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Original article

The effect of temperature and pH on biomass and bioactive compounds production in *Silybum marianum* hairy root cultures

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Abstract

Background and objectives: The seed extract of *Silybum marianum* contains seven flavonolignans known collectively as silymarin. These metabolites can be produced in hairy root cultures of *S. marianum*. The effect of different physical factors can change root biomass and silymarin production which has been investigated in the present study. **Methods**: The effect of different physical factors of temperature (30 ºC/25 ºC, 25 ºC/25 ºC and 15 ºC/20 ºC in 16 h/8 h cycle) and pH (5, 5.7, 6 and 7) were evaluated with respect to the root biomass and silymarin production in hairy root cultures of the plant. **Results**: Incubation temperature, 25 °C /25 °C promoted the silymarin production in 4-week old hairy roots (0.18 mg/g DW) as compared with the cultures treated with 15 °C/20 °C and 30 °C/25 °C (0.13 and 0.12 mg/g DW, respectively). Maximal increases in biomass and silymarin accumulation occurred in the root cultures grown in pH 5 and 25 °C/25 °C (0.45 g and 0.26 mg/g DW). The content of silybin, isosilybin, silychristin, silydianin were 0.025, 0.024, 0.061 and 0.095 mg/g DW, respectively which were higher than those grown in higher pH. **Conclusion**: The results of the present study suggest that 25 °C/25 °C and acidic environment of medium are beneficial for silymarin production using hairy root cultures. Furthermore, lipoxygenase (LOX) activity was strongly affected by pH which suggested that acidic environment may act as inducing signal for LOX activity and subsequently greater silymarin production.

Keywords: hairy root, pH, *Silybum marianum*, silymarin, temperature

Introduction

Silybum marianum (L.) Gaertn known as milk thistle belongs to Asteraceae family which has been used in medicine for more than 2000 years. The seed extract of this plant contains seven distinct flavonolignans namely silybin A, silybin B, isosilybin A, isosilybin B, silydianin, silychristin, isosilychristin; and one flavonoid taxifolin known collectively as silymarin [1,2]. The hepatoprotective role and antiproliferative effects in cultured cancer cells were recognized for this herbal medicine [3,4].

Hairy roots, caused by the infection of wounded higher plants with *Agrobacterium rhizogenes*, can stably and extensively produce secondary

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metabolites in spite of the low yield of plant secondary metabolites in plant cell cultures [5-9]. Since the secondary metabolite productivity is affected by several physical factors like temperature and pH, to develop a successful industrial-scale plant cell and tissue culture system stably producing secondary metabolites, an optimum culture system is the first requisite [10]. Several studies have indicated that the optimal temperature treatment is necessary for accumulation of biomass and production of metabolites in suspension cultures [11]. Moreover, the pH profiles are characteristic of plant cell/organ cultures that were considered as an important factor influencing cell growth and secondary metabolite accumulation [12]. Changes in medium pH can change the permeability of cell membranes, resulting in the release of products into the culture medium [13]. Abiotic and biotic stresses irritate cell defense system, resulting in damage to cells. Plant cells protect themselves from such toxic agents by engaging antioxidant enzymes. Little is known about silymarin production pathway and it is not clear what factors affect this signal transduction pathway [14]. The present article has discussed the effect of different physical factors of temperature and initial pH with respect to the root biomass and silymarin production in hairy root cultures of *S. marianum.*

Experimental

Hairy root culture

Hairy root culture of *S. marianum* used in this study was transformed by *A. rhizogenes* (AR15834), and the genetic transformation of these hairy roots was confirmed by polymerase chain reaction (PCR) according to the method described by Rahnama *et al.* [14]. All the experiments were carried out in shake-flask cultures with 100-mL Erlenmeyer flasks on orbital shaker set at 150 rpm and incubated in the dark. Each flask contained 50 mL liquid Murashige and Skoog (MS) medium (Duchefa, Netherlands) supplemented with 30 g/L sucrose and 0.1 mg/L NAA (naphthalene acetic acid) (Duchefa., Netherlands). The medium was inoculated with six 1 cm pieces of roots from 30-days old cultures [15].

