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OPTIMIZATION OF MICROPROPAGATION BY DIFFERENT CONCENTRATION OF VITAMINS AND SUCROSE IN ST. JOHN'S WORT (*HYPERICUM PERFORATUM*)

ABSTRACT

In order to approach optimal micropropagation of *Hypericum perforatum*, it will be necessary to optimize shoot proliferation stage in *in vitro* culture. This study was conducted to investigate the effects of different concentrations of B group vitamins; Thiamine HCl, Pyridoxine HCl, Nicotinic acid (control and 100 fold of MS) and Sucrose (30 and 40 g.l⁻¹) on shoot proliferation. For this purpose, Stems with one node were taken from *in vitro* shoots and cultured on MS medium. All cultures kept at 16h light/8h photoperiod and 25 ±2 °C in growth chamber. Results showed that the highest number of shoots and leaves were achieved when explants cultured in media containing 40 g.l⁻¹ sucrose with 100 fold of MS vitamins. The highest shoots and leaf length were obtained with medium supplemented with 30 g.l⁻¹ sucrose. Nicotinic acid concentrations had an important role in length of the leaves. The highest number of nodes achieved in the media containing 40 g.l⁻¹ sucrose with both concentrations of Nicotinic acid. After two month growing plantlets, light (LGN) and dark glands number (DGN) were counted. Maximum number of LGN was observed in the media containing 30 g.l⁻¹ sucrose with 100 fold of Thiamine and Pyridoxine. However, The Highest number of DGN achieved in the media containing 40 g.l⁻¹ sucrose with 100 fold of Thiamine or Pyridoxine. Increasing of sucrose and vitamins concentrations were efficiently improved *in vitro* proliferation and some morphological attributes without negative side effects. Therefore, use of high levels of sucrose and vitamins were useful on micropropagation of *Hypericum perforatum*.

Key words: *Hypericum perforatum*, nicotinic acid, pyridoxine, shoot proliferation, sucrose, thiamine

INTRODUCTION

Hypericum perforatum L. (St. John's wort) has been received considerable interest worldwide due to its biochemical characteristics and unique secondary metabolites (Radusiene and Bagdonaite, 2002; Gadzovska *et al.* 2005). It is a well-known traditional medicinal plant that has been used for centuries for the treatment of several diseases, such as skin lesions, eczema, burns, inflammations and some psychological disorders (Barnes *et al.* 2001; Sanchez *et al.* 2002). This feature is obviously attributed to the presence of a broad spectrum of secondary metabolites, mainly naphthodiantrons and phloroglucinols (Cellarova *et al.* 1994). In particular, aromatic polycyclic diones, such as hypericin and pseudohypericin have an interest as their antiviral, anticancer and antidepressant activities that have been documented previously (Sanchez *et al.* 2002). To date, field grown plant material has generally been used for commercial St. John's Wort production but the quality of these products may be affected by different environmental conditions, pollutants, fungi, bacteria, viruses, and insects which can alter the concentration of medicinal metabolites (Murch *et al.* 2000). In vitro systems have been reported as an effective tool for the development of genetically uniform plants (Santarem and Astarita, 2003; Farsad-Akhtar *et al.* 2013; Motallebi-Azar *et al.* 2011; Mahna and Motallebi-Azar, 2007; Zolfagari-Nasab *et al.* 2004; Pourjabar *et al.* 2007). Therefore, the application of in vitro techniques provides an approach for the production of standardized plant materials. In St. John's Wort micropropagation system has been used for producing of high-quality phytopharmaceutical for the treatment of neurological disorders (Murch *et al.* 2000). Plant regeneration of *H. perforatum* L. has been achieved using explants such as whole seedlings or their excised parts (Kazemiani and Motallebi-Azar, 2011; Cellarova *et al.* 1994), hypocotyl sections (Zobayed *et al.* 2004) and leaves, (Wójcik and Podstolski, 2007) using various types and concentrations of auxins and cytokinins. Production of secondary metabolites via plant cell and tissue cultures holds various advantages such as standardization, and production of pure medicinal compounds of biochemical and clinical research over conventional field grown plants (Pourjabar *et al.* 2007; Zobayed *et al.* 2004). Plant cells and organ cultures have been used in the production of useful secondary metabolites in St. John's Wort (Barnes *et al.* 2001; Bais *et al.* 2002). Kirakosyan *et al.* (2000) analyzed the relationship between biosynthesis of hypericin and in vitro development of differentiated structures in *H. perforatum*. The biosynthesis of hypericin seems to be strictly linked to the differentiation of black globules in the leaves of *H. perforatum* (Zdunek and Alfermann, 1992; Briskin and Gawienowski, 2001). Histological and ultrastructural studies have also indicated the presence of black globules in the stem and petals (Farsad-Akhtar *et al.* 2014). Morphological changes induced by carbon source and hydrolyzed casein were observed in *H. perforatum* callus culture (Kazemiani and Motallebi-Azar, 2011). The aim of

