conditions [41]. In the present study, plants rooted in PGR-free MS medium have gained significantly ($P < 0.05$) low plant height (2.23 ± 1.94 cm), with less number of roots and small root length (1.67 ± 1.53 and ~ 0.70 ± 0.61 cm, respectively), which might have restricted their establishment during acclimatization. In elongation medium plants attended significantly ($P < 0.05$) better plant height (14.33 ± 3.62 cm) with adequate average root numbers (24.33 ± 9.29) and root length (3.37 ± 0.51 cm) which is essential for better survival in in vivo conditions where plant requires its own system for PGR synthesis. Because root tips are major sites of cytokinin synthesis and young shoots are the major sites of auxin production and these signals move in specific structural pathways and by different mechanisms to regulate plant development and differentiation [42]. Observations of present study revealed that for in vitro mass multiplication of *O. vulgare*, MS medium supplemented with 4.0 µM BAP and 0.25 µM NAA is the best. Further, plant length is essential for better survival of *O. vulgare* in field conditions along with root numbers and root length. Aloni et al. [41] reported that the young shoots are the major sites of auxin production, which promotes root development and induces vascular differentiation. The differentiating protoxylem vessel elements stimulate lateral root initiation by auxin-ethylene-auxin signaling [43]. The well-developed rooting system possibly strengthened the survival of in vitro-raised plants during acclimatization, as root tips are major sites of cytokinin synthesis [42], which regulate plant development and differentiation. Therefore, planting material of in vitro-raised *O. vulgare* can be developed within two steps without rooting step i.e. mass multiplication and elongation in 4.0 µM BAP and 0.25 µM GA_3 supplemented MS medium (Fig. 1e, f). In vitro propagation methods are also being used in secondary metabolite production [44], screening of high metabolite producing cell lines [45] and studying the metabolism [46]. Thus further research needs to be done in these areas to harness the complete potential of this important species.

Phytochemical and antioxidant analysis

Polyphenols have received greater attention due to their role in several degenerative and aging-related diseases [47]. The results of present study revealed that the level of total phenolic content in different parts of mother plant i.e. leaves (MPL) and stem (MPS) and in vitro-raised plant parts i.e. leaves (IL) and stem (IS) along with in vitro growing culture (IVG) of *O. vulgare* was varied significantly ($P < 0.05$). The highest total phenolic content was observed in IL extract (16.97 ± 0.06 mg GAE/g dw). Similarly, highest total tannin content (23.55 ± 0.29 mg TAE/g dw) was observed in MPL extract and IL (23.42 ± 0.11 mg TAE/ g dw) extract, and flavonoid content in MPL extract (12.15 ± 0.02 mg QE/g dw) (Table 1). However, the total anthocyanin content was recorded maximum in IL (0.0251 mg CE/100 g dw) and least in IVG (0.0054 mg CE/100 g dw). These variation in phytochemicals content within the plant parts have also been reported by Surveswaran et al. [48] in 12 medicinal plants of the Asclepiadaceae and Periplocaceae families, and can be attributed to specific metabolic and endogenous physiological changes taking place in the plants [11].

The total antioxidant activities were determined by DPPH and ABTS assays, and the results were presented in Table 1. Among the all studied extracts, significantly ($P < 0.05$) higher DPPH radicals scavenging activity was observed in MPL extract (33.22 ± 0.14 mM AAE/g dw) and lowest in IVS extract (19.18 ± 0.14 mM AAE/g dw). However, the extracts have shown no significant ($P < 0.05$) difference in ABTS activity. The higher antioxidant activity of ex vitro plants than in vitro grown plants is an agreement with the levels of stress in different growth conditions [33, 49]. The ex vitro growing mother plant (MPL and MPS) is more vulnerable to physical, climatic and biological stresses than the in vitro-raised and greenhouse maintained plants (IL and IS), while in vitro-growing cultures are least vulnerable to these factors, as these were kept in controlled conditions and fortified with nutrient media. These stress conditions develop reactive oxygen species (ROS) and therefore plants might develop a strong antioxidant system against ROS for their survival [33, 50]. Phenolic compounds such as anthocyanin,

Table 1 Phytochemical composition in methanolic extract of *Origanum vulgare* under different growing conditions and different plant parts

