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Full Length Research Paper

Increasing gland number and red pigments in St. John's wort *in vitro* culture: Influence of mannitol, sucrose and hydrolyzed casein

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In order to develop a protocol for increasing the gland number and red pigments of *Hypericum perforatum*, this study was carried out to evaluate the effect of hydrolyzed casein (0.0 and 500 mg l^{-1}), mannitol (0.0, 5 and10 g l^{-1}) and sucrose (20 and 30 g l^{-1}) on the synthesis of these pigments and glands on the produced leaves. Leaf discs of *in vitro* plantlets, were prepared and cultured on MS medium with 0.5 mg l^{-1} BAP to induce the shoot. All the cultures were incubated in the dark at 25 ± 2°C for 1 month. In all of the treatments, callus and shoot induction were observed. Percentage of calli and leaves containing red pigments, number of glands and percentage of leaves containing gland were noted as indicating the presence of hypericin and pseudohypericin pigments. Percentage of calli and leaves containing red pigments were significantly influenced by different concentrations of the hydrolyzed casein, mannitol and sucrose. The highest percentage of calli containing red pigments was observed in the culture medium which had 500 or 0.0 mg l^{-1} hydrolyzed casein and 20 g l^{-1} sucrose, without mannitol. Glands were observed on all the produced leaves. Number of glands and percentage of leaves containing gland were significantly influenced by the different concentrations of mannitol and sucrose and their interaction. The highest number of gland and percentage of leaves containing gland was achieved when explants were cultured in medium that included 30 g l^{-1} sucrose with 5 or 10 g l^{-1} mannitol and in medium containing 20 g l^{-1} sucrose, with 5 g l^{-1} mannitol. Morphological changes induced by carbon source and hydrolyzed casein were observed and described in detail. The obtained results will be applied in experimental botany and in the technology of *H. perforatum* cultivation for pharmaceutical applications.

Key words: Hydrolyzed casein, hypericin, *Hypericum perforatum*, mannitol, pseudohypericin, sucrose.

INTRODUCTION

St. John's wort (*Hypericum perforatum* L.) which its habitat is Europe, North Africa and west Asia (Gillett and Robson, 1981) has been used as remedy in the treatments of psychological and nervous disorders, gastritis and pulmonary hemorrhage (Filandrinos et al., 2006). *Hypericum* species have some of the biological compounds like naphtodiantrones, phloroglucinols, flavonoids, procyanidins, tannins, phenyl proponoids, xanthones and other soluble compounds in water (Greeson et al., 2001). Among them, naphtodianthrones such as hypericin and pseudohypericin are of pharmaceutical interest. They are localized in the small black glandular structures located on flower petals, stamens, leaves and stems (Jensen et al., 1995). Hypericin and pseudohypericin are photodynamic pigments, produced from dimerized emodin anthrone, presumably via phenol oxidation further oxidized to hypericins (Falk, 1999). Chemical synthesis of hypericins is hardly executable and economically unprofitable. Therefore, many attempts to obtain them both *in vivo* and *in vitro* have been undertaken. All this makes topical the production of long-term cell of *H. perforatum* cultures capable of biosynthesis of secondary metabolites *in vitro* (Vinterhalter et al., 2006). Many authors focused their attention on the synthesis of bioactive compounds under different

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conditions of cultivation (dark conditions and growth regulators influenced growth and synthesis of hypericin in *H. perforatum*) and also, to their distribution in plants in different ontogenetic stages of plants having different activity of metabolism (Kirakosyana et al., 2000; Vinterhalter et al., 2006; Falk, 1999). It was also reported that the biosynthesis of hypericins is connected with the morphogenesis and formation of dark red colored oil glands on leaves of the intact plant. Stimulation of the production of hypericins by mannan in *H. perforatum* shoot cultures was also reported (Kirakosyana et al., 2000). The seedlings exposed to 25 or 50 mmol/l nickel showed a stunted growth habit and a greater proliferation of small red glands of pigmentation characteristic of hypericin in St. John's wort than those observed on the control seedlings. Nickel contamination affects growth and secondary metabolite composition of St. John's wort (Anna and Podstolski, 2007). Red-pigmented dots, characteristic for hypericin first, were observed in developing shoots and corresponding to oil glands on leaf surfaces. Results showed that low levels of BA (0.1 to 1.0 mg l^{-1}) stimulated dark oil gland formation and significantly increased hypericin and pseudohypericin production in shoots. Shoot hypericin content was related to leaf morphogenesis and apparition of dark oil glands. Such a relationship between the biosynthesis of hypericin and pseudohypericin and morphogenesis, formation of dark colored oil glands on the margins of leaves and flower petals, have already been reported for field grown plants (Zdunek and Alfermann, 1992) (Gadzovska et al., 2005). Hypericin and pseudohypericin contents were examined in compact red or brownish callus cultured with 3.0 to 5.0 mg l^{-1} BAP. Red and brown calli were obtained using 3.0 to 5.0 mg l^{-1} BAP. High BAP levels (4.0 to 5.0 mg l^{-1}) increased the naphthodianthrone production (Gadzovska et al., 2005). Callus formation and red pigmentation from leaf disc were occurred on 30 g.l^{-1} of sucrose (Ayan et al., 2005). Non significant color changes of the leaf color to red were observed at 20 g l^{-1} sucrose. More color changes (red and in some cases dark red) were occurred at 30 g l^{-1} sucrose and they became evident also in higher parts (leaves, stems) of plants. Generally, the intensity of color change to red leaves and stems appeared at lower concentrations of sucrose and PEG than necrosis of leaves and stems (the color changes in plant organs are primary effects followed by necrotization with increasing concentration of sucrose and PEG (Pavliak et al., 2007).