the present study was to evaluate the effects of different concentration of vitamins and sucrose in shoot induction in in vitro culture of *H. perforatum* for optimization of micropropagation.

MATERIALS AND METHODS

Plant materials

The sterile explants from stem segments were prepared in the Tissue Culture Laboratory of Horticultural Sciences, University of Tabriz. Each explant had 2 nodes and were cultured on MS medium supplemented with $0.5 \text{ mg} \times \text{l}^{-1}$ BAP to induce the shoot. The culture was maintained at $22 \pm 2^\circ\text{C}$ under photoperiod 16 h light/ 8 h dark (Motallebi-Azar *et al.*, 2011) for 1 month.

Media

The medium includes eight concentrations of MS vitamins: Thiamine (0.1 and $10 \text{ mg} \times \text{l}^{-1}$), Nicotinic acid (0.5 and $50 \text{ mg} \times \text{l}^{-1}$) and Pyridoxine (0.5 and $50 \text{ mg} \times \text{l}^{-1}$) and two concentrations of sucrose (30 and $40 \text{ g} \times \text{l}^{-1}$).

After one month number of shoots and leaves, length of shoot and leaves, light and dark gland number recorded. Data obtained from this study were analysed using SPSS software Ver.16. Comparison of means was done by using Duncan's multiple range tests at the 5 % probability level.

RESULTS AND DISCUSSION

In all culture media, axillary shoots induction from the bud were initiated after two weeks (Fig. 1). Dark glands appeared on leaves in each media and the number of them was different (Fig. 2). Produced shoots were growth and formed plantlet (Fig. 3).

Fig. 1. Shoot induction 1 month after culture

Fig. 2. Dark glands and light glands on leaves

Fig. 3. Number of shoots in different concentration of sucrose (right: sucrose 30 with 100 fold MS vitamins, left: sucrose 40 with 100 fold MS vitamins)

Shoot number

The shoot number was significantly affected by different concentration of sucrose and vitamins and their interaction. The highest shoot number was achieved when explants cultured into media containing 100 fold of MS vitamins (Thiamine: $10 \text{ mg} \times \text{l}^{-1}$, Nicotinic acid $50 \text{ mg} \times \text{l}^{-1}$, Pyridoxine: $50 \text{ mg} \times \text{l}^{-1}$) (Fig. 4). The shoot number significantly affected the interaction of Thiamin $\times$ Nicotinic acid, Thiamin $\times$ Pyridoxin. Roest and Bokelman (1975) have shown that the MS vitamins have improved shoot induction in *Chrysanthemum morifolium*. They showed that there is no shoot induction in treatments without vitamins. In potato, increasing vitamins concentrations have improved the shoot induction (Kazemiani *et al.* 2011). Vitamin concentrations in the media are important to improve the morphogenesis and they completely relate to the plant types and other medium components (Abrahamian and Kantharajah, 2011). It is necessary to use vitamins in culture media, however the concentrations

of vitamins are less and in the media without vitamins the quality and quantity of growth decrease.