Plant parts	TPC (mg GAE/g dw)	TTC (mg TAE/g dw)	TFC (mg QE/g dw)	DPPH (mM AAE/g dw)	ABTS (mM AAE/g dw)	TAC (mg CN/100 g dw)
MPL	14.11 ± 0.14^{b}	23.55 ± 0.29^{a}	12.15 ± 0.02^{a}	33.22 ± 0.14^{a}	1.28 ± 0.01^{a}	0.0112
MPS	7.54 ± 0.06^{c}	22.62 ± 0.15^{b}	2.78 ± 0.03^{d}	20.23 ± 0.10^{d}	1.29 ± 0.01^{a}	0.0092
IL	16.97 ± 0.06^{a}	23.42 ± 0.11^{a}	10.97 ± 0.08^{b}	32.56 ± 0.11^{b}	1.28 ± 0.02^{a}	0.0251
IS	7.27 ± 0.06^{cd}	22.21 ± 0.11^{bc}	2.09 ± 0.03^{e}	19.18 ± 0.14^{e}	1.29 ± 0.01^{a}	0.0071
IVG	7.41 ± 0.04^{cd}	21.74 ± 0.04^{c}	5.25 ± 0.02^{c}	21.79 ± 0.29^{c}	1.27 ± 0.01^{a}	0.0054

The data shown is the mean of three replicates ± SD

TPC total phenolic content, *TTC* total tannin content, *TFC* total flavonoid content, *DPPH* 2,2-diphenyl-1-picrylhydrazyl, *ABTS* 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid, *TAC* Total anthocyanin content, *MPL* mother plant leaf, *MPS* mother plant stem, *IL* in vitro-derived plant leaf, *IS* in vitro-derived plant stem, *IVG* in vitro-growing cultures, *GAE* gallic acid equivalent, *TAE* tannic acid equivalent, *QE* quercetin equivalent, *AAE* ascorbic acid equivalent, *CN* cyanidin 3-glucoside

Values followed by same letters within a column are not significantly different and separated by using Duncan's multiple range test (DMRT; $P < 0.05$)

tannin, and flavonoid have exhibited the significant antioxidant activity in different in vitro cellular models [11], and are protective against diverse reactive oxygen species (ROS) including hydroxyl radical, peroxyl radical, hypochlorous acid, superoxide anion and peroxynitrite through their scavenging [13], and stabilizing lipid peroxidation [51]. Now a days, the food sources rich in natural phenolic compounds are of considerable interest and such plants or plant products are an important parts of diets, as they can bring several antioxidant related health benefits.

Antimutagenic activity

Ultraviolet radiation produces hydroxyl (OH) radicals by peroxidizing H_2O_2, which can damage the plasmid DNA especially supercoiled form. Compounds having OH scavenging activity can avoid this process and protect plasmid DNA. Methanolic extract of different growing conditions and different plant parts of *O. vulgare* were studied for DNA protection potential. Ultraviolet radiation breaks H_2O_2 and produces OH radicals, which damages pBR322 plasmid DNA consequently degradation of supercoiled DNA takes place. However, the reaction mixture having OH scavenging material can prevent the DNA damage. The DNA damage has been prevented by the methanolic extracts of *O. vulgare* and significantly ($P < 0.05$) higher DNA damage prevention activity was observed in MPL extract (RS%, 69.45 ± 3.23) followed by IL extracts (RS%, 64.97 ± 3.04) (Fig. 3 lane 3–8). It was observed that the DNA protection activity of *O. vulgare* plant extracts was significantly ($P < 0.05$) higher than the used standard antioxidant i.e. ascorbic acid (Fig. 4, Table S3). Results of DNA prevention assay also support the phytochemical composition of methanolic extracts of *O. vulgare*. The antioxidant activity of flavonoids corresponds to their peroxyl-radicals scavenging property and by chelating iron ions [9]. Further, anticarcinogenic and antimutagenic potentials of tannins have been well documented by Amarowicz [52]. Tannins functions as primary as well as secondary antioxidants and chelate metal ions like Fe^{2+}, Zn^{2+}, Cu^{2+}, thereby delay oxidation process [53]. The inhibitory effect of the iron ions in the UV photolysis of H_2O_2 is well-studied under different UV light sources [54].