The aim of this work was to improve the production of red pigments and gland leading to the increasing production of hypericin and pseudohypericin by using a combination of different concentrations of hydrolyzed casein, sucrose and mannitol.

MATERIALS AND METHODS

The seed surfaces of *H. perforatum* were sterilized by immersion in 75% ethanol solution for 30 s, followed by immersion in a 5% (v/v) solution of sodium hypochlorite (10 min) and then, rinsed three times with sterile distilled water. Sterile seeds were germinated on hormone free MS medium in jar. Elongated shoots were used as the experimental material for subsequent experiments.

Leaf discs of *in vitro* plantlets, were prepared in the Tissue Culture Laboratory of Horticultural Sciences, University of Tabriz and were cultured after cutting to 4 to 5 mm pieces from the posterior surface of the leaf on modified MS media supplemented with 0.5 mgl^{-1} BAP and were incubated in the dark at 25°C. Every 30 days, subculture was performed. Percentage of calli and leaves containing red pigments, number of glands and percentage of leaves containing gland were noted. The usable factors were hydrolyzed casein (0.0, 500 mg l^{-1}), mannitol (0.0, 5 and10g l^{-1}) and sucrose (20 and 30 g l^{-1}). This study was carried out by factorial experiment based on completely randomized design with five replications. Each experimental unit (Petri dish) included 5 explants. Data obtained from this study were analyzed using SPSS software Ver.16. Comparison of means was done by Duncan's multiple range tests at 5% probably level.

RESULTS AND DISCUSSION

In all of the culture media supplemented with hydrolyzed casein, sucrose and mannitol (with 0.5 mgl^{-1} BAP), callus induction began in the 7th to 8th day after culture. Calli started to grow in 14th to 20th day and their diameter varied from 10 to 20 mm (Figure 1). The calli were soft, green and yellow with red or brown dots in all of the treatments (Figure 2). Godzovska et al. (2005) succeeded in calli production on culture medium including 2 mgl^{-1} BAP and the produced calli were soft, small and green or brown. Adventitious shoot induction was observed in all of the treatments in 20 to 30 days after culture. After induction and development of shoot, calli growth stopped and lateral shoots were produced after one month on the induced shoots. The result of this process was cluster or mass shoots on the calli. Shoot regeneration was successfully done in all of the treatments. Shoots were green and were sometimes with red dots (Figure 3). In a few explants, direct shoot induction were observed (Figure 4). Glands were observed in all of the leaves. Glands diameter were 0.1 to 0.2 mm (Figure 5). The mean number of gland on each leaf varied with 7 to 10.

Effects of different concentrations of hydrolyzed casein, sucrose and their interaction was found to be insignificant for calli containing red pigments. Calli containing red pigments were significantly influenced by different concentrations of mannitol and mannitol × sucrose and mannitol × sucrose × hydrolyzed casein interactions ($p < 0.01$). The highest percentage of calli containing red pigments was observed in the culture medium supplemented with 500 or 0.0 mgl^{-1} hydrolyzed casein and 20 gl^{-1} sucrose without mannitol. Therefore, the highest percentage of calli containing red pigments was observed in the media supplemented with 500 or 0.0 mgl^{-1} hydrolyzed casein. So, it was not necessary to add hydrolyzed casein for improving the production of calli containing red pigments. On the other hand, calli containing red