Fig. 4. Effect of different concentration of vitamins on shoot numbers

Pyridoxine was affected the shoot numbers ($p < 0.01$). Kazemiani *et al.* (2011) have reported that the increasing pyridoxine concentration, improved the shoots number in Potato callus cultures. In all treatments with $40 \text{ g} \times \text{l}^{-1}$ sucrose, the axillary shoots were observed in highly compact. Increasing the sucrose concentration cause the highest number of shoots (Fig. 5). In *Alocasia amazonica*, the highest number of shoots was obtained in 6 and 9% sucrose (Jo *et al.* 2009). In *Chlorophytum arundinaceum*, the best shoot induction was obtained in modified MS media with 4% sucrose (Samantaray and Maiti, 2010). Optimum shoot induction in *Hypericum perforatum* callus culture was obtained in 20 and $30 \text{ g} \times \text{l}^{-1}$ with mannitol (5 and $10 \text{ g} \times \text{l}^{-1}$) and Hydrolyzed casein ($500 \text{ g} \times \text{l}^{-1}$) (Motallebi-Azar and Kazemiani, 2011). After two months, the number of shoots were 288.95 in media with $40 \text{ g} \times \text{l}^{-1}$ sucrose and $50 \text{ mg} \times \text{l}^{-1}$ Pyridoxine. Santarem and Astarita (2003) reported 60 shoots after two months in modified MS with $4.5 \mu\text{M} \times \text{l}^{-1}$ BAP, $0.05 \mu\text{M} \times \text{l}^{-1}$ NAA.

Fig. 5. Effect of different concentration of sucrose on shoot number

Shoots length

In all cultures, the axillary shoots were growing, but in $30 \text{ g} \times \text{l}^{-1}$, the length of shoots were higher than $40 \text{ g} \times \text{l}^{-1}$ sucrose (Fig. 6). The Shoot length was significantly influenced by sucrose concentration and Nicotinic acid, Thiamin concentration and their interaction (Fig. 7). The maximum shoot length was observed on $30 \text{ g} \times \text{l}^{-1}$ sucrose with $10 \text{ mg} \times \text{l}^{-1}$ Thiamine, $50 \text{ mg} \times \text{l}^{-1}$ Nicotinic acid (100 fold MS concentration). Results showed that the increasing sucrose concentration causes the highest number of shoots, but avoided increasing the length of shoots. When sucrose concentration increased, BAP increase the apical dominance and the shoot number. Increasing Thiamine and Nicotinic acid concentration significantly affected the shoot length. Thiamine improved the growth in culture media (Hall, 1999). In peanuts embryo cultures, $0.01 \text{ mg} \times \text{l}^{-1}$ Nicotinic acid increased the length of shoots and they were 3 fold higher than media without Nicotinic acid after four weeks (Barwale and Widholm, 1986). In our cultures the highest shoot length (7.37 cm) observed in media containing $30 \text{ g} \times \text{l}^{-1}$ sucrose with 100 fold MS vitamins after two months (Fig. 8).

Different concentration of Thiamin, Nicotinic acid and sucrose

Fig. 6. Effect of different concentration of Thiamin, Nicotinic acid and sucrose on shoot length

Fig. 7. Compact growth in 40 g.l⁻¹ sucrose (right), length growth in 30 g × l⁻¹ sucrose (left)

Fig. 8. The Highest length in 30 g × l⁻¹ sucrose with 100 fold MS vitamins

Leaves number

ANOVA Table showed that the sucrose concentration and vitamins and their interaction significantly influenced the leaves number. The highest leaves number was obtained in $40 \text{ g} \times \text{l}^{-1}$ sucrose with 100 fold MS vitamins (Fig. 9). Increasing sucrose and vitamin concentration, improved the growth and caused the optimum number in this culture.