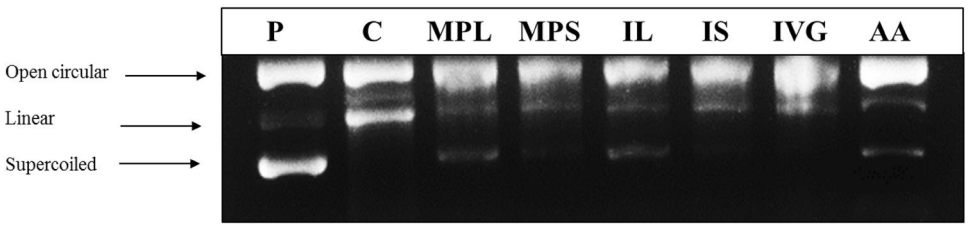

Fig. 3 Agarose gel view of H_2O_2 induced DNA damage prevention activity of different treatments. Lane 1 (P): non irradiated control (pBR322 + PBS); lane 2 (C): irradiated control (pBR322 + H_2O_2); lane 3–6: protecting effect of different extracts (1 mg/mL of dw) and lane 7: effect of ascorbic acid (1 mg/mL) on DNA damage. *MPL* mother plant leaf, *MPS* mother plant stem, *IL* in vitro-raised plant leaf, *IS* in vitro-raised plant stem, *IVG* in vitro-growing cultures

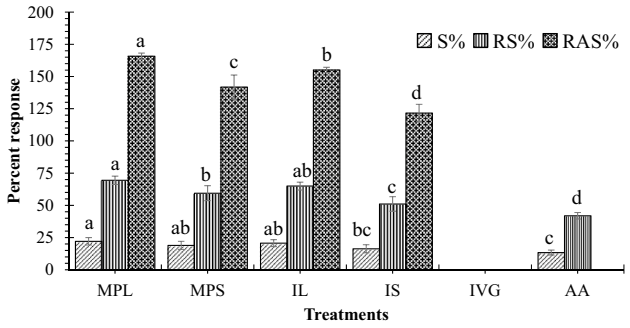

Fig. 4 Modulating effect of different methanolic extracts of *O. vulgare* on H_2O_2 induced DNA damage. *S* supercoiled pBR322 plasmid DNA, *RS* relative supercoiled pBR322 plasmid DNA, *RAS* relative ascorbic acid supercoiled pBR322 plasmid DNA, all treatments were exposed to the UV light and containing 1xPBS + pBR322 plasmid DNA + H_2O_2 + 1 mg/mL plant extracts (*MPL* mother plant leaf, *MPS* mother plant stem, *IL* in vitro-raised plant leaf, *IS* in vitro-raised plant stem, *IVG* in vitro-growing cultures and *AA* ascorbic acid). Vertical bars, representing mean ± SD, followed by same letters between treatments (represented by same pattern) are not significantly different and separated by using Duncan's multiple range test (DMRT; $P < 0.05$)

Phenolic profile of different plant parts in different growing conditions

The phenolic profiles of different plant parts in different growing conditions of *O. vulgare* were identified by HPLC-DAD and 13 phenolic compounds were detected in these samples (Table 2). The presence of individual phenolic compound varied significantly ($P < 0.05$) in different growing conditions and plant parts. A maximum number of phenolic compounds were detected in leaf extracts as compared to stem and in vitro-growing cultures (Table 2). These variations in the phytochemicals might be due to the level of hormonal content, specific metabolic and endogenous physiological changes taking place in the plants exposed to different growing conditions [11]. Among all the detected phenolic compounds chlorogenic acid was detected the maximum in MPL and MPS extracts (1.50 ± 0.08 and 1.30 ± 0.17 mg/g dw), respectively. The concentration of catechin was high in IL, IS and IVG extracts (2.48 ± 0.16; 2.63 ± 0.02 and 2.83 ± 0.42 mg/g dw), respectively (Table 2). The *trans*-cinnamic acid was detected only in MPL extract, while gallic acid was present in all the extracts. Moreover, the concentration of gallic acid varied significantly ($P < 0.05$) among the growth conditions (Table 2). The maximum number of polyphenolics were detected in mother plant, but their concentration was observed significantly ($P < 0.05$) higher in plants growing inside culture room i.e. IVG (Table 2). The HPLC chromatograms of phenolic compounds derived from methanolic extracts of different plant parts and growing stages of *O. vulgare*, detected in different wavelengths, are presented in Figure S1 of the supporting information.

