Bulgarian Journal of Agricultural Science, 20 (Supplement 1) 2014, 46–50
Agricultural Academy

EFFECT OF PLANT GROWTH REGULATORS ON GROWTH PATTERNS AND ENZYMATIC ANTIOXIDANT ACTIVITIES IN *HYPERICUM CALYCINUM* SHOOT CULTURES

G. TRENEVA[1], Y. MARKOVSKA[1], E. WOLFRAM[2] and K. DANOVA[3*]
[1] *Sofia University "St. Kliment Ohridski", Faculty of Biology, BG – 1164 Sofia, Bulgaria*
[2] *Zürcher Hochschule für angewandte Wissenschaften (ZHAW), Life Sciences und Facility Management, Institut für Biotechnologie, Wädensvil, Switzerland*
[3] *Bulgarian Academy of Sciences, Institute of Organic Chemistry with Centre of Phytochemistry, BG – 1113 Sofia, Bulgara*

Abstract

TRENEVA, G., Y. MARKOVSKA, E. WOLFRAM and K. DANOVA, 2014. Effect of plant growth regulators on growth patterns and enzymatic antioxidant activities in *Hypericum calycinum* shoot cultures. *Bulg. J. Agric. Sci.,* Supplement 1: 46–50

Hypericum calycinum L. (section Ascyreia) is a species of the *Hypericum* genus, relative to the medicinal plant *H. perforatum* (St. Jonn's worth). Unlike the widely studied *H. perforatum,* it is characterized by the lack of hypericins production. However, research has revealed its commensurable antidepressant activity, as compared with the latter species, and shown the presence of polyphenolics with marked radical scavenging activity. In order to develop an *in vitro* system with the potential of a novel source of bioactive constituent's characteristic for the *Hypericum* genus, we initiated *in vitro* cultures of *H. calycinum*. In the present work we studiy the effect of exogenous BA and IBA treatments on the developmental patterns, enzymatic and non-enzymatic antioxidant defence of hypericin non-producing *Hypericum calycinum* shoot cultures. It was shown that supplementation of PGR led to stimulation of phenylalanine ammonia-lyase and superoxide dismuthase, as well as non-enzymatic antioxidant glutathione. However, they inhibited glutathione reductase, as well as lowered the levels of flavonoids, ascorbate and dehydroascorbate in comparison with plant growth regulators-free control. Further on, it was established that elevation of IBA concentration slightly stimulated axillary shoot formation and shoot length, but inhibited polyphenolic levels *in vitro.* These results are in agreement with our previous results of interrelations between biomass formation and polyphenolics production in other *Hypericum* species *in vitro.* An *in vitro* culture system optimization is in progress in order to increase biomass production and retain biosynthetic capacity of the species.

Key words: Hypericum calycinum, shoot culture, enzymatic antioxidant defense, polyphenolics *in vitro*
Abbreviations: BA – benzyl adenine, IBA – indole-3-butyric acid, PGR – plant growth regulators, DPPH - 2,2-diphenyl-1-picrylhydrazyl, NO – nitric oxide, BAS – Bulgarian Academy of Sciences, PAL – Phenylalanine ammonia-lyase, SOD – superoxide dismuthase, GR – glutathione reductase, APX – ascorbate peroxidase, CAT – catalase, AsA – ascorbate, DHA – dehydrosacorbate, GSSG – oxidized glutathione, GSH – reduced glutathione, NADPH – Reduced nicotinamide adenine dinucleotide phosphate, KH_2PO_4 – potassium phosphate monobasic, $MgCl_2$ – magnesium chloride, EDTA – Ethylenediaminetetraacetic acid, NaAA – sodium ascorbate, H_2O_2 – hydrogen peroxide, DTNB – 5,50-dithiobis – (2-nitrobenzoic acid).

