

# *In vitro* plant regeneration of *Ocimum basilicum* L. is accelerated by zinc sulfate

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**Abstract** This article describes a fast, simple, and efficient plant regeneration protocol for *Ocimum basilicum* L. Two sets of experiments were performed. The first set was performed on Murashige and Skoog (MS) medium lacking zinc sulfate heptahydrate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) and containing varying concentrations of indole-3-acetic acid (IAA). The second set used three different concentrations (8.6, 12.9, and 17.2  $\text{mg L}^{-1}$ ) of  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  along with varying concentrations of IAA. In the first set, without zinc sulfate, an IAA concentration of 1.0  $\text{mg L}^{-1}$  (MS4) was found to be most effective, producing a mean of 12.6 roots per hypocotyl explant, while shoots were not produced. In the second set, with zinc sulfate, a combination of 12.9  $\text{mg L}^{-1}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  + 1.0  $\text{mg L}^{-1}$  IAA (MS11) produced significantly more shoots per explant (15 shoots) than a combination of 12.9  $\text{mg L}^{-1}$   $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  + 0.5  $\text{mg L}^{-1}$  IAA (MS10), which produced only six shoots. Later, the plantlets were successfully acclimatized (100%) and finally transferred to the greenhouse (*ex vitro*). In the *O. basilicum* plants grown using MS11 medium, total phenolic content and rosmarinic acid content were estimated from stem, shoot tip, and old leaf tissue of *in vitro* plantlets, *ex vitro* plantlets, and flowering plants. The highest amounts of total phenolic content (131.8  $\text{mg gallic acid equivalent g}^{-1}$  DW)

and rosmarinic acid (13.0  $\text{mg g}^{-1}$  DW) were obtained in the old leaf tissue at flowering time. This rapid regeneration protocol for *O. basilicum* L. represents a major improvement over the conventional protocols for plant regeneration and propagation of this species.

**Keywords** *Ocimum basilicum* · Shoot regeneration · Total phenolic content · Rosmarinic acid content

## Introduction

*Ocimum basilicum* L. (Lamiaceae) is an aromatic herb native to tropical Asia that is commonly used in Indian and Southeast Asian cuisine, especially in Thai stir-fries. *O. basilicum* is well-known as a source of essential oils and also as a spice ingredient, imparting flavor and delicacy to the prepared food. Many Indians consume small quantities of young basil leaves either as an offering after divine worship in temples or as a food additive (Archana and Namasivayam 2002). The domestic herb is much appreciated for its beauty and fragrance as an ornamental plant. The leaves of *O. basilicum* are rich in rosmarinic acid, a predominant phenolic acid detected in the methanolic extract of the leaves and stems (Hakkim *et al.* 2007), which have numerous dots of oil glands that secrete strongly scented volatile oils.

Regarding its medicinal properties, the plant is considered to be stomachic, anthelmintic, antipyretic, diaphoretic, expectorant, carminative, stimulant, and pectoral (Kirtikar and Basu 2003) as well as having antiseptic, antiallergic, and anticancer effects (Hakkim *et al.* 2007). It is also used to treat purulent discharge of the ear, bronchitis, hiccup, and diseases of the heart and brain (Siddique and Anis 2007). Several studies have established that the compounds present in basil oil have potent antioxidant, antiaging, anticancer, antiviral, and

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antimicrobial properties. In India, *O. basilicum* plants have been traditionally used for the supplementary treatment of stress, asthma, and diabetes (Dube *et al.* 1989).

The conventional method for the propagation of *O. basilicum* is via seeds. However, poor seed germination restricts its multiplication by this method. As a consequence of cross-pollination of the plant, the seedling progeny shows wide variation (Heywood 1993). The present article is the first report on the *in vitro* multiplication of *O. basilicum* through direct shoot regeneration techniques and thus offers an effective alternative method of propagation of this important multipurpose medicinal plant. Recent reports on plant tissue culture techniques have focused on facilitating plant propagation and production of *Ocimum* (Sahoo *et al.* 1997; Begum *et al.* 2002; Kintzios *et al.* 2003; Rady and Nazif 2005; Gopi and Pomurugan 2006; Siddique and Anis 2007; Kiferle *et al.* 2011), but none of these reports used a combination of  $ZnSO_4 \cdot 7H_2O$  plus indole-3-acetic acid (IAA) for direct plant regeneration.

IAA is the most common naturally occurring phytohormone, and it is predominantly produced in the cells of the apex (*i.e.*, bud) and very young leaves. IAA synthesis can take place through several independent biosynthetic pathways. The most common route starts from tryptophan, but there is also a biosynthetic pathway independent of tryptophan (Zhao 2010). IAA has many different effects, such as inducing cell elongation and cell division, which ultimately contribute to plant growth and development. On a larger scale, IAA serves as a signaling molecule necessary for the development of plant organs and coordination of plant growth.

