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Original Article

The effect of BA and IBA on the secondary metabolite production by shoot culture of *Thymus vulgaris* L.

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***Abstract*:**
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In vitro shoots of the common thyme (*Thymus vulgaris* L.) were established, and the effects of different concentrations of the cytokinin BA and the auxin IBA on secondary metabolite production were investigated. The highest number of shoots was obtained through cultivation on the MS medium containing 2 mg/L BA and 0.1 mg/L IBA and 4 mg/L BA and 0.1 mg/L IBA (14.3 and 13.3 respectively). Quantitative changes in chlorophyll *a*, chlorophyll *b* and carotenoid content were recorded in response to the effect of varying concentrations of growth regulators in the medium. Furthermore, after addition of plant growth regulators (0.5 mg/L BA + 0.1 mg/L IBA) to the medium, elicitation of phenolic compounds was recorded in plantlets. The concentration range of BA from 2 to 4 mg/L improved the production of flavonoids (0.61 and 0.64 mg/g FW). In contrast, plantlets cultivated on the same treatment, showed a decrease in monomeric anthocyanins .

Key words: common thyme, *in vitro*, micropropagation, secondary metabolite production

Introduction

The common thyme *or Thymus vulgaris* L., a member of the Lamiaceae family, is an aromatic herb growing up to 30 cm in height and flowering from July to September (Gruenwald et al., 2004). It is native to southern Europe, where it is often cultivated as a culinary herb. Thyme is used as spice and medicinal herb almost everywhere in the world (Morales, 2002). Many pharmacological *in vitro* experiments carried out during the last decade revealed well defined pharmacological properties of both, the thyme essential oil and the plant extracts. The non-medicinal use of thyme is worthy of attention, because thyme is used in the food and aroma industries and it is widely used as a culinary ingredient and it serves as a preservative for foods especially due to its antioxidant effect (Zarzuelo & Crespo, 2002).

The active substances in the thyme essential oil are thymol, carvacrol, p-cymene, β - pinene, γ-terpinene, β-caryophyllene, 1-borneol, 1, 8-cineole, etc (Juven et al., 1994). Recent studies showed that thyme essential oils have strong antibacterial, antifungal, and antioxidative properties (Cruz et al., 1989; Piccaglia et al., 1993; Consentino et al., 1999; Karaman et al., 2001; Vardar et al., 2003, Prabuseenivasan et al., 2006; Budka & Khan, 2010). Essential oils of different species of genus *Thymus* inhibit a broad spectrum of bacteria, with Gram-positive bacteria generally being more susceptible than Gram-negative bacteria (Blakeway, 1986; Farag et al., 1986; Deans & Ritchie, 1987; Knobloch et al., 1988). Recently, the antibacterial property of thyme oil against some important food-borne pathogens, like *Salmonella enteritidis, Escherichia coli, Staphylococcus aureus, Listeria monocytogenes,* and

Campylobacter jejuni, has been tested (Smith-Palmer et al., 1998).

Several *in vitro* and *in vivo* screenings have shown that volatile oils may be used against fungal diseases, especially those from plants of the genus *Thymus* (Blakeway, 1986; Farag et al., 1986; Deans & Ritchie, 1987). A few studies demonstrate the antiviral effects of thyme extracts (Herrmann & Kucera, 1967).

Micropropagation techniques play an important role in conservation and genetic improvement of pharmaceutical and medicinal plants. Today, there is a tendency to develop *in vitro* systems of production as an alternative to field production of plants used as a source of active compounds. Such system is not limited by sources of nutrients or climate changes and can thus be better controlled. For effective induction of secondary metabolite production, plant growth regulators (PGRs) are required (Zhong et al., 1996; Al-Sane' et al., 2005; Shilpashree & Ravishankar, 2009), since they have significant effects on the metabolism of secondary metabolites. The quality, as well as the quantity, of plant growth regulators, plays a major role in the production capability of a given *in vitro* culture. PGRs affect cell multiplication and division, which increases production of secondary metabolites (Kurz & Constabel, 1979; Staba, 1980). The effects of different PGRs on secondary metabolite production have previously been studied and confirmed in vrious plants (Furuya et al., 1971; Ikuta et al., 1975; Ikeda et al., 1976; Ikuta & Itokawa, 1982; Nair et al., 1992; Weathers et al., 2005; Khan et al., 2008).