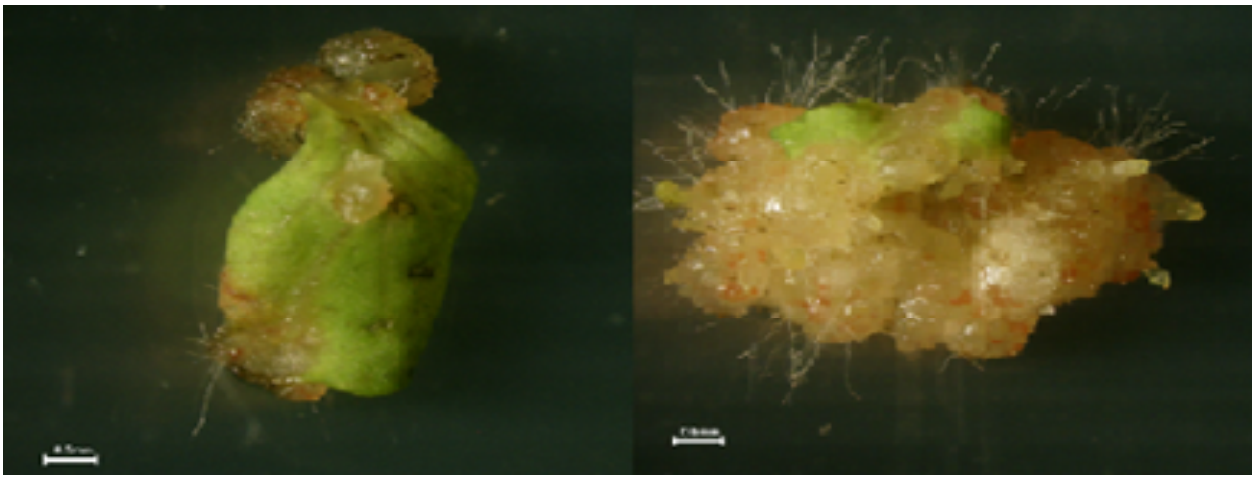

Figure 1. Callus diameter (Left: 5 mm and right: 20 mm)

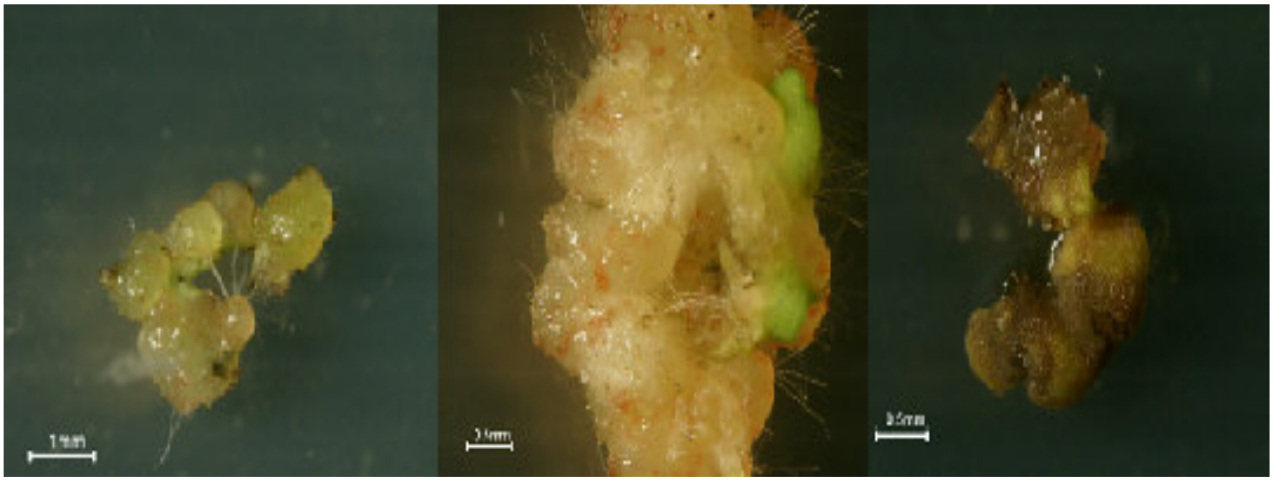

Figure 2. Callus color (Left: green, center: yellow with red dotes; right: brown).

