

Morphogenetic and biosynthetic potential of *in vitro* grown *Hypericum perforatum* under stress and normal conditions

M. Altaf Wani¹, G.R. Lawania², R.A. Bhat³, Iffiat Fayaz¹, A. Nanda³ and Gazenfar Gani³

¹Division of Plant Breeding and Genetics, SK University of Agricultural Sciences and Technology of Kashmir, Srinagar, India.

²Division of Plant Breeding and Genetics, Allahabad Agricultural Institute-Deemed University, Allahabad, India. ³Division of Floriculture, SK University of Agricultural Sciences and Technology of Kashmir, Srinagar, India.

Abstract

Three different strains of *Hypericum perforatum* viz. HP-1, HP-2, and HP-3 were subjected to different levels of saline stress (0.25, 0.5, 0.75 and 1.0% with NaCl) and high pH regime (8.5, 9.0, 9.5 and 10.0 with NaOH). Gradual loss in callus growth was observed in all the three strains in response to both kinds of stress. However, high pH showed more drastic effect than saline stress. All the three strains showed higher content of pseudohypercin than hypercin. Change in hypercin production was negligible, however remarkable change was observed in pseudohypercin production in response to both kinds of stress. HP-2 strain produced higher content of hypercin than HP-1 and HP-3 strains under normal as well as under stressfull regime. Proteins were affected qualitatively as well as quantitatively. Maximum numbers of proteins were isolated from control cultures at the retention time of five minutes. Among the three strains maximum numbers of proteins were isolated from HP-3 strain. High pH reduced number of proteins to 12 and 3 while salinity increased number of proteins to 42 and 52 in HP-1 and HP-2, respectively due to accumulation of low molecular weight proteins in response to saline stress.

Key words: *Hypericum perforatum*, hypercin, pseudohypercin, hyperforin, HPLC, saline stress

Abbreviations: PMSF- Phenylmethylsulphonyl fluoride, LC/MS-Liquid chromatography/Mass spectrometry

Introduction

Among the medicinal plants which recieved great attention during the recent years from pharmaceutical industry at global level, *Hypericum perforatum* ranks very high due to its secondary metabolites (hypercin and pseudohypercin) which are found to be anti depressant in action (Wentworth and Agostini, 2000). Besides, anti depressant activity *H. perforatum* is gaining popularity due to its antiviral (Lopez and Hudson, 1991), anti retroviral (Lavie *et al.*, 1989) and antibiotic action (Fritz and Schleicher, 1993).

Although vast information is available on cell cultures of *H. perforatum*, however, investigations are required to understand the biosynthetic pathway of hypercin, pseudohypercin and hyperforin production, enhancement of production by media manipulations and induction of gene/ protein alterations by environmental stress (Ramgopal and Carr, 1991; Sairam and Aruna, 2004). Abiotic stress like salinity, pH changes and drought conditions highly influence biomass production, accumulation of secondary metabolites and yield of the most plants. The fast expanding research in plant molecular biology has given several clues in understanding how plant responds under stressful regime (Cushman *et al.*, 1990). Great deal of success has been achieved in unveiling gene / protein alterations associated with preparation of plant against the abiotic stress.

Owing to the medicinal value of *H. perforatum* an experiment was carried out at IIIM (CSIR), Jammu to find out the morphogenetic and biosynthetic potential of *H. perforatum* under saline and alkaline stress and to unveil the alternations in protein profiles in response to stress.

Materials and methods

The calli resulted from shoot tip cultures were transferred to stress induced (saline and alkaline) solid MS culture media and cultivated on a constant temperature of 25±2 °C with 16 hour photoperiod. High pH stress was induced by adjusting pH of culture media to 8.5 (T-1), 9.0 (T-2), 9.5 (T-3) and 10.0 (T-4) with NaOH and saline stress was induced by incorporating different concentrations of NaCl₂ 0.25% (T-6), 0.5% (T-7), 0.75% (T-8) and 1.0% (T-9). The calli developed on this high pH and saline stress media were analyzed for presence and quantification of hypercin, pseudohypercin and hyperforin.

Morphological analysis of callus cultures: Growth was measured in terms of fresh callus weight. All the replicates of all the treatments were harvested at the same time. All cultures were also visually screened for red colour formation and for friability at different stages of growth to assess pigment formation.