The aim of this study was to develop an efficient protocol for mass multiplication of *T. vulgaris*, through manipulation of different concentrations of plant growth regulators, and to examine the relationship between PGRs and the production and accumulation of antioxidants, polyphenols and chlorophyll.

Materials and methods

Plant Material and Treatments - Commercially purchased seeds of *T. vulgaris* L. (Sjemenarna Ljubljana d.d., Slovenija) were surface-sterilised with 20% commercial bleach for 15 min followed by 3x5 min rinsing in sterile distilled water and were then germinated on a MS basal medium (Murashige & Skoog, 1962).

Shoot multiplication was investigated on the MS basal medium (BH; control) or on media supplemented with 0.5, 2, 4 mg/L BA and 0.1 mg/L IBA (BI1; BI2 and BI4).

The pH of all media was adjusted to 5.8 with 1 M KOH or 1 M HCl before autoclaving at 121°C for 20 minutes. All cultures were incubated at 24±1°C under cool white fluorescent lights (3000 lux) and with the 16 h light photoperiod. The aerial parts of *T. vulgaris* were collected for analysis.

Photosynthetic pigments – Extraction was undertaken in accordance to Porra et al. (1989). Quantification was done by spectrophotometric determination of absorbance on 663 nm (chlorophyll *a*), 646 nm (chlorophyll *b*) and 440 nm (carotenoids). The concentrations of photosynthetic pigments were expressed as mg of pigments per g of fresh weight (mg/g FW).

Secondary Metabolite Production – Samples used for the analysis of concentrations of phenols, flavonoids and anthocyanins were frozen in liquid nitrogen and stored at -20°C. Extraction of phenols and flavonoids was performed in 100% methanol. Total phenol content was determined according to Wolfe et al. (2003), with Folin-Ciocalteu reagent through spectrophotometric readings of absorbance at 765 nm. Total phenol content was expressed as mg of catechin per g of FW. Total flavonoid content was analysed according to Ordon et al. (2006) by absorbance readings at 415 nm. Total flavonoid content was expressed as mg of ruthin per g of FW. Monomeric anthocyanins were determined according to Mancinelli (1984). Extraction was performed in acidified methanol (1% HCL in methanol), and the concentration was expressed as mg of cyanidin 3-glucoside per L of extract.

Observations and Statistical Analysis - Each treatment had 5 replicates containing 10 explants and all experiments were repeated three times. All analyses were done after 30 days of cultivation with no subcultivation. The data was analysed using SPSS 15.0 by employing parametric (Pearson correlation, one tailed) and non-parametric (Kruskal-Walis analysis) tests.

Results and discussion

Shoot Multiplication – The induction of shoot formation started after a week of culture cultivation with initiation of shoot meristemswhich was followed by growth of shoot buds. No multiplication was recorded on the MS medium without plant growth regulators (control) (**Fig. 1a**), while a high rate of root induction was observed on the same medium. Shoot apices grown in the presence of 0.5 mg/L BA and 0.1 mg/L IBA, produced on average 8.2 axillary buds at the base of the first pair of leaves (**Tab. 1**, **Fig. 1b**). Treatments with higher concentrations of BA (2 mg/L) increased the number of shoots, but shoots were much shorter and

Table 1. The induction of adventive shoots formation in *Thymus vulgaris* L.

Treatment	Number of shoots per explant	Rate of regeneration (%)	Induction of root formation (%)
BH	2.9 (±0,71)	100	81.25
BI1	8.2 (±0,56)*	100	0
BI2	14.3 (±0,82)*	100	0
BI4	13.3 (±0,84)*	100	0

The results represent mean values of three replications (±standard deviation) after 30 days on the MS basal medium with no plant growth regulators and with addition of different concentrations of BA and IBA.
*statistically important differences at 0.01 level with respect to control.
BH 0 m g/L BA + 0 mg/L IBA; **BI1** 0.5 mg/L BA + 0.1 mg/L IBA; **BI2** 2 mg/L BA + 0.1 mg/L IBA; **BI4** 4 mg/L BA + 0.1 mg/L IBA;

Table 2. The effect of BA and IBA on photosynthetic pigments in shoot cultures of *T. vulgaris*