Different concentration of Thiamin, Nicotinic acid, pyridoxine and sucrose

Fig. 9. Effect of different concentration of Thiamin, Nicotinic acid, pyridoxine and sucrose on leaf number

Leaf length

In *Hypericum perforatum*, biological compounds such as hypericin and pseudohypericin are localized in smaller glandular structures on the leaves and the length of leaves is important in flavonoids concentrations (Kazemiani and Motallebi-azar, 2011). Increasing in sucrose concentration influenced leaves number in $40 \text{ g} \times \text{l}^{-1}$ sucrose but the leaf length was less than $30 \text{ g} \times \text{l}^{-1}$ sucrose. There are some reports about carbon source effect on plant morphogenesis *in vitro* cultures (Motallebi-Azar and Kazemiani, 2011; Fuentes *et al.* 2000; Karhu, 1997; George, 1993). When the medium supplemented with $30 \text{ g} \times \text{l}^{-1}$, the highest leaves length was obtained correlated with Thiamin and Nicotinic acid concentrations. After two months, the highest length was 5.3 mm in $30 \text{ g} \times \text{l}^{-1}$ sucrose with 100 fold MS vitamins (Fig. 10).

Fig. 10. Effect of different concentration of Thiamin and sucrose on leaf length

Nude number

Data analyses showed that the node's number were significantly affected by sucrose and Nicotinic acid interaction. However, no significant differences were observed among different concentrations of sucrose. When the medium was containing $40 \text{ g} \times \text{l}^{-1}$ sucrose with $10 \text{ mg} \times \text{l}^{-1}$ Thiamin, $50 \text{ mg} \times \text{l}^{-1}$ Nicotinic acid (100 fold MS) and $0.5 \text{ mg} \times \text{l}^{-1}$ Pyridoxine the highest number of nuds were obtained (Fig. 11). A thiamin necessity in *in vitro* cultures observes specially in cytokine deficiency. Nicotinic acid and pyridoxine is necessary in *Haplopappus gracilis in vitro* culture (Abrahamian and Kantharajah, 2011).

Fig. 11. Effect of different concentration of nicotinic acid and sucrose on nod numbers

Ligh glands number

The ligh glands are located on leaves in *Hypericum perforatum* and have some flavnoieds (Curtis and Lersten, 2002). Light glands were significantly influenced by vitamin concentrations and sucrose interactions (Fig. 12). The highest number of light glands were obtained in the media include $30 \text{ g} \times \text{l}^{-1}$ sucrose correlated with high Thiamin and Pyridoxine concentrations (Fig. 13). There were 1.97 light glands on 1 mm^2 each leaf. Thiamin improves the secondary metabolite synthesis in plants (Abrahamian and Kantharajah, 2011). Light glands have oils and Hyperphorin (Farsad-Akhtar *et al.* 2014). So, it is important to use Thiamin in *Hypericum in vitro* cultures.

Fig. 12. Effect of different concentration of thiamin, nicotinic acid and sucrose on light gland number

Fig. 13. Effect of different concentrations nicotinic acid and pyridoxine on light gland number

Dark glands number

Hypericum species have some biological compounds like naphthodiantrones. Red-pigmented dots, characteristic for hypericin first, were observed in developing shoots and corresponding to oil glands on leaf surfaces (Curtis and Lersten, 2002). Biosynthesis of hyperlinks is connected with the morphogenesis and the formation of dark red colored oil glands on the leaves of the intact plant (Zdunk and Alfermann, 1992). ANOVA table showed that number of dark glands was significantly influenced by vitamins combinations and sucrose interactions. The highest number of dark glands was obtained when explants were cultured in media containing $40 \text{ g} \times \text{l}^{-1}$ sucrose and correlated with high Nicotinic acid concentration (Fig. 14). The morphogenesis related with carbon source and sucrose is the commonly applied in *in vitro* culture. In addition vitamins are necessary for enzymatic systems. Increasing vitamin concentration in media supplemented with $40 \text{ g} \times \text{l}^{-1}$ sucrose improved the dark glands number (3.8 on 1 mm^2 each leaf). Farsad-Akhtar *et al.* (2014) reported 0.3 dark gland number in *Hypericum perforatum* *in vitro* culture supplemented with growth regulators. Also reported, there were significantly relate between dark gland number and Hypericin content in *Hypericum perforatum*.