Level of pH and temperature regime

In the first experiment, effect of temperature on hairy root growth and silymarin accumulation was studied and cultures were maintained at differential temperatures such as 30 $^{\circ}$C/25 $^{\circ}$C, 25 $^{\circ}$C/25 $^{\circ}$C and 15 $^{\circ}$C/20 $^{\circ}$C in 16 h/8 h cycle for 30 days. In the second experiment, the media pH was adjusted to 5, 5.7, 6 and 7 before autoclaving and cultures were harvested after 30 days.

Extraction and determination of silymarin

Hairy roots harvested from the shake-flasks and dried by tissue paper were measured in terms of fresh weight (FW). Lyophilized powdered hairy root samples were measured in terms of dry weight (DW) (Freez dryer, Operon Co., Korea). The samples were defatted with petroleum ether. The flavonolignans were extracted from the dried residue with 10 mL of methanol at 40 ºC for 8 h. The methanol solution was concentrated to a dry residue. The extract was dissolved in 2 mL of methanol and kept at 4 ºC in darkness [15,16]. Silymarin was quantified by high performance liquid chromatography (HPLC) analysis as described by Hasanloo *et al.* on a Knauer liquid chromatography equipped with a Knauer injector with a 20 µL loop, a Nucleosil C18 (250 × 4.6 mm, 5 µ) column, Knauer K2600A UV detector) [17,18]. The mobile phase consisted of the solvents; acetonitrile: water (40:60) with 10% (v/v) H_3PO_4, (pH 2.6). All solvents and chemicals were of HPLC grade (Merck). The elution time and flow rate were 30 min and 1 mL/min and peaks were detected at 288 nm. Identification was achieved by comparison of retention times (Rt) of standards of silymarin, silycrstin (SCN), silydianin (SDN), silybin (SB), taxifolin (TXF) and a standard mixture of silymarin [17,18]. Standards of silymarin, SB and TXF were purchased from Sigma-Aldrich (Germany); SCN

and SDN were obtained from Phytolab (Germany) [17,18].

Antioxidant enzymes activities assay

Guaiacol peroxidase (G-POD) and ascorbate peroxidase (APX) were extracted by homogenising the hairy root (0.5 g FW) with 0.05 M phosphate buffer (pH 7.0) containing 1 mM sodium bisulphite, and 5% polyvinylpyrrolidone. The homogenate was centrifuged for 30 min at 15,000 g and 4 °C and then the supernatant was collected as the crude enzyme extract. G-POD activity, with guaiacol as a substrate, was assayed by the method of Chance and Maehlyin a reaction mixture, containing enzyme extract, 0.1 M phosphate buffer (pH 7.0), 70 mM H_2O_2, 10 mM guaiacol and double distilled water [19]. The increase in absorbance at 470 nm due to the guaiacol oxidation was recorded for 3 min. APX activity, with ascorbate as the substrate, was assayed by the method of Nakano and Asada in a reaction mixture, containing enzyme extract, 2 mM H_2O_2 and 0.5 mM ascorbate [20]. The decrease in absorbance at 290 nm due to the ascorbate oxidation was recorded for 3 min. The results were reported base on ΔOD/min g FW.

Extraction and assay of lipoxygenase (LOX)

For the lipoxygenase activity assay, the roots were homogenized in an ice bath with 0.1 M Trise HCl buffer (pH 8.5) containing 1% polyvinylpyrrolidone (PVP; w/v), 1 mM $CaCl_2$, 5 mM dichlorodiphenyltrichloroethane (DTT), and 10% (v/v) glycerol. The homogenate was centrifuged at 11000 g for 20 min at 4 ºC, and the supernatant was used as the enzyme extract. Lipoxygenase was assayed according to Axelroad *et al*. [21]. Fifty mg of linoleic acid was added to 50 mg Tween 20 and mixed with 10 mL of Na_2HPO_4 buffer (0.1 M, pH 8.7) by stirring and ultrasonic dispersion. The solution was cleared by addition of 250 mL of 1 M NaOH and diluted to 25 mL with the buffer. One mL of the enzyme reaction mixture contained 50 mL enzyme extract, 0.95 mL Na_2HPO_4 buffer and 5 mL of substrate solution. The increase in absorbance was monitored at 234 nm. Total protein was assayed according to Bradford and the results reported base on ΔOD/mg protein min [22].

Statistical analysis

The data are given as the mean of at least three replicates. Statistical analysis was performed with SAS software (version 6.2) using ANOVA method with Duncan test set at $\alpha' \leq 0.05$.