Micronutrients are essential requirements for the balanced growth and development of plants (Graham *et al.* 2001). Among the metals, zinc is one of the most essential and is supplied in the form of zinc sulfate heptahydrate ( $ZnSO_4 \cdot 7H_2O$ ). Zinc regulates many physiological and metabolic processes in plants (Ramesh *et al.* 2004). It plays a vital role in cell division, cell expansion, protein synthesis, and metabolism of carbohydrates, nucleic acids, and lipids (Lepp 1981). The most distinct zinc deficiency symptoms, namely stunted growth and small leaf size, are presumably related to disturbances in the metabolism of auxins and in the generation of IAA. It has been established that tryptophan is the most likely precursor for the biosynthesis of IAA (Hossain *et al.* 1997; Saeki *et al.* 2000), and there are several reports indicating that zinc is required for the synthesis of tryptophan (Oguchi *et al.* 2004a, b). In addition, zinc is a constituent of a metalloenzyme or a cofactor for several enzymes such as anhydrases, dehydrogenases, oxidases, and peroxidases and plays an important role in regulating nitrogen metabolism, cell multiplication, photosynthesis, and auxin synthesis in plants (Shier 1994). Further, zinc finger transcription factors (ZF-TFs) are required for plant morphogenesis and organogenesis (Kobayashi *et al.* 1998; Yanagisawa 2004). As zinc is considered to be an

essential element for plant growth and development (Shier 1994; Welch and Shuman 1995), it must be present in low concentrations in all media used for *in vitro* plant regeneration. Optimization of zinc content has resulted in a very significant improvement in the regeneration of a range of monocots and dicots (Pande *et al.* 2000; Dahleen and Bregitzer 2002). Based on this literature, a systematic investigation of the effect of changing concentrations of zinc sulfate and IAA on the *in vitro* regeneration of *O. basilicum* was undertaken.

This report describes a simple and effective *in vitro* protocol for the direct regeneration of *O. basilicum* plantlets from hypocotyl segments, with an emphasis on total phenolic content and rosmarinic acid content of the plant.

## Materials and Methods

**Plant material, surface sterilization, and seed germination conditions** Commercially available seeds of *O. basilicum* L. were purchased from a local market in Istanbul, Turkey. The seeds were surface-disinfected with 100 mL of 20% commercial bleach (Domestos<sup>®</sup>, Unilever, Istanbul, Turkey) along with five drops of Tween 20 (Merck, Darmstadt, Germany) for 10 min by using a sonicator and then rinsed three times with sterile distilled water. An average of 20–25 seeds were aseptically cultured in 100-mm × 15-mm Petri dishes containing 30 mL of MS medium (Murashige and Skoog 1962; Duchefa Biochemie, Haarlem, The Netherlands) containing 3% (*w/v*) sucrose (Duchefa Biochemie, Haarlem, The Netherlands). The medium pH was adjusted to 5.8 using either 0.1 N HCl or 0.1 N KOH. The resultant medium was then solidified with 0.8% (*w/v*) agar (Duchefa Biochemie, Haarlem, The Netherlands) after autoclaving at 121°C and 1.06 kg cm<sup>-2</sup> pressure for 15 min. The seeds were kept in the dark at 23 ± 1°C for 2 d and then transferred to a 16-h photoperiod provided by a cool-white fluorescent light (Philips Master, Warsaw, Poland) with irradiance at 50 μmol photons m<sup>-2</sup> s<sup>-1</sup> at a relative humidity of 60%.

**Establishment of cultures** Hypocotyl segments (5–8 mm long) of *O. basilicum* excised from aseptically germinated 1-mo-old seedlings were used as explants for direct shoot regeneration. After 6 wk, plantlets with shoots and roots were transferred to capped bottles (Magenta™ B-cap, Sigma-Aldrich<sup>®</sup>, St. Louis) and observed for up to 8 wk. The explants were cultured on MS medium containing various concentrations of  $ZnSO_4 \cdot 7H_2O$  and/or IAA (Duchefa Biochemie, Haarlem, The Netherlands) (Table 1). The cultures were incubated in a growth chamber under the conditions described above. Each experiment was performed three times, each using 15 replicates (*i.e.*, a total of 45 explants per treatment). Both the frequency (%) of explants developing shoots with roots and