Treatment	Chlorophyll *a* (mg/g)	Chlorophyll *b* (mg/g)	Total Chlorophylls (mg/g)	Carotenoids (mg/g)
BH	0.29 (±0.02)	0.16 (±0.01)	0.45 (±0.02)	0.51 (±0.03)
BI1	0.27*(±0.01)	0.40* (±0.02)	1.25* (±0.03)	0.20* (±0.03)
BI2	0.24*(±0.24)	0.39* (±0.01)	1.14* (±0.01)	0.22* (±0.01)
BI4	0.10*(±0.10)	0.17* (±0.02)	0.48* (±0.01)	0.65* (±0.02)

The results represent mean values of three replications (±standard deviation) after 30 days on the MS basal medium with no plant growth regulators and with addition of different concentrations of BA and IBA.
*statistically important differences at 0.01 level with respect to control.
BH 0 m g/L BA + 0 mg/L IBA; **BI1** 0.5 mg/L BA + 0.1 mg/L IBA; **BI2** 2 mg/L BA + 0.1 mg/L IBA; **BI4** 4 mg/L BA + 0.1 mg/L IBA;

Table 3. The Effect of BA and IBA on secondary metabolite production in shoot cultures of *T. vulgaris*

Treatment	Phenols (mg/g)	Flavonoidis (mg/g)	Monomeric anthocynins (mg/L)
BH	2.09 (±0.14)	0.50 (±0.01)	6.62 (±0.32)
BI1	3.05* (±0.11)	0.55 (±0.02)	3.38*(±0.05)
BI2	1.70* (±0.01)	0.61*(±0.01)	2.95*(±0.01)
BI4	2.32 (±0.04)	0.64*(±0.01)	2.13* (±0.04)

The results represent mean values of three replications (±standard deviation) after 30 days on the MS basal medium with no plant growth regulators and with addition of different concentrations of BA and IBA.
*statistically important differences at 0.01 level with respect to control.
BH 0 m g/L BA + 0 mg/L IBA; **BI1** 0.5 mg/L BA + 0.1 mg/L IBA; **BI2** 2 mg/L BA + 0.1 mg/L IBA; **BI4** 4 mg/L BA + 0.1 mg/L IBA;

had developed necrosis (**Fig. 1c**). On the medium with 4 mg/L BA and 0.1 mg/L IBA, an average of 13.3 shoots was obtained directly from the apical or the axillary buds (**Tab. 1**, **Fig. 1d**). Similar results were observed in previous studies on the common thyme (Beheshti & Morteza, 2005). A high capability of explant multiplication was observed on the medium with the highest cytokinin/auxin ratio (2 and 4 / 0.1 mg/L) and leads us to consider these combinations as efficient promoters of *T. vulgaris* multiplication.

Photosynthetic pigments – The content of the total chlorophylls ranged from 0.45 mg/g FW for control to 1.25 mg/g FW for BI1 treatment (**Tab. 2**). The increase in the total chlorophyll content was a result of a significant increase in chlorophyll *b* content, while chlorophyll *a* content decreased significantly when compared to the control. According to our results an increase in the cytokinin concentration in the medium (BI1 and BI2) significantly increases the amount of chlorophyll *b* produced, except for BI4 medium. On the other hand it shows reverse correlation with the chlorophyll *a* content. It was not possible to establish a direct link between concentrations of PGRs used and the content of photosynthetic pigments in all cases (Stetler & Laetsch, 1965; Edelman & Hanson, 1971). Cytokinins as well as auxins can improve concentration of photosynthetic pigments (Verma & Sen, 2008; Parsaeimehr et al., 2010; Vamil et al., 2010). Furthermore, it was shown that the

biosynthesis pathway of chlorophyll is highly insensitive to light and cytokinins (Yaronskaya et al., 2006). Changes in the content of chlorophylls, caused by PGRs, could, most likely, be related to growth rate and primary and secondary metabolic activities (Lichtenthaler, 1987).

Secondary Metabolite Production – The total phenol content was significantly higher on BI1 treatment, while total flavonoid content was higher on all treatments when compared with the control (**Tab. 3**). It was previously reported that the maturation of cells always corresponds to an increase in secondary metabolites (Shetty & Labbe, 1998; Biondi et al., 2002; Parsaeimehr et al., 2010). On the other hand, the quality and the quantity of applied plant growth regulators have a significant effect on the metabolism of secondary metabolites (Khan et al., 2008). In plant cell cultures the production of secondary metabolites supports cell multiplication and division, hence plant growth regulators are bound to have a major role in determining the production of potentiality of a given culture. During our research presence of higher concentrations of BA promoted shoot proliferation and flavonoid production. Accumulation of flavonoids is usually correlated to morphogenesis during plant development (Pasqua et al., 2003). However, no such positive influence of higher BA concentrations was observed for phenol content. Only the BA concentration that tested the lowest (0.5 mg/L) induced phenol accumulation.