Results and Discussion

Figure 1 shows the changes in root growth and silymarin production in relation to temperature regimes. The dry weight of hairy roots was 0.26 g in the cultures incubated under 25 °C/25 °C and 15 °C/20 °C in 16 h/8 h cycle which was higher than that of the treatment under 30 °C/25 °C (0.09 g).

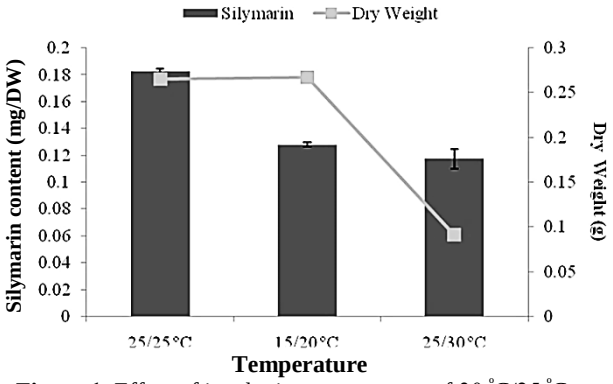

Figure 1. Effect of incubation temperature of 30 °C/25 °C, 25 °C/25 °C and 15 °C/20 °C (with 16 h/8 h cycle) on biomass and silymarin production in hairy root cultures of *Silybum marianum*

Furthermore, there was a slight improvement in the production of silymarin under 25 °C/25 °C (0.18 mg/g DW) which was plummeted to 0.12 and 0.13 mg/g DW under 15 °C/20 °C and 30 °C/25 °C, respectively. The highest TXF, SDN and SBN accumulation were obtained under 25 °C /25 °C (0.045, 0.059 and 0.021 mg/g DW, respectively; table 1).

Table 1. Effect of incubation temperature of 30 °C/25 °C, 25 °C/25 °C and 15 °C/20 °C (with 16 h/8 h cycle) on flavonolignan content (mg/g DW) in hairy root cultures of *Silybum marianum*

Growth temperature (ºC)	Flavonolignans (mg/g DW)				
	TXF	SCN	SDN	SB	ISB
15/25	0.017±0.003	0.053±0.004	0.037±0.002	0.013±0.002	0.013±0.002
25/25	0.045±0.003	0.047±0.000	0.059±0.003	0.021±0.002	0.01±0.001
25/30	0.023±0.006	0.045±0.010	0.044±0.010	0.009±0.001	0.01±0.002

ISB - isosilybin; SBN - silybin; SCN - silychristin; SD - silydianin; TXF - taxifolin

Table 2. Effect of pH levels (5, 5.7, 6 and 7) on flavonolignan content (mg/g DW) in hairy root cultures of *Silybum marianum*

pH	Flavonolignans (mg/g DW)				
	TXF	SCN	SDN	SB	ISB
5	0.044±0.002	0.061±0.001	0.095±0.003	0.025±0.002	0.024±0.004
5.7	0.045±0.003	0.047±0.000	0.059±0.003	0.021±0.002	0.01±0.001
6	0.038±0.001	0.039±0.003	0.046±0.014	0.021±0.001	0.009±0.001
7	0.024±0.001	0.04±0.004	0.082±0.001	0.017±0.001	0.012±0.002

ISB - isosilybin; SBN - silybin; SCN - silychristin; SD - silydianin; TXF - taxifolin

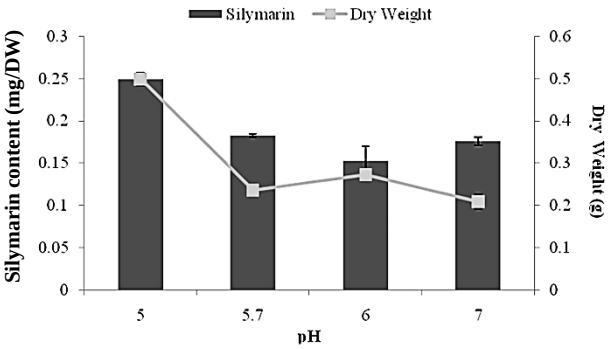

Figure 2. Effect of pH levels (5, 5.7, 6 and 7) on biomass and silymarin production in hairy root cultures of *Silybum marianum*