**Table 1** Media used for direct shoot and root induction from hypocotyl explants of *O. basilicum* L.

Treatment	Composition <sup>z</sup> Micronutrient (mg L <sup>-1</sup> ) and plant hormone (mg L <sup>-1</sup> )
MS0	MS
MS1	MS – ZnSO <sub>4</sub>
MS2	MS – ZnSO <sub>4</sub> + IAA (0.1)
MS3	MS – ZnSO <sub>4</sub> + IAA (0.5)
MS4	MS – ZnSO <sub>4</sub> + IAA (1.0)
MS5	MS + IAA (0.1)
MS6	MS + IAA (0.5)
MS7	MS + IAA (1.0)
MS8	MS + ZnSO <sub>4</sub> (12.9)
MS9	MS + ZnSO <sub>4</sub> (12.9) + IAA (0.1)
MS10	MS + ZnSO <sub>4</sub> (12.9) + IAA (0.5)
MS11	MS + ZnSO <sub>4</sub> (12.9) + IAA (1.0)
MS12	MS + ZnSO <sub>4</sub> (17.2)
MS13	MS + ZnSO <sub>4</sub> (17.2) + IAA (0.1)
MS14	MS + ZnSO <sub>4</sub> (17.2) + IAA (0.5)
MS15	MS + ZnSO <sub>4</sub> (17.2) + IAA (1.0)

IAA quantities are always added

<sup>z</sup> MS0 (control): MS as published and without any plant growth regulators.

MS1 – MS4: ZnSO<sub>4</sub>·7H<sub>2</sub>O (8.6 mg L<sup>-1</sup>) was completely eliminated from the MS medium

MS5 – MS7: MS as published (it contains 8.6 mg L<sup>-1</sup> of ZnSO<sub>4</sub>·7H<sub>2</sub>O)

MS8 – MS11: MS + added ZnSO<sub>4</sub>·7H<sub>2</sub>O (4.3 mg L<sup>-1</sup>); Net ZnSO<sub>4</sub>·7H<sub>2</sub>O in the medium = (8.6 + 4.3) = 12.9 mg L<sup>-1</sup>

MS12 – MS15: MS + added ZnSO<sub>4</sub>·7H<sub>2</sub>O (8.6 mg L<sup>-1</sup>); Net ZnSO<sub>4</sub>·7H<sub>2</sub>O in the medium = (8.6 + 8.6) = 17.2 mg L<sup>-1</sup>

the mean number of shoots with roots per explant were recorded after 8 wk of culture. After a period of 12 wk, the developed plantlets were placed in pots containing a mixture of soil/manure/moss/sand (1:2:2:1 [w/w/w/w]), incubated in the growth chamber for 15 d, and transferred to greenhouse (*ex vitro*) conditions. Samples were collected for the determination of total phenolic content and rosmarinic acid content from the stem, shoot tip, and old leaf tissue of *in vitro* and *ex vitro*, and plants at the flowering stage (after 8, 16, and 21 wk, respectively).

**Sampling and extraction** Dried samples (stem, shoot tip, or old leaf) were collected from the MS11 treatment and then powdered using a mortar and pestle and screened through a 380- $\mu$ m sieve. Fifty milligrams of the fine powder was extracted with 2 mL of methanol (Sigma-Aldrich<sup>®</sup>) and kept at room temperature (20  $\pm$  3°C) for 2 d in dark condition. The resulting mixture was centrifuged at 13147  $\times$  g (14,000 rpm) for 10 min. This extract was taken for total phenolic content determination.

For the purpose of rosmarinic acid content determination, 50 mg of the fine powdered was extracted twice with 2 mL of methanol in a 2.5-mL microcentrifuge tube using ultrasonication at room temperature (20  $\pm$  3°C) for 30 min. The resulting mixture was centrifuged at 13147  $\times$  g (14000 rpm) for 5 min. The supernatant was then transferred into a new 2.5-mL microcentrifuge tube. A 50- $\mu$ L sample of extract was transferred into a new microcentrifuge tube, brought to a volume of 1000  $\mu$ L with methanol, and mixed by vortexing for 1 min. The mixture was filtered through a 0.22- $\mu$ m Millex Millipore syringe filter (Merck, Istanbul, Turkey).

**Determination of total phenolic content** The total phenolic content in the extract was determined following a modified Folin–Ciocalteu method (Marigo and Boudet 1979). Briefly, a 20- $\mu$ L aliquot of the extract was placed in a 2-mL microcentrifuge tube containing 1.58 mL of water and 100  $\mu$ L of Folin–Ciocalteu reagent (AppliChem, Ankara, Turkey). The microcentrifuge tube was allowed to stand for 5 min, and then 300  $\mu$ L 20% Na<sub>2</sub>CO<sub>3</sub> (w/v) (Merck, Darmstadt, Germany) was added into the tube. After about 20 min at 40°C, the absorbance of the solution was measured using a spectrophotometer (Hitachi U-1900 UV–vis, Tokyo, Japan) at  $\lambda$  = 750 nm. The total phenolic content was expressed as (milligrams gallic acid equivalent)/(gram dry weight) or milligrams GAE per gram DW.