The concentration of monomeric anthocyanins was significantly lower on all treatments in comparison to the control (**Tab. 3**). Anthocyanins exhibited positive correlation for chlorophyll *a* and carotenoids, but a negative correlation for flavonoids, with the significance at the 0.01 level.

The reverse relation between the *in vitro* shoot proliferation and the production of anthocyanins production, which was found in this study, is a general complication found in *in vitro* secondary metabolites production. Several studies dealing with this problem observed that 2.4-D or NAA increase anthocyanin production and decrease growth rate. On the other hand some of the studies observed that BA or KIN enhance production of anthocyanins, while inhibiting cell growth (Meyer & Van Staden, 1995).

The difficulties expressed through the reverse relationship between the production of anthocyanins and cell growth can cause problems in all secondary products induction media and additional research is needed to establish specific requirements of each plant species.

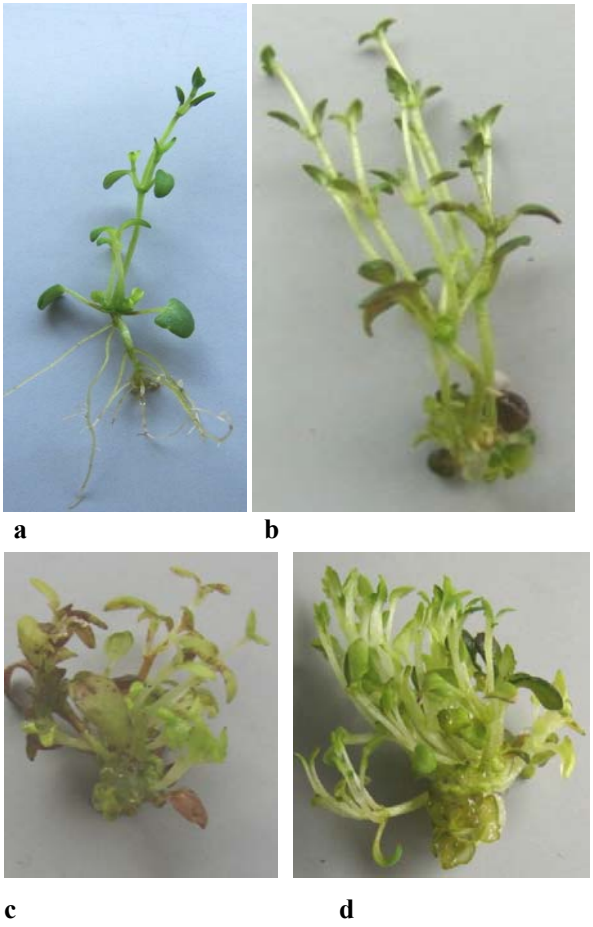

Figure 1. Induction of adventive shoots formation on MS medium with BA and IBA

a – BH 0 m g/L BA + 0 mg/L IBA; **b – BI1** 0.5 mg/L BA + 0.1 mg/L IBA; **c – BI2** 2 mg/L BA + 0.1 mg/L IBA; **d – BI4** 4 mg/L BA + 0.1 mg/L IBA;

Conclusion

Our research shows that the shoot multiplication of common thyme is depended upon the treatment that is used. The highest number of adventive shoots was obtained on the MS media supplemented with 2 or 4 mg/L BA and 0.1 mg/L IBA, which employed the highest concentration of cytokinins. An increase in the concentration of cytokinins above 2 mg/L was followed by a decrease in photosynthetic pigments content, and total monomeric anthocyanins content. Cytokinins (0.5 mg/L) in combination with low auxin levels (0.1 mg/L) can enhance production of phenolic compounds, and higher concentrations of cytokinins can induce an increase in total

flavonoid content. This study successfully established the optimal conditions for the *in vitro* growth of common thyme, with a potential for secondary metabolite production. Further investigations are necessary in order to increase the production of secondary metabolites by *in vitro* cultures.

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