Hairy root cultures were grown in four different pH levels (5, 5.7, 6 and 7) under 25 °C/25 °C for 30 days (figure 2). It can be seen that the highest dry biomass (0.50 g) was recorded when the hairy roots were grown in pH 5 compared to pH 5.7, 6 and 7 (0.24, 0.27 and 0.21 g). Maximum silymarin concentration (0.25 mg/g DW) was obtained in cultures treated with pH 5, whereas the silymarin accumulation was 0.18, 0.15 and 0.18 mg/g DW in pH 5.7, 6 and 7, respectively. The content of silybin, isosilybin, silychristin, silydianin were 0.025, 0.024, 0.061, 0.095 mg/g DW, respectively in hairy root cultures with pH 5 which were higher than those grown in higher pH (table 2).

G-POD and APX activities were measured in hairy root cultures grown in different pH levels (5, 5.7, 6 and 7) (table 3). G-POD and APX were 3.82 and 1.45 ΔOD/min g FW in the cultures treated with pH 5 which were lower than that of treated cultures in pH 5.7, 6 and 7. In order to establish the involvement of LOX in the lipid peroxidation process, its activity in the samples treated with different pH levels measured (figure 3). It was found that the LOX activity was greatly stimulated at pH 5 (59.07 ΔOD/min mg protein), while it was significantly plunged to 5.46, 6.36 and 2.79 ΔOD/min mg protein in hairy roots cultured at pH 5.7, 6 and 7, respectively.

For optimization of silymarin production by hairy root cultures of *S. marianum*, the effect of different temperature regimes and pH levels was investigated in flasks. As it was indicated, the silymarin content was optimal under 25 °C/25 °C in 16 h/8 h cycle which is similar to a previous report on anthocyanin production by suspended

Table 3. Effect of pH levels (5, 5.7, 6 and 7) on guaiacol peroxidase and ascorbate peroxidase (ΔOD/min g FW) activities in hairy root cultures of *Silybum marianum*

Antioxidative enzyme activity (ΔOD/ming FW)	pH			
	5	**5.7**	**6**	**7**
G-POD activity	3.82±0.31	14.85±0.23	8.95±0.57	7.18±0.70
APX activity	1.45±0.13	8.95±0.68	6.76±2.60	3.36±0.14

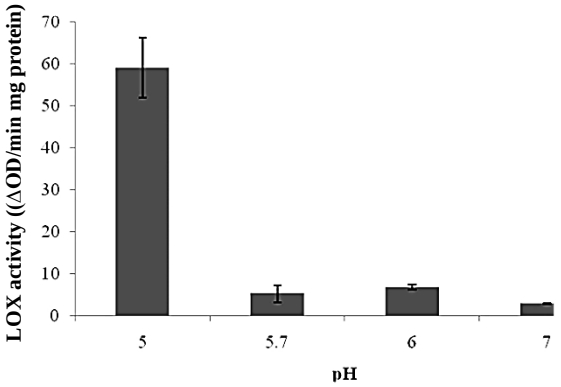

Figure 3. Effect of pH levels (5, 5.7, 6 and 7) on lipoxygenase activity in hairy root cultures of *Silybum marianum*

cultures of *Perilla frutescens* [9]. The lower level of silymarin was observed in relatively high temperature regime of 30 °C/25 °C which resembled the remarkable reduction of anthocyanin production at the high temperature of 28 ºC [11]. In contrary to our findings, ajmalicine production was found to be optimal at 27.5 °C in *Catharantus roseus* suspension cultures [21] and also Yu *et al*. revealed that biomass accumulation and ginsenoside production appeared to be optimal under 20 ºC/13 ºC day (12 h)/night (8 h) cycle [24]. Wu and Zhong suggested that the optimal temperature for ginseng cells is in the range of 24-28 ºC for most strains, and the growth is either slow at lower temperatures or completely inhibited at higher temperatures [25]. Moreover, the acidic environment of root cultures in pH 5 was beneficial for the root growth and silymarin accumulation. These results are in agreement with the earlier research by Merkli *et al*. where an improved growth and greater biosynthesis of diosgeninin hairy root cultures of *Trigonella foenum-graecum* was observed at pH 5 as compared to pH level of 5.5 and 5.9 [6]. Furthermore, a low pH (4.6) could enhance root growth and plumbagin accumulation in 20-day *Plumbago indica* roots [26]. However, pH shift was used by Taya *et al.* and Mukundan *et al*. to release products normally stored in vacuoles, which can be attributed to cell damage, probably at the vacuolar membrane [27, 28]. For instance, short exposure to reduce pH was beneficial for releasing up to 50% of the betalains to culture medium of *Beta vulgaris* [28]. From the results on ginseng hairy root culture [12], the initial pH of the medium should be controlled in the range of 6.0-6.5 before autoclaving, whereas hairy root growth and ginsenoside production were strongly inhibited when the initial pH was below 4.0 or above 7.0. When the pH is too low, levels of aluminum and hydrogen in the medium are toxic to the root systems of ginseng. If the pH is too high, micronutrients may be bound in forms unavailable for hairy root uptake.