Biochemical analysis of callus cultures

Sample extraction for biochemical analysis: Air-dried callus of *H. perforatum* was extracted with chloroform and then filtered. The powder from filter paper was dissolved in acetone and concentrated on rotawave to dryness. This dried material was then dissolved in methanol and analyzed by HPLC after filtration.

HPLC analysis: Presence and quantification of hypercins and pseudohypercins was determined by an HPLC using Gilson system (Gilson Medical Electronics, France) equipped with 321-model pump, operated at room temperature (25±2°C). Separations were performed on Lichosphere RPC-18 (5 µm, 4.6 x 150 mm) and detection was achieved with a UV detector set at 589 nm.

The chromatographic data was recorded and processed on uni-point system software. Isocratic mobile phase was used with methanol, ethyl acetate and water (pH 2.5) in the ratio of 67:16:17 respectively. pH of water was adjusted to 2.5 with O-phosphoric acid. The flow rate was kept constant at 1 mL min⁻¹ and injection volume 25 µL using six point calibration curve generated with authentic hypericin and pseudohypericin.

Sample extraction for protein analysis by LC/MS: Fresh callus was homogenized with 2-3 volume of 0.1M buffer carrying 5mM EDTA. A pinch of PMSF was added during grinding. The homogenized sample was centrifuged at 4°C @ 10,000 rpm for 20 minutes. The protein from supernatant was precipitated with 5 volumes of chilled (-20°C) acetone and all the samples were kept at -20°C for 1-2 hours. The samples were again centrifuged at 4°C @ 10000 rpm for 10 minutes to separate the precipitated proteins from the solution. The acetone was evaporated from the sample and the precipitate was dissolved in 500µL of HPLC grade water. The protein solution was then filtered through 0.2 mesh micro filter.

LC/MS analysis: The extracted samples were analyzed before LC/MS using AGILENT 1100 series HPLC and squin 300 (Bruker/Mass spectrometer) connected by ESI (Electro Spray Ionization) interface using DAD (Diode Array Detector) at wavelength of 280.8 nm. The mobile phase was used with 0.05% TFA in acetonitrile and 0.05% TFA in water. RP-8 column with diameter of 5µm and length of 4x250 mm and flow rate of 0.5 mL min⁻¹ was used. The temperature of column was kept at 30°C.

Results

Response of callus to high pH and NaCl stress: Calli of all three strains investigated showed similar response in terms of callus weight however gradual decrease in callus growth was observed in stress cultures (saline and high pH) as compared to control cultures (devoid of stress). Among the two stresses high pH stress showed more drastic effect than saline stress. No callus growth was observed at pH 9.5 (T₃) and pH 10 (T₄). Further callus showed slight increase in compactness towards the higher concentration of NaCl and decrease in compactness towards the increase in pH.

The visual examination of callus revealed dark brown to reddish brown callus and the intensity of red colour formation showed a slight increase with time. Among the three strains HP-2 strain showed more tendency towards red colour formation. No remarkable effect was shown by high pH on colour formation but NaCl showed slight increase in red colour formation. (Table 1)

Accumulation of hypericin and pseudo hypericin in response to stress: Calli of all the three strains investigated showed

Table 1. Effect of saline and high pH on fresh callus weight/flask (Mean value ± Standard error)

Treatment	HP-1 (g)	HP-2 (g)	HP-3 (g)
T ₁	3.68±0.30	3.89±0.60	3.19 ±0.08
T ₂	1.55±0.18	1.88±0.04	1.03±0.25
T ₃	-	-	-
T ₄	-	-	-
T ₅	5.62±0.22	5.49±0.27	5.27±0.33
T ₆	4.45±0.24	3.87±0.32	3.61±0.33
T ₇	3.89±0.13	2.69±0.28	3.24±0.30
T ₈	3.15±0.35	3.15±0.15	3.77±0.46
T ₉	-	-	-

production of hypericin and pseudohypericin under normal (devoid of stress) as well as under stress conditions (saline and high pH). In the present study, HP-2 strain produced higher content of hypericin (0.272) than HP-1 (0.188) and HP-3 (0.196) under control conditions. There was no remarkable effect of stress on accumulation of hypericin; however pseudohypericin production was highly influenced by both kinds of stress indicating that abiotic stresses has differential effect on biosynthesis of different metabolites. (Table 1).

Inability of undifferentiated cultures to produce flavonoids under both normal and stress conditions: HPLC analysis of all the three strains revealed no signs of hyperforin production from the undifferentiated cultures where as field grown plants showed presence of hyperforin particularly at the flowering stage.