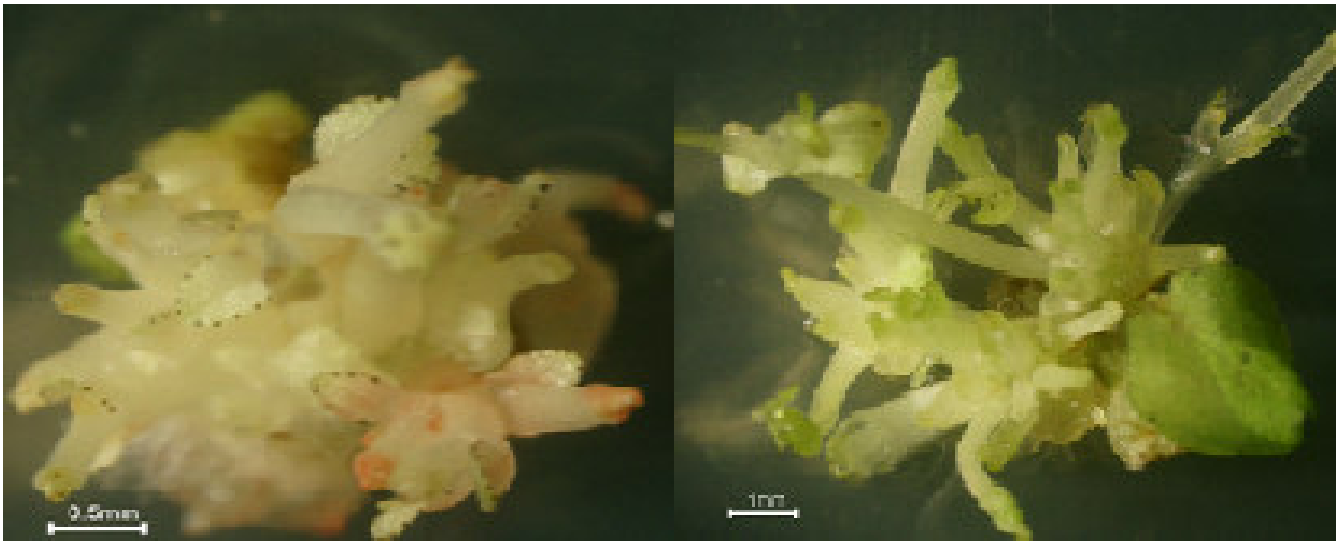

Figure 3. Color of shoots (Left: yellow with red spots; right: green).

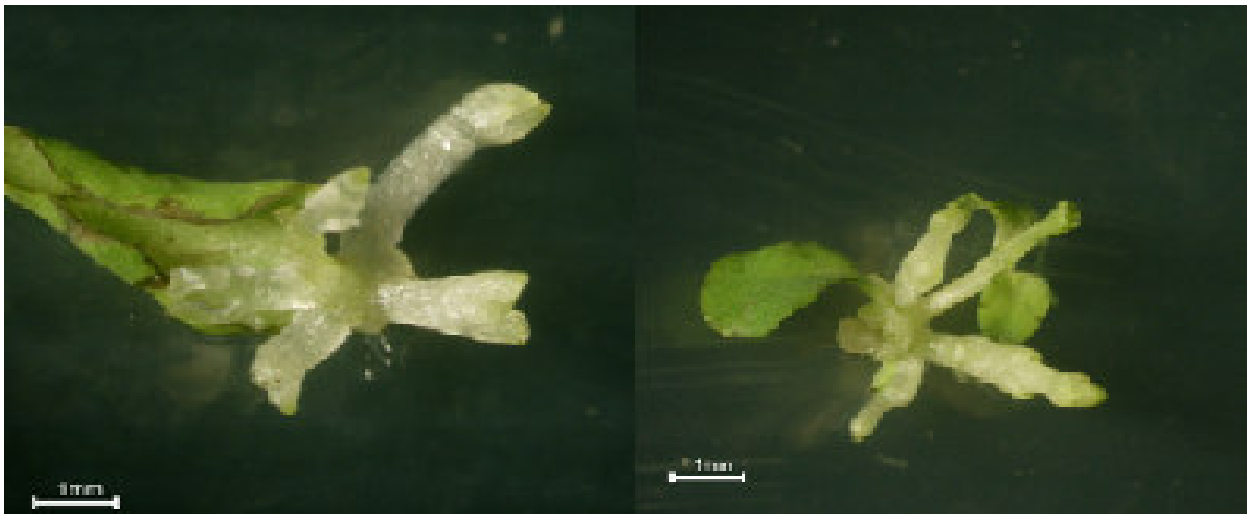

Figure 4. Shoot direct regeneration.