**Determination of rosmarinic acid using HPLC** Three extracts were prepared from each sample and analyzed using HPLC (Agilent 1100 series, Waldbronn, Germany). A 20- $\mu$ L aliquot of the filtrate (*vide supra*, “Sampling and extraction” section) was injected into the HPLC system for analysis of rosmarinic acid. The quantification of rosmarinic acid was done by comparing the sample to a pure standard (Sigma-Aldrich<sup>®</sup>, Steinheim, Germany). For the HPLC analysis, the selected wavelength was 320 nm for UV detection. Elution was carried out at a flow rate of 1.0 mL min<sup>-1</sup> at 20°C using a binary pump solvent system. An autosampler (Waters Autosampler 717 Plus) was used for injecting 20  $\mu$ L of the sample (the filtrate mentioned above) into an Inertsil ODS-3, 4.6-mm  $\times$  150-mm column (GL Sciences Inc., Tokyo, Japan). Two mobile phases, A and B, were used. Mobile phase A was 2% (v/v) aqueous acetic acid, while mobile phase B was 70% acetic acid:30% acetonitrile (7:3, [v/v]). Rosmarinic acid was eluted with gradients A and B as follows: 0–3 min: 30% B, 70% A; 3–9 min: 40% B, 60% A; 9–12 min: 50% A, 50% B, 12–24 min: 60% B, 40% A; and 24–35 min: 70% B, 30% A. All reagents used in the HPLC runs were obtained from Sigma-Aldrich<sup>®</sup>.

**Statistical analysis** The collected data were statistically analyzed using a computer program (SPSS Statistics, version

17.0, SPSS Inc., Chicago, IL). The experimental results were subjected to an analysis of variance (ANOVA) (Maxwell and Delaney 2004) and Duncan's multiple range test (Duncan 1955). The mean  $\pm$  SE (standard error) were subjected to Duncan's multiple range test at  $p < 0.05$ .

## Results and Discussion

The present article describes the first systematic study that aims to unravel the effects of zinc sulfate heptahydrate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ) and IAA on total phenolic content and rosmarinic acid accumulation in *in vitro* cultures of *Ocimum basilicum* L. For this purpose,  $\text{ZnSO}_4$ , IAA, and  $\text{ZnSO}_4$  + IAA combinations were tested for direct shoot induction.

### Induction of direct organogenesis and plant regeneration

One week after culture initiation, microshoot-like structures were clearly visible from the cut end of the hypocotyl explants cultured on MS medium containing various concentrations and combinations of  $\text{ZnSO}_4$  and IAA. During the following 2 wk, small microshoot-like structures developed on the cut surface of the hypocotyl segments. Well-developed shoots were observed within 4 wk of culture initiation. The absence of any callus formation indicated that the process of shoot development was direct, with the appearance of root formation within 4 wk (Fig. 1a). The presence of both  $\text{ZnSO}_4$  and IAA in the medium improved shoot and root formation simultaneously (Fig. 1b, c), and finally, whole plantlets were developed (Fig. 1d). These plantlets gradually formed many shoots after an additional 1-mo culture on the same media without any subculturing (Fig. 1e).

For those treatments that produced both shoots and roots, there were no appreciable differences in morphology of the plantlets induced by different concentrations of IAA in combination with  $\text{ZnSO}_4$  (Table 2). All shoots developed into normal plantlets when transferred to the same medium. Finally, plantlets were transferred to a mixture of soil, manure, moss, and sand (1:2:2:1 [w/w/w/w]), where they continued growing well and acclimating under the greenhouse conditions (Fig. 1f-h). Eventually, all the plantlets were established in the field, with 100% survival.

**Effect of  $\text{ZnSO}_4$  alone** Hypocotyl segments of *O. basilicum* excised from 1-mo-old seedlings were cultured on MS medium in the presence or absence of  $\text{ZnSO}_4$ . Following a 1-mo-old culture period on medium MS0 containing zinc sulfate but no IAA, hypocotyl explants became necrotic and no morphogenic change was observed. Variations of  $\text{ZnSO}_4$  concentration alone produced no effect on morphogenesis of hypocotyl explants (Table 2). Thus, it can be inferred that zinc sulfate in the absence of IAA is ineffective on the *in vitro* cultures of *O. basilicum*.