**E-mail: k_danova@abv.bg*

Introduction

St. Jonhn's Wort (*Hypericum perforatum*) is a medicinal plant species of high economic value and used in phytopharmaceutical preparations worldwide (Bäcker et al., 2006; Azizi et al., 2011). The wide array of its pharmacological activities is due to the complexity of the *Herba Hyperici* extract comprising different classes of compounds as phenolics, flavonoids, terpenoids, phloroglucinols, as well as naphthodianthrones hypericin and pseudohypericin (Butterweck and Schmidt, 2007). *H. calycinum* (belonging to the more primitive Ascyrea section) is a hypericin non-producing species. Phytochemical work has revealed the presence of leucocyanidin, quercetin, hyperoside, rutin and luteolin in this species (Kitanov and Blinova, 1987). A phloroglucinol derivative with certain fungicide and antimalarial activities has been reported as well (Decosterd et al., 1991). Interestingly, a comparative study between *H. perforatum* and *H. calycinum* has proven the commensurable antidepressant activity of these two species (Öztürk et al*.,* 1996). Newer report has established the presence of cafeic acid derivatives, as well as several flavonoid and flavanol compounds (Kirmizibekmez et al., 2009). These compounds showed strong DPPH and moderate NO scavenging activities in a concentration dependent manner.

A wide array of research has been conducted on the investigation of pharmacologically relevant secondary metabolites production by different *Hypericum* species in the controlled environment of plant cell tissue and organ culture (Kartning et al., 1996; Kirakosyan et al., 2004; Cui et al., 2010; Savio et al., 2012). In spite of the piling number of works on this topic, only scarce attention has been given to representatives of sections of the genus, other than *Hypericum* or *Taeniocarpium* (Danova, 2014). Research has shown that species of the *Hypericum* genus are susceptible to the effect of exogenously applied plant growth regulators in terms of morphogenic response and production of secondary metabolites *in vitro* (Danova et al., 2010a, 2010b and references cited within). In addition, our previous research on *H. calycinum* has proven its high biosynthetic capacity in respect to polyphenolics production *in vitro* (Danova et al., 2012a). This motivated the present work to develop an *in vitro* system for the production of secondary metabolites of the species as a novel source of secondary metabolites characteristic for the genus. Therefore the effect of BA and IBA on biosynthetic capacity and physiological status of *in vitro* cultured *H. calycinum* were studied.

Materials and Methods

Plant material

H. calycinum L was collected at the site of ornamental plants arrangements on the territory of the Seaside Garden – Varna, the plant was identified by Prof Dimitar Dimitrov, National Museum of Natural History, BAS, voucher specimen was deposited at Herbarium of Institute of Biodiversity and Ecosystem Reseach, BAS, SOM 165 685. Shoot cultures were established from surface sterilized stem segments as previously described (Danova, 2010). For the purpose of the present experiment PGR-free control plants were maintained in the basic Murashige and Skoog basic medium (Hri_0). For the study of PGR effects the following media were used 0.2 $mg.L^{-1}$ BA + 0.1 $mg.L^{-1}$ IBA (Hri_1), 0.2 $mg.L^{-1}$ BA + 0.5 $mg.L^{-1}$ IBA (Hri_2), 0.5 $mg.L^{-1}$ BA + 0.1 $mg.L^{-1}$ IBA (Hri_3) and 0.5 $mg.L^{-1}$ BA + 0.5 $mg.L^{-1}$ IBA (Hri_4). All media were supplemented with 3% sucrose, 0.65% agar, pH was set at 5.8, media autoclaved at 121ºC for 20 min and grown at 25ºC at 16 h photoperiod for 4 months.

Determination of enzymatic activities

Enzyme extraction was performed after Yuan et al. (2002) as follows: 0.2 g FW of the whole shoots were ground with 0.05 g polyvinylpyrrolidone into fine powder with liquid nitrogen in 4 ml of 100 mM potassium phosphate buffer pH 7.2 containing 2 mM EDTA and 8 mM mercaptoethanol. After centrifugation at 15 000 rpm for 25 min at 4ºC aliquots of the supernatant were immediately used for the below described assays.

Phenylalanine ammonia lyase (PAL EC 4.3.1.24, Yuan et al., 2002) 0.02 M phenylalanine is added to the obtained protein extracts, then borate buffer (pH 8.8) and water are added and after incubation at 30ºC, absorption is measured at 290 nm.

Glutathione reductase (GR EC 1.8.1.7, Sherwin and Farrant 1998) was measured at 340 nm after incubation of the supernatant with buffered reaction mixture of the protein extract with 300 mM KH_2PO_4 (pH 7.5), 3 mM $MgCl_2$, 0.1 mM EDTA, 10 mM GSSG, 0.15 mM NADPH.

Ascorbate peroxidase (APX EC 1.11.1.11, Nakano and Asada, 1981) was measured at 290 nm after incubation of the protein extract with 50 mM KH_2PO_4 (pH 7.0), 0.5 mM NaAA, 0.1 mM H_2O_2.