Figure 5. Gland diameter 0.1 to 0.2 mm.

pigments were decreased with increasing amounts of mannitol and minimum calli containing red pigments was observed in the medium containing 10 g l^{-1} mannitol. Calli containing red pigments were not produced in the medium containing 20 g l^{-1} sucrose and 10 g l^{-1} mannitol without the hydrolyzed casein. The reason was probably due to a reduction in the energy source and increase in osmotic pressure. The media without hydrolyzed casein and mannitol (20 or 30 g l^{-1} sucrose) supplied relatively cells requirements for secondary metabolites production, because the calli containing red pigments in the thesis culture media was significantly more than that of the media that included mannitol (Figure 6). In the medium containing 500 mg l^{-1} hydrolyzed casein, the highest percentage of calli containing red pigments was observed in the medium containing 20 g l^{-1} sucrose without

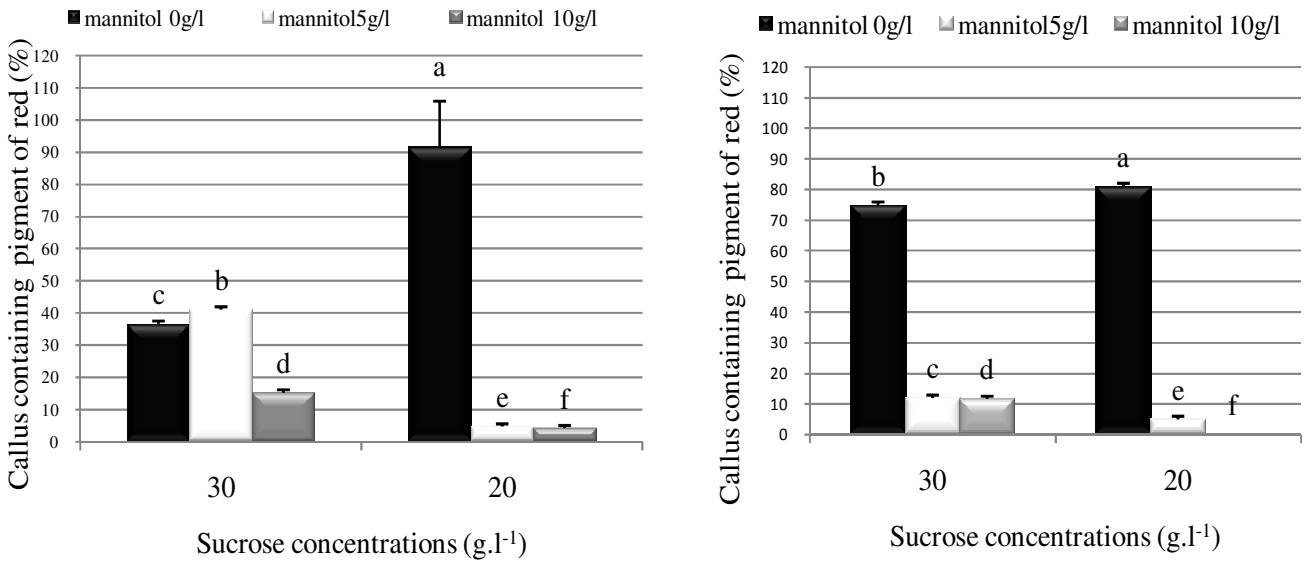

Figure 6- Percentage of calli containing red pigments in different concentrations of sucrose and mannitol as well as hydrolysis casein (right: medium without hydrolyzed casein, left: medium with 500 mg.l-1 hydrolyzed casein).

mannitol. This study showed that calli containing red pigments was reduced in the media containing osmotic materials (mannitol) (Figure 6). Ayan et al. (2005) reported that hypericin is the only pigment that supplies the red color in response to methanol extraction and the majority of calli were green and friable exhibiting dense red pigmented areas that indicate the presence of hypericin. Callus formation and red pigmentation from leaf disc were observed on 30 gl^{-1} of sucrose (Ayan et al., 2005).