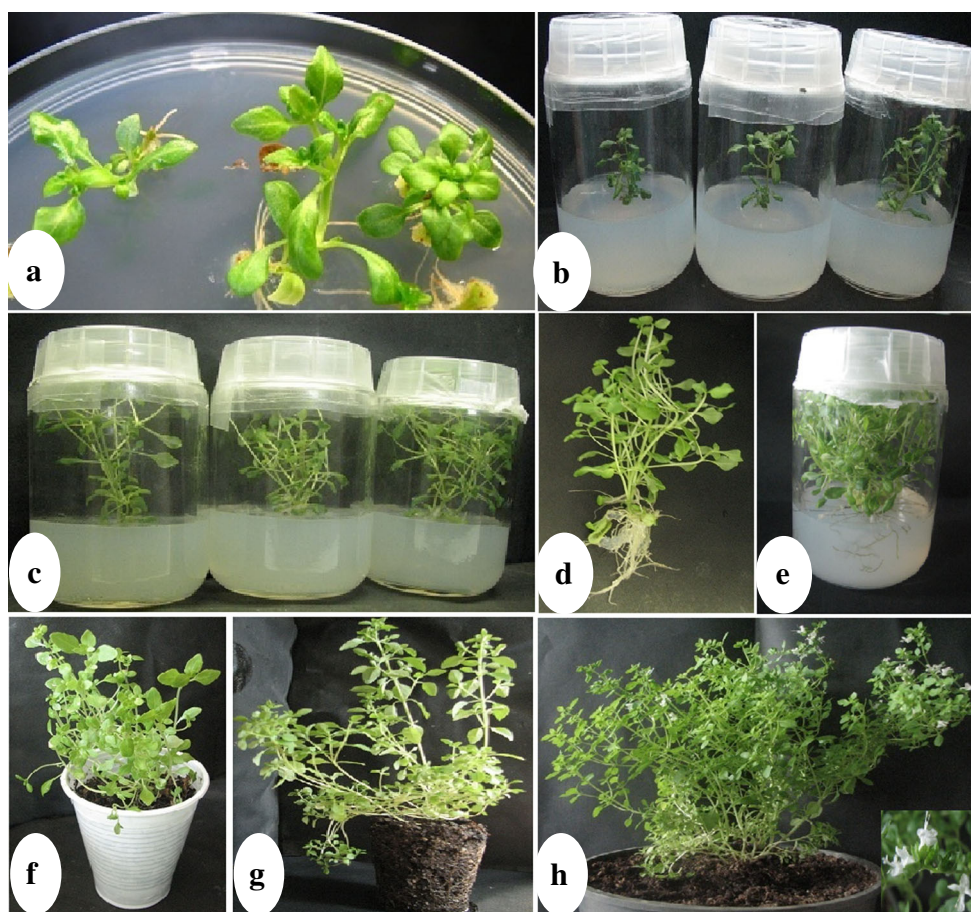
**Effect of IAA alone** The highest number of roots was obtained with an IAA concentration of  $1.0 \text{ mg L}^{-1}$  (using medium MS4, no  $\text{ZnSO}_4$ ), which produced a mean of 12.6 roots per hypocotyl explant and mean root length was 0.9 cm (Table 2). In comparison, MS2 (no  $\text{ZnSO}_4$ ) and MS3 (no  $\text{ZnSO}_4$  + IAA) produced longer roots (1.5 cm long) but mean number of roots was much less (Table 2).

**Effect of  $\text{ZnSO}_4$  plus IAA** MS media supplemented with  $\text{ZnSO}_4$  (net concentration  $12.9 \text{ mg L}^{-1}$ ) plus IAA (0.1, 0.5, and  $1.0 \text{ mg L}^{-1}$ ; in media MS9, MS10 and MS11, respectively) stimulated formation of shoots (20.7, 47.3, and 57.7%, respectively, for each IAA concentration) and roots (86.6, 71.0, and 100%, respectively, for each IAA concentration). However, when the net  $\text{ZnSO}_4$  concentration was increased to  $17.2 \text{ mg L}^{-1}$  and tested in combination with IAA (0.1, 0.5, and  $1.0 \text{ mg L}^{-1}$ ), only the  $0.1 \text{ mg L}^{-1}$  IAA treatment (MS13) induced shoots (38.5%) and roots (71.0%). The other two IAA concentrations (0.5 and  $1.0 \text{ mg L}^{-1}$ ) in combination with  $17.2 \text{ mg L}^{-1}$   $\text{ZnSO}_4$ , as well as all three IAA concentrations (0.1, 0.5, and  $1.0 \text{ mg L}^{-1}$ ) in combination with  $8.6 \text{ mg L}^{-1}$   $\text{ZnSO}_4$  (the  $\text{ZnSO}_4$  concentration in the regular MS medium), induced only roots. It is noteworthy that 15 shoots per hypocotyl explant were induced by the best treatment (MS11), which contained  $12.9 \text{ mg L}^{-1}$   $\text{ZnSO}_4$  plus  $1.0 \text{ mg L}^{-1}$  IAA (Table 2). The explants continued to simultaneously form shoots and roots on the same (MS11) medium in a Petri dish (Fig. 1a). This effect was more pronounced in screw-capped bottles, which are larger and have more headspace volume (Fig. 1b, c). For each monthly subculture, 15 shoots could be recovered; so up to the fourth subculture, 60 shoots could be collected from a single hypocotyl segment during a 4-mo period (Fig. 1e). These well-developed plantlets were later transplanted *ex vitro* inside the greenhouse.