Catalase (CAT EC 1.11.1.6, Aebi, 1984) protein extract is incubated in a reaction mixture of K_2HPO_4 (pH 7) and 15 mM H_2O_2 and absorption is measured at 240 nm.

Superoxide dismuthase (SOD, EC 1.15.1.1, Marklund and Marklund, 1974) protein extract was incubated with 50 mM Tris-succinate buffer (pH 8.2) and 8 mM pyrogallol. Absorption was measured at 412 nm.

Non-enzymatic antioxidants assays

Ascorbate and dehydrosacorbate (Foyer et al., 1983), for ascorbate reduction of the absorption is measured at 265 nm after incubation of the protein extract with 100 mM KH_2PO_4 (pH 5.6), 0.2 mM ascorbate and 5µl ascorbate oxidase. For dehydroascorbate reaction mixture contains 100 mM KH_2PO_4 (pH 8.5), 0.2 mM ascorbate and 10mM GSH. Reduction of the absorption – at 265 nm.

Oxidized and reduced glutathione (Griffith 1980), the assay was based on sequential oxidation of glutathione by DTNB and reduction by NADPH in the presence of GR. For determination of the quantities of GSH plus GSSG and GSSG separately, the extract was processed and subsequently assayed as per the method given earlier (Fadzilla et al., 1997). One sample was used to determine the concentrations of GSH and GSSG and another one pretreated with 2-vinylpyridin (99%, HPLC grade) for masking GSH by derivatization and allow determination of GSSG alone. In each case the assay mixture in 1 ml contained 125 mM potassium phosphate buffer and 6.3 mM EDTA pH 6.5, 0.3 mM NADPH, 3 mM DTNB and 0.01 ml of the supernatant. The reaction was initiated by the addition of 10 µl of GR (5 U/ml) and the change in absorbance at 412 nm was recorded. Standard curves were generated with reduced and oxidized glutathione. The results were expressed per gram FW.

Polyphenolics determination

100 mg air dried plant material, were extracted with hot 80% (v/v) ethanol and then centrifuged at 15 000 rpm for 15 min. Total phenolics were determined by the Folin & Ciocalteu's colorimetric method of Singleton et al. (1999), and flavonoids – by the colorimetric method of Zhishen (1999).

Determination of morphometric parameters

The number of axillary shoots, as well as the leaf couples per shoot formed was recorded from plant specimens of 20 separate culture vessels. Index of compactness (IC) was calculated as the number of leaf couples per 1 cm of shoot length. The obtained data was processed to distinguish between shoots with length lower and higher than 1 cm. The frequency of occurrence of each of the two types of shoot lengths was separately presented.

Statistical evaluation of data

The average sample sizes and measurement repetitions are specified in each paragraph of Materials and Methods above. The means were compared by t test of unequal variances at $P \leq 0.05$. Unless otherwise stated, differences were considered statistically significant at $P \leq 0.05$.

Results and Discussion

Effect of PGR on enzymatic and non-enzymatic antioxidant defence in H. calycinum shoots

BA and IBA acid affected significantly the studied enzymatic antioxidant activities of *H. calycinum in vitro.* Their supplementation led to stimulation of PAL and SOD activities, as well a raise of the levels of non-enzymatic antioxidants as oxidised glutathione and dehydroascorbate. On the contrary, in the same Hri_1 treatment, GR, as well as APX activities, and ratios of reduced/oxidised glutathione and ascorbate/dehydroascorbate were shown to be inhibited in comparison with plant growth regulators-free control (Table 1). A complex interplay between factors of enzymatic and non-enzymatic antioxidant defence in the plant organism underlines the molecular mechanisms overcoming environmental stress. In a study, concerning the related *H. perforatum* species, physiological stress (caused by elevation of temperature to 35°C) has been shown to increase leaf total peroxidase activity (as well as hypericin, pseudohypericin and hyperforin concentrations) in the pot cultured shoot tissues of the plant (Zobayed et al., 2005). Plant cell tissue and organ culture development has been shown to induce oxidative stress on the plant organism (Benson, 2000). In our previous studies on *Hypericum* tissue culture it was shown that lowering of enzymatic activities *in vitro* (caused by long-term post thaw recovery after cryopreservation, or even the long-term storage of the stock shoot cultures of the plant) was accompanied by elevation of low molecular non-enzymatic antioxidants *in vitro* (Danova et al., 2012b). Surprisingly, in the present study, in spite of affecting enzymatic activities *in*