The shoot containing red pigments was significantly influenced by different concentrations of mannitol, sucrose, hydrolyzed casein and their interaction ($p < 0.01$). The highest percentage of shoot containing red pigments was observed in culture medium supplemented with 30 gl^{-1} sucrose, without hydrolyzed casein and mannitol and in medium containing 500 mgl^{-1} hydrolyzed casein. The highest percentage of shoot containing red pigments was observed in 20 gl^{-1} sucrose, without mannitol. Significant differences were observed among the different concentrations of mannitol for shoot containing red pigments. So, the highest percentage of shoot containing red pigments was observed in the culture medium supplemented with 30 gl^{-1} sucrose without hydrolyzed casein. But when the medium was without hydrolyzed casein, in the high levels of mannitol, percentage of shoot containing red pigments decreased, so the percentage of shoot containing red pigments was zero in 10 g l^{-1} mannitol. In the media without hydrolyzed casein, the percentage of shoot containing red pigments was zero in the media containing 20 g l^{-1} sucrose in all concentrations of mannitol (Figure 7). In the media containing 500 mg l^{-1} hydrolyzed casein, the highest percentage of shoot containing red pigments was observed in 20 and 30 l^{-1} sucrose without mannitol. But significant differences were observed among the 20 and 30 g l^{-1} sucrose,for shoot containing red pigments. The highest percentage of shoot containing red pigments was observed in 20 g l^{-1} sucrose. So, we can conclude that adding hydrolyzed casein as a source of amino acids, in low concentrations of sucrose, increased percentage of shoot containing red pigments. However, percentage of shoot containing red pigments reached zero with increasing mannitol in both concentrations of sucrose. Generally, the intensity of color change to red leaves and stems appeared at lower concentrations of saccharose and PEG than the necrosis of leaves and stems (the color changes in plant organs are primary effects followed by necrotization with increasing concentration of saccharose and PEG) (Pavliak et al., 2007).

Effects of different concentrations of hydrolyzed casein and their interaction was found to be insignificant for number of glands and the percentage of leaves containing gland. Hydrolyzed casein as an organic complex can be effective on *in vitro* morphogenesis, however, in this work, using it was not effective for number of glands and the percentage of leaves containing gland. Since there is no report on using hydrolyzed casein in medium for number of glands and the percentage of leaves containing gland on *H. perforatum*, then, this study showed that using 500 mg l^{-1} of this material cannot be effective in increasing gland production. Number of glands and the percentage of leaves containing gland was significantly influenced by different concentrations of mannitol, sucrose and their interaction ($p < 0.01$). Percentage of leaves containing gland in culture media containing 30 g l^{-1} sucrose was significantly more than that of the medium that included 20 g l^{-1} sucrose. Addition

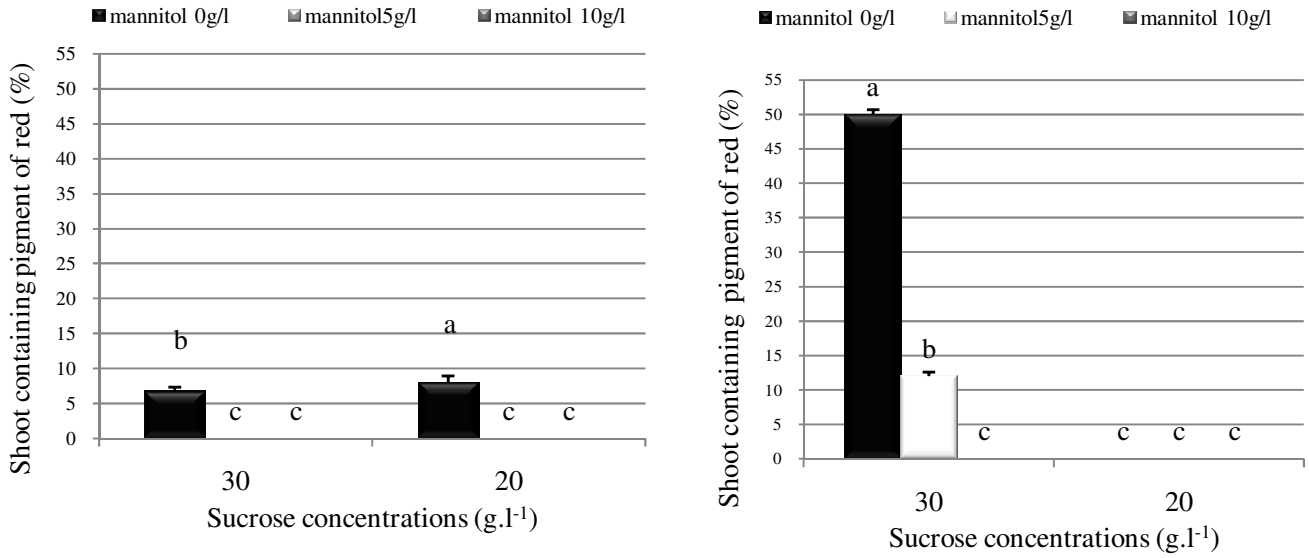

Figure 7. Percentage of shoots containing red pigments in different concentrations of sucrose and mannitol as well as hydrolysis casein (right: medium without hydrolyzed casein, left: medium with 500 mg.l-1 hydrolyzed casein).