*O. basilicum* has been shown to induce good shoot regeneration on MS medium supplemented with 6-benzylaminopurine (BAP) plus auxins (indole-3-butyric acid [IBA] or IAA), as reported by various authors (Sahoo *et al.* 1997; Phippen and Simon 2000; Begum *et al.* 2002). In the present study, the use of  $\text{ZnSO}_4$  ( $12.9 \text{ mg L}^{-1}$ ) plus IAA ( $1.0 \text{ mg L}^{-1}$ ) was sufficient to produce the maximum number of shoots (15 shoots) from the hypocotyl explants. Ekmekci and Aasim (2014) reported that hypocotyl explants of *O. basilicum* produced the maximum number of shoots per explant (5.17) on MS medium containing  $2 \text{ mg L}^{-1}$  thidiazuron (TDZ). Similarly, TDZ ( $4 \text{ mg L}^{-1}$ ) was also found to be the best cytokinin for maximum callus and shoot induction in basil (Phippen and Simon 2000). Nevertheless, the results presented in this article suggest that the presence of  $\text{ZnSO}_4$  plus auxin, without added cytokinin, was sufficient and efficient for shoot regeneration in *O. basilicum*. Based on this finding, it can be hypothesized that  $\text{ZnSO}_4$  is involved either directly or indirectly in regulating cytokinin and



**Figure 1** Direct shoot and plant regeneration from hypocotyl explants of *Ocimum basilicum* L. (a) Shoot and root induction on MS11 medium ( $12.90 \text{ mg L}^{-1} \text{ ZnSO}_4$  plus  $1.0 \text{ mg L}^{-1} \text{ IAA}$ ) after 6 wk. (b–d) Simultaneous development of shoots and roots, enabling production of whole plantlets (after 8 wk). (e) Plantlets gradually formed many shoots after further culture on the same medium without subculturing (after 11 wk). (f, g) Plantlets growing in the soil (after 16 wk). (h) Well-developed plant (flowering time; after 21 wk). *Inset* shows flowering.



subsequent shoot regeneration in *O. basilicum*. The results here confirm that the presence of  $\text{ZnSO}_4$  plus IAA in the culture medium is an essential requirement for shoot induction and/or plant regeneration in *O. basilicum*.

**Total phenolic content in *O. basilicum*** The total phenolic content in tissues varied widely: from 23 to  $71 \text{ mg GAE g}^{-1} \text{ DW}$  (*in vitro*), from 54 to  $127 \text{ mg GAE g}^{-1} \text{ DW}$  (*ex vitro*), and from 88 to  $132 \text{ mg GAE g}^{-1} \text{ DW}$  (flowering time) (Fig. 2a). Thus, the lowest total phenolic content ( $23 \text{ mg GAE g}^{-1} \text{ DW}$ ) was found in the *in vitro*-grown stem, while old leaf tissue collected during flowering time contained the highest phenolic content ( $132 \text{ mg GAE g}^{-1} \text{ DW}$ ). A similar observation was made in buckwheat (*Fagopyrum esculentum*), in which the identified phenolic content levels varied significantly among plant organs and vegetative growth stages (Syta *et al.* 2014). Moreover, variations in the concentration of phenolic contents in plants during their phenological cycle were also reported by Çirak *et al.* (2007). These authors found that among different tissues samples, the fully opened flower had higher phenolic content than stem, leaf, and other reproductive parts. Data published in the literature and the experimental findings here confirm that it would be appropriate to use tissues at flowering

time for screening of secondary metabolite levels in *O. basilicum*.