Table 1

Parameters of enzymatic and non-enzymatic antioxidant defense in *H. calycinum* shoot cultures

	Medium abbreviation	
	Hri_0	Hri_1
PAL [µmol.mg.min^{-1}]	15.26 ± 0.6	19.97 ± 1.3
SOD [ÄE.mg.min^{-1}]	0.16 ± 0.01	0.76 ± 0.1
GSSG [nmol.gFW^{-1}]	31.66 ± 1.7	75.00 ± 2.9
DHA [nmol.gFW^{-1}]	6.39 ± 0.6	15.28 ± 1.7
CAT [nmol/mg/min]	2.10 ± 0.2	1.80 ± 0.4
GR [nmol/mg/min]	2.08 ± 0.2	1.32 ± 0.2
APX [nmol/mg/min]	27.46 ± 4.7	23.45 ± 4.1
GSH [nmol/gFW]	12.44 ± 0.4	10.11± 1.4
AsA [nmol/gFW]	4.36 ± 0.59	2.83 ± 0.4
AsA/DHA	0.40 ± 0.03	0.13 ± 0.02
GSH/GSSG	0.68 ± 0.07	0.19 ± 0.04

± values represent standard error of the mean of six measurements

Table 2

Total phenolic, flavonoid content, as well as proportion of total flavonoids as part of phenolics in the shoot cultures of *H. calycinum*

Medium abbreviation	Total phenolic content, mg.g^{-1}	Total flavonoid content, mg.g^{-1}
Hri_0	132.46 ± 2.8	28.29 ± 0.6
Hri_1	131.18 ± 4.3	25.75 ± 0.9
Hri_2	108.67 ± 3.1	21.22 ± 0.8
Hri_3	121.95 ± 5.5	23.75 ± 1.6
Hri_4	121.87 ± 2.3	24.72 ± 0.3

± values represent standard error of the mean of six measurements

vitro, supplementation of 0.2 mg.L^{-1} BA + 0.1 mg.L^{-1} IBA (Hri_1) only slightly affected the levels of polyphenolics in *H. calycinum* (Table 2). Unlike for *H. calycinum*, in our previous studies on hypericin producing *H. richeri,* in Hri_1 supplemented medium levels of polyphenolics decreased 1.4 times (Wolfram et al., 2013). Further increase of IBA concentration to 0.5 mg/l, led to a drop of polyphenolics, and after increasing BA supplementation to 0.5 mg/l, polyphenolic levels were slightly elevated, without reaching the levels of PGR-lacking control.

Effect of PGR on growth patterns of H. calycinum in vitro

H. calycinum, grown in PGR-free medium displayed a multiplication index of 2-3 shoots per explant (data not shown). The increase of IBA concentration from 0.1 to 0.5 mg.L^{-1} (in combination with both 0.2 mg.L^{-1} and 0.5 mg.L^{-1} BA), led to stimulation of axillary shoots formation, increased shoot length and number of leaf couples per shoot (Table 3). Interestingly, media supplementations Hri_2 and Hri_4, characterized with predominance of shoots with length exceeding 1 cm were also characterized with a drop of polyphenolics, as compared with other media supplementations. These results are in agreement with our previous results of interrelations between stimulation of biomass formation and lowering of polyphenolics production in other *Hypericum* species *in vitro* (Danova et al., 2012). While for *H. richeri,* the studied here media supplementations stimulated axillary shoot formation 5–9 times (Wolfram et al., 2013), for *H. calycinum* this effect was less pronounced, displaying axillary shoot stimulation of 2–3 times compared to the PGR-free control.

Conclusions

In accordance to literature data on *Hypericum* species, as well as to our own previous research on hypericin producing *H. rumeliacum,* there is relation between the enzymatic and non-enzymatic antioxidant defense in the representatives of the *Hypericum* genus. The production of condensed naphthodianthrones hypericin and pseudohypericin, however seem to strongly affect the growth patterns and biosynthetic capacity in terms of polyphenolic production *in vitro.* The higher polyphenolic productivity of hypericn non-producing *H. calycinum* as compared with the hypericin producing species studied by us is less affected by the effect of PGR. Work is in progress in order to evaluate the potential phytopharmaceutical value of the spectrum of polyphenolics produced by *H. calycinum in vitro*.