To determine how acidic environment could stimulate silymarin accumulation in hairy root cultures, the antioxidative enzymes and lipoxygenase activities were assayed in pH-treated hairy root cultures. Environmental stresses result in an increased formation of toxic active oxygen species which may cause breakdown of membranes and lipid peroxidation [29, 30]. The results obtained in this study indicated that the G-POD and APX were not activated in the cultures maintained at pH 5 in comparison to other treated roots. Although, induced activity of LOX in acidic medium suggested that lipid peroxidation was initiated by LOX under this condition, which converts fatty

acids to their corresponding hydroperoxides leading to accumulation of silymarin. The present results support the hypothesis that the jasmonate signaling pathway is involved in the induced production of silymarin by activation of lipoxygenase in acidic media. In the present work, based on the above results we propose that the biomass and silymarin accumulation is a pH and temperature dependent process.

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Declaration of interest
The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

References

[1] Davis-Searles PR, Nakanishi Y, Kim NC, Graf TN, Oberlies NH, Wani MC, Wall ME, Agarwal R, Kroll DJ. Milk thistle and prostate cancer: differential effects of pure flavonolignans from *Silybum marianum* on antiproliferative end points in human prostate carcinoma cells. *Cancer Res*. 2005; 65 (10): 4448-4457.

[2] Kim NC, Graf TN, Sparacino CM, Wani MC, Wall ME. Complete isolation and characterization of silybins and isosilybins from milk thistle (*Silybum marianum*). *Org Biomol Chem*. 2003; 1(19): 1684-1689.

[3] Flora K, Hahn M, Rosen H, Benner K. Milk thistle (*Silybum marianum*) for the therapy of liver disease. *Am J Gastroenterol*. 1998; 93(2): 139-143.

[4] Tyagi A, Bhatia N, Condon MS, Bosland MC, Agarwal C, Agarwal R. Antiproliferative and apoptotic effects of silibinin in rat prostate cancer cells. *Prostate*. 2002; 53(3): 211-217.

[5] Rhodes MJC, Robins RJ, Hamill JD, Parr AJ, Hilton MH, Walton NJ. Properties of transformed root culture. *Phytochem Soc Eur*. 1990; 1(3): 201-225.

[6] Merkli A, Christen P, Kapetanidis I. Production of diosgenin by hairy root cultures of *Trigonella foenum-graecum* L. *Plant Cell Rep*. 1997; 16(9): 632-636.

[7] Kittipongpatana N, Hock RS, Porter JR. Production of solasodine by hairy root, callus, and cell suspension cultures of *Solanum aviculare* Forst. *Plant Cell Tiss Org*. 1998; 52(3): 133-143.

[8] Hasanloo T, Eskandari S, Kowsari M. Trichoderma strains- *Silybum marianum* hairy root cultures interactions. *Res J Pharmacogn.* 2015; 2(2): 33-46.

[9] Hasanloo T, Eskandari S, Najafi F. Chitosan (middle-viscous) as an effective elicitor for silymarin production in *Silybum marianum* hairy root cultures. *Res J Pharmacogn*. 2014; 1(1): 9-13.

[10] Jeong CS, Chakrabarty D, Hahn EJ, Lee HL, Paek KY. Effects of oxygen, carbon dioxide and ethylene on growth and bioactive compound production in bioreactor culture of ginseng adventitious roots. *Biochem Eng J*. 2006; 27(3): 252-263.