Fig. 14. Effect of different concentration of vitamins and sucrose on dark gland number

CONCLUSION

Optimisation of shoot induction and an increasing glands number in *in vitro* cultures are controlled by different factors including: carbon concentration, vitamin concentrations and other growth regulators. Using different concentrations of sucrose and vitamins showed that high levels of sucrose and vitamins had positive effects on shoot induction and gland formation. The using of the high

levels of sucrose had a negative effect on shoot length. Therefore, the using of $30 \text{ g} \times \text{l}^{-1}$ sucrose was useful to shoot and leaves length. Increasing of vitamin concentrations was improved efficiently dark and light gland number in *in vitro* culture.

REFERENCES

- Abrahamian P., Kantharajah A. 2011. Effect of :Vitamins on in vitro organogenesis of plant. *American journal of plant Sciences*. 2: 669-674.
- Bagdonaite E., Janulis V., Ivanauskas L., Labokas J. 2002. Ex situ studies on chemical and morphological variability of *Hypericum perforatum* L. in Lithuania. *Biologija journal*. 53: 63-70.
- Bais P.H., Ravishankar G.A. 2002. Role of polyamines in the ontogeny of plants and their biotechnological applications. *Plant Cell, Tissue and Organ Culture*. 69: 1-34.
- Barnes J., Anderson A., Phillipson D. 2001. St. John's wort (*Hypericum perforatum* L.) A review of its chemistry, pharmacology and clinical properties. *Journal of Pharmacy and Pharmacology*. 53: 583-600.
- Barwale U.B., Kerns H.R., Widholm J.M. 1986. Plant regeneration from callus cultures of several soybean genotypes via embryogenesis and organogenesis. *Planta*. 167: 473-481.
- Briskin D.P., Gawienowski M.C. 2001. Differential effects of light and nitrogen on production of hypericins and leaf glands in *Hypericum perforatum*. *Plant Physiology Biochemistry*. 39: 1075-1081.
- Cellarova E., Daxnerova Z., Kimakova K., Haluskova J. 1994. The variability of the hypericin content in the regenerants of *Hypericum perforatum*. *Acta Biotechnology*. 14: 267-274.
- Curtis J.D., Lersten N.R. 2002. Internal secretory structure in *Hypericum* (Gluciaceae): *H. Perforatum* L. and *H. balearicum* L. *New Phytologist*. 14: 571-580.
- Farsad Akhtar N., Aharizad S., Mohammadi S.A., Motallebi-Azar A., Movafeghi A., Banan Khojasteh S.M. 2013. In vitro shoot regeneration and hypericin production in four *hypericum perforatum* genotypes. *International Journal of Agriculture: Research and Review*. 3: 887-893.
- Farsad Akhtar N., Aharizad S., Mohammadi S.A., Motallebi-Azar A., Movafeghi A. 2014. The study of relationship between morphological traits and hypericins content in *Hypericum perforatum*. *Technical Journal of Engineering and Applied Sciences*. 4: 45-47.
- Fuentes S.R.L., Calheiros M.B.P., Manetti-Filho J., Vieira, L.G.E. 2000. The effects of silver nitrate and different carbohydrate sources on somatic embryogenesis in *Coffea canephora*. *Plant Cell, Tissue and Organ Culture*. 60: 5-13.
- Gadzovska S., Maury S., Ounnar S., Riguezza M., Kascakova S., Refregiers M., Spasenoski M., Joseph C., Hagege D. 2005. Identification and quantification of hypericin and pseudohypericin in different *Hypericum perforatum* L. *in vitro* cultures. *Plant Physiology Biochemistry*. 43: 591-601.
- George E.F. 1993. Plant propagation by tissue culture. part1. The technology ExegeticsEngland. Potato. Phd Thesis, Punjab Agricultural University Ludhiana (pb) India.
- Hall R.D. 1999. Plant Cell Culture Protocols. Humana Press Ottawa.
- Jo E.A., Park K.Y. 2009. In vitro sucrose concentration affect growth and acclimatization of *Alocasia Amazonica* plantlets. *Plant Cell, Tissue and Organ culture*. 96: 307-315.
- Karhu S.T. 1997. Sugar use in relation to shoot induction by sorbitol and cytokinin in apple. *Journal of American Society Horticultural Science*. 122: 476-480.
- Kazemiani S., Motallebi-Azar A. 2011. Increasing gland number and redpigments in St. John's wort *in vitro* culture: Influence of mannitol, sucrose and hydrolyzed casein. *African Journal of Biotechnology*. 10: 10-29.
- Kirakosyan A., Hayashi H., Inoue K., Charchoglyan A., Vardapetyan H. 2000. Stimulation of the production of hypericins by mannan in *Hypericum perforatum* shoot cultures. *Phytochemistry*. 53: 345-348.
- Mahna N., Motallebi-Azar A. 2007. In vitro micropropagation of apple cv. Golden Delicious. *Comm. Appl. Bio. Sci*. 72: 235-238.
- Motallebi-Azar A., Kazemiani S., Kiumarsie F., Mohaddes N. 2011. Shoot proliferation from node explants of potato (*Solanum tuberosum* cv. Agria). II. effect of different concentrations of NH_4NO_3 , hydrolyzed casein and BAP. *Romanian Biotech. Let*. 16: 6199-6204.
- Motallebi-Azar A., Mahna N., Markafshi A.S. 2011. Shoot regeneration from leaf segments of peach $\times$ almond hybrid, GF677 rootstock. *Advances in Agriculture Botany*. 3: 168-177.