Acknowledgements

This work was supported by the Swiss Enlargement Contribution in the framework of the Bulgarian-Swiss Research Programme (BSRP, grant No. IZEBZ0_142989, DO2-1153) and and EMAP (FP7-PEOPLE-2009-IRSES) N°247548

References

Aebi, H., 1984. Catalase *in vitro*. *Methods of Enzymology*, **105:** 121–126.

Azizi, M., A. Ghani, T. Ebadi and S. Crockett, 2011. The *ex situ* comparison of two improved St. John's Wort (*Hypericum perforatum*) cultivars with an Iranian wild population. *Acta Horti*culturae, **925**: 163–170.

Bäcker, W., H. Bart, F. Bischoff, S. Grabley, R. Goedecke, W. Johannisbauer, V. Jordan, R. Stockfleth, J. Strube and V. Wiesmet, 2006. Phytoextrakte – Produkte und Prozesse. Available online: http://www.dechema.de/dechema_media/Downloads/Extraktion/Phytoextrakte.pdf

Table 3

Growth patterns in *H. calycinum* shoot cultures in BA and IBA supplemented media

	Axillary shoots	Axillary shoots < 1 cm				Axillary shoots > 1 cm			
		frequency, %	length, cm	Leaf couples	IC	frequency, %	length, cm	Leaf couples	IC
Hri_1	6.5 ± 1.5	81.8	0.5 ± 0.03	3.9 ± 0.1	0.12 ± 0.06	18.2	1.3 ± 0.2	6.7 ± 0.5	0.2 ± 0.03
Hri_2	6.8 ± 2.2	68.3	0.6 ± 0.02	4.4 ± 0.2	0.13 ± 0.003	31.7	2.8 ± 0.4	10.1 ± 0.6	0.26 ± 0.02
Hri_3	5.5 ± 0.8	71.7	0.4 ± 0.03	3.7 ± 0.5	0.11 ± 0.002	28.6	1.7 ± 0.5	7 ± 0.6	0.25 ± 0.06
Hri_4	7.3 ± 1.1	69	0.5 ± 0.02	4.1 ± 0.3	0.13 ± 0.01	31	2.6 ± 0.3	9.0 ± 1.6	0.3 ± 0.04

Benson, E. E., 2000. Special symposium: *in vitro* plant recalcitrance do free radicals have a role in plant tissue culture recalcitrance? *In Vitro Cell Dev. Biol. Plant.,* **36**: 163–170.

Butterweck, V. and M. Schmidt, 2007. St. John's wort: role of active compounds for its mechanism of action and efficacy. *Wien Med. Wochenschr.*, **157:** 356–361.

Cui, X. H., H. N. Murthy, C. H. Wu and K. Y. Paek, 2010. Sucrose-induced osmotic stress affects biomass, metabolite, and antioxidant levels in root suspension cultures of *Hypericum perforatum* L. *Plant Cell Tiss. Organ Cult.*, **103**: 7–14.

Danova, K., 2010. Production of polyphenolic compounds in shoot cultures of Hypericum species characteristic for The Balkan Flora. *Botanica Serbica*, **34**: 29–36.

Danova, K., E. Čellárová and V. Kapchina-Toteva, 2010. Impact of growth regulators on *in vitro* regeneration of *Hypericum rumeliacum* Boiss. *Journal of Environmental Prot. and Ecol.*, **11:** 1285–1292.

Danova, K., E. Čellárová, A. Macková, Z. Daxnerová and V. Kapchina-Toteva, 2010. *In vitro* culture of *Hypericum rumeliacum* Boiss. and production of phenolics and flavonoids. *In Vitro Cellular and Developmental Biology – Plant,* **46**: 422–429.

Danova, K., B. Nikolova-Damianova, R. Denev and D. Dimitrov, 2012a. Influence of vitamins on polyphenolic content, morphological development, and stress response in shoot cultures of *Hypericum* spp. *Plant Cell Tiss. Organ Cult.,* **110:** 383–393.

Danova, K., 2014. Biotechnological Utilization of the Indigenous Biosynthetic Capacity of Medicinal and Aromatic Plants: Experience in the Genera *Hypericum Pulsatilla* and Essential Oil Bearing *Artemisia alba* Characteristic for the Balkan Region. Chapter 14, In: J.N. Govil (Editor), *Recent Progress in Medicinal Plants Biotechnology and Genetic Engineering* II & Vol.39, Studium Press LLC, USA, pp. 355–392.