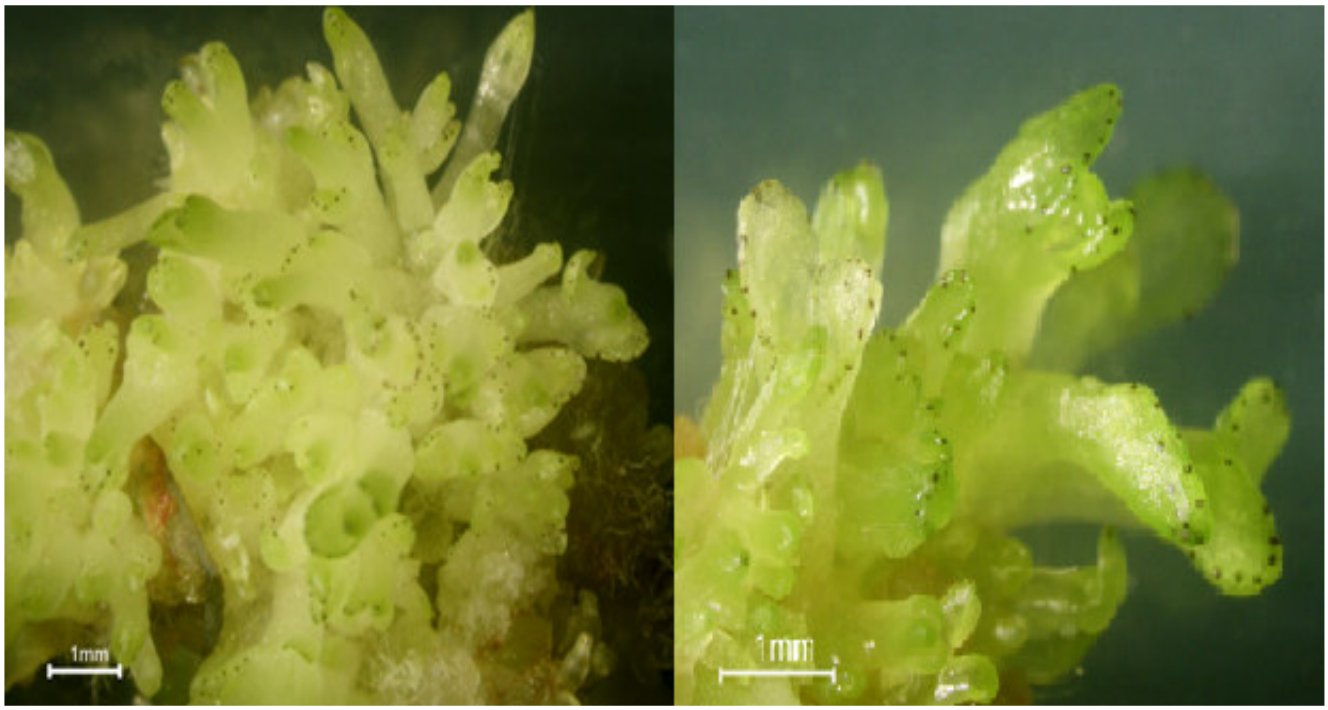

Figure 8. Leaves containing gland in medium containing 5 or 10 g.l^{-1} mannitol and 20 or 30 g.l^{-1} sucrose.

of mannitol to the media that contained 30 g l^{-1} sucrose affected gland number. Although, insignificant differences were observed among 5 and 10 g l^{-1} mannitol for gland number, in the medium containing 20 g l^{-1} sucrose, the highest gland number was observed in 5 g.l^{-1} mannitol (Figures 8, 9 and 10). Thus, it can be concluded that the presence of one carbohydrate source as nutrition was (30 g l^{-1} sucrose) enough in medium; using osmotic materials (alcohol sugars such as mannitol) will improve gland number in *H. perforatum*. The results showed that low levels of BA (0.1 to 1.0 mg l^{-1}) stimulated dark oil gland formation and significantly increased hypericin and pseudohypericin production in the shoots.