[11] Zhong JJ, Yoshida T. Effects of temperature on cell growth and anthocyanin production by suspension cultures of *Perilla frutescens* cells. *J Ferment Bioeng*. 1993; 76(6): 530-531.

[12] Sivakumar G, Yu KW, Hahn EJ, Paek KY. Optimization of organic nutrients for ginseng hairy roots production in large-scale bioreactors. *Curr Sci*. 2005; 89(4): 641-649.

[13] Saenz-Carbonell LA, Maldonado-Mendoza IE, Moreno-Valenzula O, Ciau-Uitz R, Lopez-Meyer M, Loyola-Vargas VM. Effect of the medium pH on the release of secondary metabolites from roots of *Datura stramonium*, *Catharanthus roseus*, and *Tagetes patula* cultured *in vitro*. *Appl Biochem Biotech*. 1993; 38(3): 257-267.

[14] Rahnama H, Hasanloo T, Shams MR, Sepehrifar R. Silymarin production in hairy root culture of *Silybum marianum* (L.) Gaertn. *Iran J Biotechnol.* 2008; 6(2):113-118.

[15] Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*. 1962; 15(3): 473-497.

[16] Cacho M, Moran M, Corchete P, Fernandez-Tarrago J. Influence of medium composition on the accumulation of flavonolignans in cultured cells of *Silybum marianum* (L.) Gaertn. *Plant Sci*. 1999; 144(2): 63-68.

[17] Hasanloo T, Khavari-Nejad RA, Majidi E, Shams-Ardakani MR. Analysis of flavonolignans in dried fruits of *Silybum marianum*(L.) Gaertn from Iran. *Pak J Biol Sci*. 2005; 8(12): 1778-1782.

[18] Hasanloo T, Khavari-Nejad RA, Majidi E, Ziai SA, Shams-Ardakani MR. Determination of flavonolignan of dried fruits of *Silybum marianum* L. Gaertn collected from different areas of Iran by spectrophotometer, TLC and HPLC. *J Med Plant*. 2005; 4(1): 25-32.

[19] Chance B, Maehly AC. *Assay of catalases and peroxidases*. In: Colowick SP, Kaplan NO, editors. *Methods in enzymology*. New York: Academic Press, 1955.

[20] Nakano Y, Asada K. Purification of ascorbate peroxidase in spinach chloroplasts; its inactivation in ascorbate depleted medium and reactivation by monodehydroascorbate radical. *Plant Cell Physiol*. 1987; 28(1): 131-140.

[21] Axelroad B, Cheesbrough TM, Laakso S. Lipoxygenase from soybeans. *Method Enzymol*. 1981; 17(2): 441-451.

[22] Bradford MA. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72(1-2): 248-254.

[23] Ten Hoopen HJG, Vinke JL, Moreno PRH, Verpoorte R, Heijnen JJ. Influence of temperature on growth and ajmalicine production by *Catharanthus roseus* suspension cultures. *Enzyme Microb Tech*. 2002; 30(1): 56-65.

[24] Yu KW, Murthy HN, Hahn EJ, Paek KY. Ginsenoside production by hairy root cultures of *Panax ginseng*: influence of temperature and light quality. *Biochem Eng J*. 2005; 23(1): 53-56.

[25] Wu J, Zhong JJ. Production of ginseng and its bioactive components in plant cell culture: current technological and applied aspects. *J Biotechnol*. 1999; 68(2-3): 89-99.

[26] Gangopadhyay M, Sircar D, Mitra A, Bhattacharya S. Hairy root culture of *Plumbago indica* as a potential source for plumbagin. *Biol Plantarum*. 2008; 52(3): 533-537.

[27] Taya M, Mine K, Kino-Oka M, Tone S, Ichi T. Production and release of pigments by culture of transformed hairy root of red beet. *J Ferment Technol*. 1992; 73(1): 31-36.

[28] Mukundan U, Bhide V, Singh G, Curtis WR. PH-mediated release of betalains from transformed root cultures of *Beta vulgaris* L. *Appl Microbiol Biot*. 1998; 50(4): 241-245.

[29] Mittler R. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci*. 2000; 7(9): 405-410.

[30] Mittler R, Vanderauwera S, Gollery M, Breusegem FV. Abiotic stress series. Reactive oxygen gene network of plants. *Trends Plant Sci*. 2004; 9(10): 490-498.