**Rosmarinic acid accumulation** The HPLC-measured rosmarinic acid content in *O. basilicum* ranged from  $0.5$  to  $13.0 \text{ mg g}^{-1} \text{ DW}$ . Rosmarinic acid levels in old leaf tissue collected during flowering time were remarkably higher than other tissues (Fig. 2b). These observations are in agreement with those of De-Eknamkul and Ellis (1985), who reported that the yield of both biomass and rosmarinic acid responded synchronously to changes in macronutrient and phytohormone concentrations in *Anchusa officinalis*. Moreover, different auxins produced quantitatively different responses. The auxin 2,4-dichlorophenoxyacetic acid (2,4-D) was found to delay the initiation and reduce the subsequent rate of rosmarinic acid synthesis in *Anchusa officinalis* (De-Eknamkul and Ellis 1985), whereas the same auxin had the opposite effect in *Coleus blumei* cultures (De-Eknamkul and Ellis 1988). Also, it was reported that  $\alpha$ -naphthaleneacetic (NAA) yielded the highest rosmarinic acid accumulation in cultures of *Anchusa officinalis* (De-Eknamkul and Ellis 1988) and *Solenostemon scutellarioides* (Sahu *et al.* 2013). Thus, it may be concluded that the effect of auxins on rosmarinic acid accumulation is species dependent.

**Table 2** Effect of IAA alone, ZnSO<sub>4</sub> alone and (ZnSO<sub>4</sub> + IAA) combination in MS medium for shoot and root induction from hypocotyl explants of *Ocimum basilicum* L.

Treatment <sup>z</sup>	Frequency (%) of explants producing shoots <sup>y</sup>	Mean number of shoots per hypocotyl segment <sup>x</sup>	Frequency (%) of explants producing roots <sup>y</sup>	Mean number of roots per hypocotyl segment <sup>x</sup>	Mean root length (cm) <sup>x</sup>
MS0	0 <sup>c</sup>	0 <sup>c</sup>	0 <sup>d</sup>	0 <sup>g</sup>	0 <sup>c</sup>
MS1	0 <sup>c</sup>	0 <sup>c</sup>	71.1 <sup>c</sup>	1.3 ± 0.2 <sup>gf</sup>	2.4 ± 0.3 <sup>a</sup>
MS2	0 <sup>c</sup>	0 <sup>c</sup>	85.9 <sup>b</sup>	1.7 ± 0.3 <sup>gf</sup>	1.5 ± 0.3 <sup>abc</sup>
MS3	0 <sup>c</sup>	0 <sup>c</sup>	100 <sup>a</sup>	3.0 ± 0.5 <sup>dgfe</sup>	1.5 ± 0.2 <sup>abc</sup>
MS4	0 <sup>c</sup>	0 <sup>c</sup>	100 <sup>a</sup>	12.6 ± 1.6 <sup>a</sup>	0.9 ± 0.4 <sup>b</sup>
MS5	0 <sup>c</sup>	0 <sup>c</sup>	71.8 <sup>c</sup>	2.2 ± 0.3 <sup>gfe</sup>	1.0 <sup>abc</sup>
MS6	0 <sup>c</sup>	0 <sup>c</sup>	100 <sup>a</sup>	6.0 ± 0.4 <sup>dc</sup>	1.5 <sup>abc</sup>
MS7	0 <sup>c</sup>	0 <sup>c</sup>	100 <sup>a</sup>	10.3 ± 1.5 <sup>ab</sup>	2.0 <sup>ab</sup>
MS8	0 <sup>c</sup>	0 <sup>c</sup>	0 <sup>d</sup>	0.0 ± 0.0 <sup>g</sup>	0 <sup>c</sup>
MS9	20.7 <sup>d</sup>	3.0 ± 0.5 <sup>bc</sup>	86.6 <sup>b</sup>	4.4 ± 0.4 <sup>dfe</sup>	0.7 ± 0.2 <sup>bc</sup>
MS10	47.3 <sup>b</sup>	6.0 ± 0.5 <sup>b</sup>	71.0 <sup>c</sup>	8.7 ± 0.3 <sup>bc</sup>	1.0 ± 0.3 <sup>abc</sup>
MS11	57.7 <sup>a</sup>	15.0 ± 1.6 <sup>a</sup>	100 <sup>a</sup>	3.0 ± 0.5 <sup>dgfe</sup>	1.2 ± 0.06 <sup>abc</sup>
MS12	0	0 <sup>c</sup>	0 <sup>d</sup>	0.0 ± 0.0 <sup>g</sup>	0 <sup>c</sup>
MS13	38.5 <sup>c</sup>	2.0 ± 0.4 <sup>c</sup>	71.0 <sup>c</sup>	4.3 ± 0.6 <sup>dfe</sup>	1.3 ± 0.1 <sup>abc</sup>
MS14	0 <sup>c</sup>	0 <sup>c</sup>	100 <sup>a</sup>	5.4 ± 0.3 <sup>dce</sup>	1.1 ± 0.1 <sup>abc</sup>
MS15	0 <sup>c</sup>	0 <sup>c</sup>	100 <sup>a</sup>	3.0 ± 0.5 <sup>dgfe</sup>	0.8 ± 0.1 <sup>bc</sup>