Danova, K., B. Nikolova-Damianova, R. Denev and Y. Markovska, 2012b. Impact of pre-culture on short- and long-term *in vitro* recovery of the biosynthetic potential and enzymatic and non-enzymatic antioxidant defense of *Hypericum rumeliacum* Boiss. after cryostorage. *Plant Growth Regulation*, **68:** 447–457.

Decosterd, L. A., E. Hoffmann, R. Kyburz, D. Bray and K. Hostettmann, 1991. A new phloroglucinol derivative from *Hypericum calycinum* with antifungal and *in vitro* antimalarial activity. *Planta Medica*, **57:** 548–551.

Fadzilla, N. M., R. P. Finch and R. H. Burdon, 1997**.** Salinity, oxidative stress and antioxidant responses in root culture of rice. *J. Exp. Bot.*, **48**: 325–331.

Foyer, C., J. Rowell and D. Walker, 1983. Measurement of the ascorbate content of spinach leaf protoplasts and chloroplasts during illumination. *Planta*, **157**: 239–244.

Griffith, O. W., 1980. Determination of glutathione disulphide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.,* **106**: 207–212.

Kartning, T., I. Göbel and B. Heydel, 1996. Production of hypericin, pseudohypericin and flavonoids in cell cultures of various *Hypericum* species and their chemotypes. *Planta Med.,* **62**: 51–53.

Kirmizibekmez, H., C. Bassarello, S. Piacente, E. Celep, I. Atay, G. Mercanoğlu and E. Yeşilada, 2009. Phenolic compounds from *Hypericum calycinum* and their antioxidant activity. *Natural Product Communications*, **4**: 531–534.

Kirakosyan, A., T. M. Sirvent, D. M. Gibson and P. B. Kaufman, 2004. The production of hypericins and hyperforin by *in vitro* cultures of St. John's wort (*Hypericum perforatum*). *Biotechnol. Appl. Biochem.*, **39**: 71–81.

Kitanov, G. M. and K. F. Blinova, 1987. Modern state of the chemical study of species of the genus *Hypericum*. *Chemistry of Natural Compounds*, **23**: 151–166.

Marklund, S. and G. Marklund, 1974. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase, *Eur. J. Biochem.*, **47:** 469-474.

Nakano, Y. and K. Asada, 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol.*, **22**: 867–880.

Savio, L. E. B., L. V. Astarita and E. R. Santarém, 2012. Secondary metabolism in micropropagated *Hypericum perforatum* L. grown in nonaerated liquid medium. *Plant Cell Tiss. Organ Cult.*, **108**: 465–472.

Sherwin, H. W. and J. M. Farrant, 1998. Protection mechanisms against excess light in the resurrection plants *Craterostigma wilmsii* and *Xerophyta viscosa*. *Plant Growth Reg.*, **24**: 203–210.

Singleton, V. L., R. Orthofer and R. M. Lamuela-Raventós, 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Method Enzymol.,* **299**: 152–178.

Öztürk, Y., S. Aydin, R. Beis, K. H. C. Başer and H. Berberoğlu, 1996. Effects of *Hypericum perforatum* L and *Hypericum calycinum* L extracts on the central nervous system in mice. *Phytomedicine*, **3**: 139–146.

Wolfram, E., L. Hostettler, S. Peter, M. Todorova, A. Trendofilova and K. Danova, 2013. Phytochemical assessment of the effect of stimulated *in vitro* multiplication on the metabolic profile of *in vitro* cultured *Hypericum richeri* and *Artemisia alba.* *Planta Med.,* **79**: PN119.

Yuan, Y. J., C. Li, Z. D. Hu, J. C. Wu and A. P. Zeng, 2002. Fungal elicitor induced cell apoptosis in suspension cultures of *Taxus chinensis* var. *mairei* for taxol production. *Process Biochem.,* **38**:193–198.

Zhishen, J., T. Mengcheng and W. Jianming, 1999. The determination of flavonoid content in mulberry and their scavenging effects on superoxide radicals. *Food Chem.,* **64**: 555–559.

Zobayed, S. M. A., F. Afreen and T. Kozai, 2005. Temperature stress can alter the photosynthetic efficiency and secondary metabolite concentrations in St. John's wort. *Plant Physiology and Biochemistry*, **43**: 977–984.