Table 2 HPLC based quantitation results of individual phenolic and flavonoid compounds of *Origanum vulgare*

SN	Compounds (mg/g dw)	MPL	MPS	IL	IS	IVG
1	3-Hydroxy Benzoic acid	0.25 ± 0.02^{c}	0.55 ± 0.130^{b}	0.96 ± 0.16^{a}	0.13 ± 0.001^{c}	–
2	3-hydroxy cinnamic acid	0.26 ± 0.02^{a}	–	0.05 ± 0.004^{b}	–	–
3	Caffeic acid	–	0.02 ± 0.001^{b}	–	0.006 ± 0.0007^{c}	0.10 ± 0.007^{a}
4	Catechin	0.18 ± 0.012^{b}	–	2.48 ± 0.164^{a}	2.63 ± 0.02^{a}	2.83 ± 0.42^{a}
5	Chlorogenic acid	1.50 ± 0.08^{b}	1.30 ± 0.171^{b}	1.93 ± 0.08^{a}	–	–
6	Ellagic acid	0.23 ± 0.01^{a}	0.05 ± 0.004^{c}	0.10 ± 0.0002^{b}	–	0.24 ± 0.02^{a}
7	Ferulic acid	–	0.31 ± 0.04^{a}	0.22 ± 0.017^{b}	0.06 ± 0.006^{c}	–
8	Gallic acid	0.21 ± 0.01^{b}	0.14 ± 0.02^{b}	0.16 ± 0.02^{b}	0.13 ± 0.02^{b}	0.42 ± 0.05^{a}
9	p-Coumaric acid	0.01 ± 0.003^{c}	0.015 ± 0.002^{c}	0.08 ± 0.026^{b}	0.47 ± 0.03^{a}	–
10	Phloridzin	0.12 ± 0.02^{b}	–	1.03 ± 0.17^{a}	0.01 ± 0.0008^{b}	0.82 ± 0.04^{a}
11	Rutin	1.28 ± 0.05^{a}	0.54 ± 0.04^{b}	–	–	–
12	Trans cinnamic acid	1.16 ± 0.03^{a}	–	–	–	–
13	Vanillic acid	0.11 ± 0.01^{c}	0.11 ± 0.006^{c}	0.17 ± 0.02^{b}	–	0.53 ± 0.03^{a}

HPLC high performance liquid chromatography; *MPL* mother plant leaf; *MPS* mother plant stem; *IL* in vitro-derived plant leaf; *IS* in vitro-derived plant stem; *IVG* in vitro-growing cultures

Values followed by same letters within a row are not significantly different and separated by using Duncan's multiple range test (DMRT; $P < 0.05$). Concentrations of phenolic compounds are in mg/g of dry weight

– not detected

Conclusion

The current investigation emphasized on (i) the efficient regeneration system, which is necessary for germplasm maintenance and to expand production of elite germplasm for agriculture, present study suggest this protocol as an alternative method for micropropagation and germplasm conservation. Also, this can contribute to the large-scale production of *O. vulgare* for commercial cultivation. (ii) in medicinal plants, their contents of active ingredients and owned function are the basis of their activities, the determination of phytochemicals, polyphenols, antioxidant and antimutagenic activity of different plant parts and growing stages, suggested that the extent of these nutritional and anti-nutritional properties varied among growth stages, which can be utilized for the appropriate harnessing of the therapeutic potential of *O. vulgare*. Therefore, the successive yield loss during vegetative propagation and poor seed germination in nature can be addressed through micropropagation technique without compromising the culinary and therapeutic potential of *O. vulgare*. Finally, the present study can find its application in industrial use as an effective *O. vulgare* quality plant material development both in terms of mass propagation and secondary metabolite accumulation.

Acknowledgements Authors thank Director G. B. Pant National Institute of Himalayan Environment and Sustainable Development, for his encouragement and facilities. Authors also thank Head, Department of Biotechnology, Bhimtal Campus, Kumaun University Nainital for facilities and encouragement during the initial stage of experimentation. Colleagues of Biodiversity Conservation and Management theme are thanked for cooperation and help during the study. Anonymous reviewers are gratefully acknowledged for providing useful inputs to improve the manuscript draft.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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