- Murch S.J., Choffe K.L., Victor J.K.R., Slimmon, T.Y., Krishnaraj S., Saxena P.K. 2000. Thidiazuron-induced plant regeneration from hypocotyl cultures of St. John s wort (*Hypericum perforatum* cv Anthos). *Plant Cell Reports*. 19: 576-581.
- Pourjabar A., Mohammadi S.A., Khosroshali M., Motallebi-Azar A., Ziai S.A. 2010. Optimization of in vitro culture of Milk Thistle and analysis of somaclonal variation by RAPD markers. *J. Agri. Sci*. 19: 1-14.
- Radusiene J., Bagdonaite E. 2002. Phenotypic Variation in *Hypericum Perforatum* L. and *H.maculatum* crantz the Wild Populations Lithuania. The Haworth Press.
- Roest S., Bokelmann G.S. 1975. Vegetative propagation of *Chrysanthemum morifolium* Ram. *in vitro. Science Horticulture*. 3: 317-330.
- Samantaray S., Maiti M. 2010. Factors influencing rapid clonal propagation of *Chlorophytum arundinaceum* (Liliales: Liliaceae), an endangered medicinal plant. *Revista de Biologia Tropical*. 59: 435-445.
- Sanchez C.C., Prado B., Rabanal R.M. 2002. Antidepressant effects of the methanol extract of several *Hypericum* species from the Canary Islands. *Journal of Ethnopharmacology*. 79: 119-127.
- Santarem E.R., Astarita L.V. 2003. Multiple shoot formation in *Hypericum perforatum* L. and hypericin production. *Brazilian Journal of Plant Physiology*. 15: 43-47.
- Wojcik A., Podstolski A. 2007. Leaf explant response in *in vitro* culture of St. John s wort (*Hypericum perforatum* L.). *Acta Physiology Plant*. 29: 151-156.
- Zdunek K., Alfermann W. 1992. Initiation of shoot organ cultures of *Hypericum perforatum* and formation of hypericin derivatives. *Planta Medica*. 58: 621-625.
- Zobayed S.M.A., Murch S.J., Rupasinghe H.P.V., Saxen P.K. 2004. *In vitro* production and chemical characterization of St. John,s wort (*Hypericum perforatum* L. cv "New Stem"). *Plant Science*. 166: 333-340.
- Zolfagari-Nasab R., Khosroshahli M., Girigorian V., Motallebi-Azar A. 2004. Investigation on in vitro propagation of apricot x Plum natural hybrid. *Iranian J. Hort. Sci. Tech*. 5: 81-92.