A total of 45 explants were maintained in each treatment, and data were recorded up to 8 wk

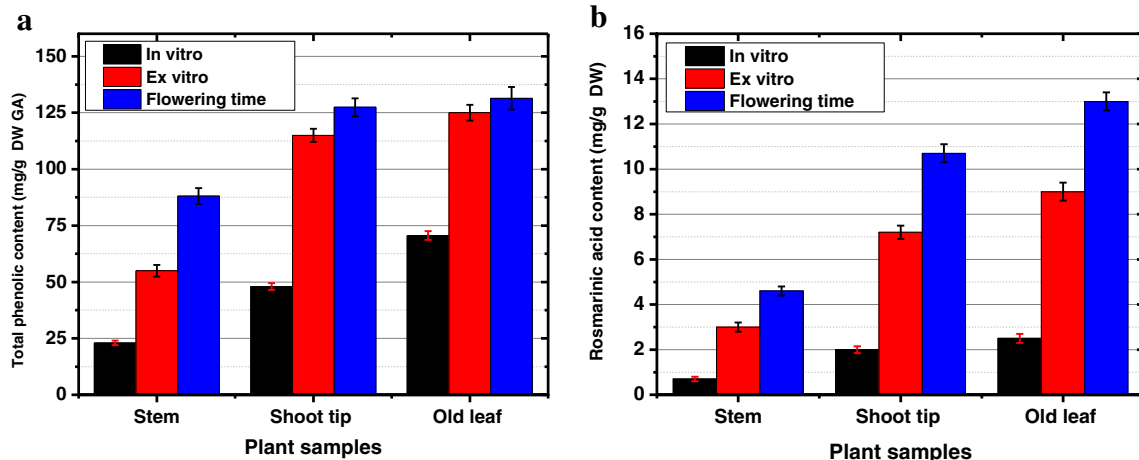
<sup>z</sup> As in Table 1

<sup>y</sup> Mean frequencies of explants with the same letter within the same column are not significantly different according to Duncan's multiple range test at *p* < 0.05

<sup>x</sup> Means ± SE (standard error) with the same letter within the same column are not significantly different according to Duncan's multiple range test at *p* < 0.05

The present study shows that the accumulation of rosmarinic acid is fivefold higher in old leaf tissue at flowering time than in old leaf tissue from *in vitro*-grown samples (Fig. 2b). The results reported here are in accordance with those of Kintzios *et al.* (2003), who reported that rosmarinic

acid increased at the flowering stage in leaves of acclimatized plants, with levels reaching up to 20 mg g<sup>-1</sup> DW. A similar observation was also reported in *O. basilicum* (Kiferle *et al.* 2011). According to Juliani *et al.* (2008), the concentration of rosmarinic acid in basil leaves increased during flowering



**Figure 2** Total phenolic content (a) and total rosmarinic acid content (b) in stem, shoot tip, and old leaf of *O. basilicum* L. grown on MS medium with a final concentration of 12.90 mg L<sup>-1</sup> ZnSO<sub>4</sub> plus 1.0 mg L<sup>-1</sup> IAA

(medium MS11). Material was sampled *in vitro* (8 wk), *ex vitro* (16 wk), and at flowering time (21 wk).

relative to that at the vegetative stage. The present study revealed that the manipulation of the culture medium composition was a feasible option for improving plantlet production of *O. basilicum*, providing a source for isolation of rosmarinic acid.

## Conclusions

The major problem with the use of Lamiaceae species for pharmaceutical purposes is the wide plant-to-plant variability due to genetic and biochemical heterogeneity. This article describes a protocol for rapid plant regeneration through direct shoot regeneration in *O. basilicum*, which was developed by testing variable concentrations of  $ZnSO_4 \cdot 7H_2O$  and IAA on MS medium. This method can be used for the production of elite/quality plant materials at a much faster rate than the conventional indirect regeneration protocols of the species. Plant propagation *via* direct organogenesis is a valuable method for the clonal propagation and *ex vitro* conservation of *O. basilicum* genetic resources. The present investigation elucidates that cultures of *O. basilicum* can serve as a potential source of secondary metabolites under suitable conditions.

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