The number of shoots containing gland (after 3 months)

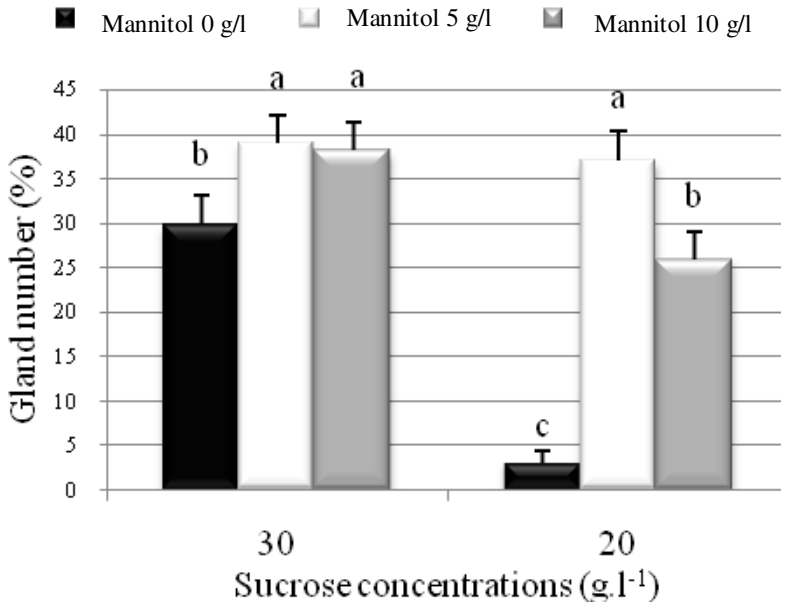

Figure 9. Number of gland in different concentrations sucrose and mannitol.

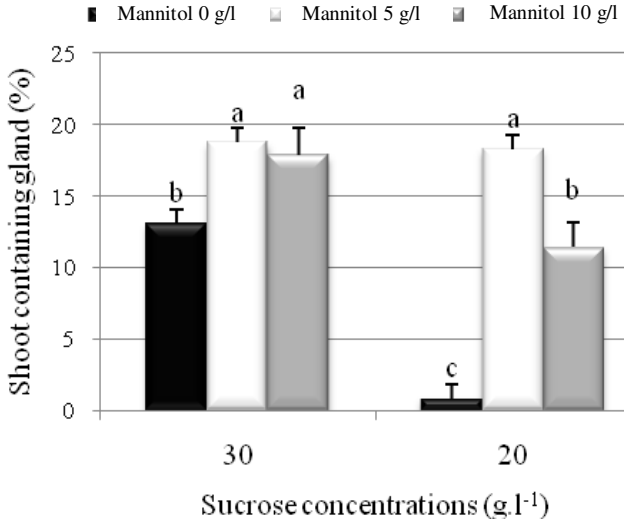

Figure 10. Number shoots containing gland in different concentrations sucrose and mannitol.

were affected by mannitol concentrations ($P < 0.01$). The highest number of shoots containing gland was observed in the medium containing 5 and 10 g l^{-1} mannitol indicating that mannitol increased osmotic pressure, in response it increased the number of dark gland in the leaf surfaces (Figures 11 and 12).

Conclusions

In this study, we produced mass or cluster shoot containing gland without using high amount of hormones. It was observed that adding different concentrations of mannitol and sucrose affected the calli and shoot containing red pigments, number of glands and the percentage of leaves containing gland. Hydrolyzed casein had no significant effect on number of glands and the percentage of leaves containing gland. On the other hand, number of shoots containing gland increased by using mannitol. The addition of mannitol in proper concentration in the medium had positive effect on calli and shoot containing red pigments, number of glands and

Figure 11. Shoots containing red pigments (after 3 months).

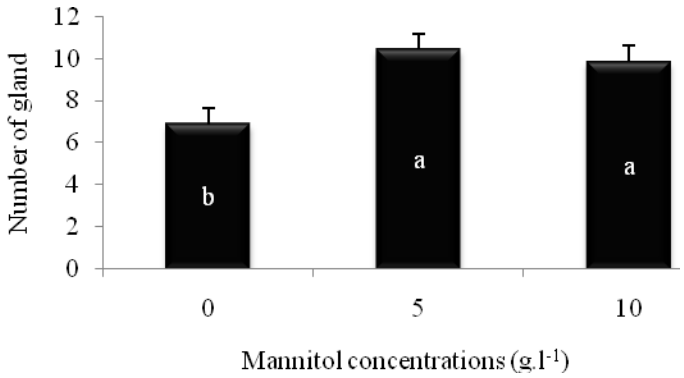

Figure 12. Number of gland in different concentrations mannitol.

the percentage of leaves containing gland. Using sucrose in the media as nutrient source was found to be essential.

The results improved our knowledge of the behavior of *H. perforatum* under osmotic pressure. The results could be very helpful in experimental botany and in *in vitro* production of *H. perforatum* for the pharmaceutical industry and traditional medicine.

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