Effect of stress on protein profile: Nine selected samples analyzed by LC/MS showed proteins of varied molecular weights (1,000-12,000 Daltons). Maximum numbers of protein were isolated from saline cultures (NaCl stress) within the retention time of 5 minutes. Among the three strains, maximum number of protein was isolated from HP-3 strain. High pH and salinity showed significant effect on the number of proteins. High pH reduced the number of protein to 12 and 3 while salinity increased the number of protein to 42 and 52 in HP-1 and HP-2 strain, respectively. The qualitative change in proteins was also indicated by change in polarity and difference in retention time. Saline stress in present study altered the protein profile by formation of proteins at different retention times and accumulation of low molecular weight protein in response to saline stress. (Table 3).

Discussion

Our results showed that the calli of all three strains used in this study had potential for accumulating hypericins and pseudohypericins under normal (devoid of stress) as well as under stress conditions (saline and high pH). Mosen *et al.* (1993) reported the formation of

Table 2. Effect of saline and high pH stress on total hypericin and Pseudohypericin content

Treatment	HP-1 (%)		HP-2 (%)		HP-3 (%)	
	Hypericin	Pseudohypericin	Hypericin	Pseudohypericin	Hypericin	Pseudohypericin
T ₁	0.180	0.213	0.176	0.278	0.180	0.310
T ₂	0.172	0.229	0.184	0.246	0.176	0.164
T ₃	-	-	-	-	-	-
T ₄	-	-	-	-	-	-
T ₅	0.188	0.574	0.272	0.590	0.196	0.573
T ₆	0.178	0.413	0.184	0.451	0.176	0.459
T ₇	0.176	0.203	0.178	0.369	0.192	0.328
T ₈	0.184	0.178	0.176	0.196	0.180	0.164
T ₉	-	-	-	-	-	-

Table 3: Effect of saline and high pH stress on protein profile

Treatment	Strain	Retention time	Molecular weight of proteins (Daltons x 1000)									
			12	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	10-11
T ₅	HP-1	5 min	-	-	8	-	1	5	1	1	4	2
		25min	-	-	-	-	-	-	-	-	-	-
		60min	-	-	-	-	-	-	-	-	-	-
	HP-2	5 min	2	1	-	2	-	1	4	4	2	6
		25min	-	-	-	-	-	-	-	-	-	-
		60min	-	-	-	-	-	-	1	-	-	-
	HP-3	5 min	1	2	2	4	9	6	9	6	8	5
		25min	-	1	-	-	-	-	-	-	1	-
		60min	-	-	-	-	-	-	-	-	-	-
T ₂	HP-1	5 min	2	-	1	-	-	1	1	3	3	-
		25min	-	-	-	-	-	-	-	-	1	-
		60min	-	-	-	1	-	-	-	-	-	-
	HP-2	5 min	-	1	-	-	1	1	-	-	-	-
		25min	-	-	-	-	-	-	-	-	-	-
		60min	-	-	-	-	-	-	-	-	-	-
	HP-3	5 min	-	2	-	3	3	1	1	4	2	2
		25min	-	-	-	-	-	-	-	-	-	-
		60min	-	-	-	-	-	-	-	-	-	-
T ₈	HP-1	5 min	-	-	-	-	-	3	-	-	-	-
		25min	-	-	-	-	-	1	-	-	-	-
		60min	3	1	1	15	3	-	8	5	6	4
	HP-2	5 min	-	-	-	-	-	-	-	-	-	-
		25min	8	-	-	1	2	-	7	11	15	5
		60min	-	-	-	-	-	6	-	-	-	-
	HP-3	5 min	-	-	-	1	1	-	-	-	-	-
		25min	-	-	-	-	-	6	-	-	-	-
		60min	-	-	-	-	-	-	-	-	-	-

hypericin and other metabolites in undifferentiated cultures of *H. perforatum*. The calli of HP-2 strain accumulated highest content of hypericin than HP-1 and HP-3 strain under control conditions. Cambell and May (1992) also confirm the difference in hypericin content of different strains of *H. perforatum*.

Karting *et al.* (1996) reported that field grown plants of *H. perforatum* produce hyperforin particularly at the flowering stage but the production of hyperforin from undifferentiated cultures of *H. perforatum* has not been documented so far. Similar results were revealed during the present investigations thus confirms the inability of undifferentiated cultures of *H. perforatum* to biosynthesize hyperforin. This inability of undifferentiated cultures to synthesize particular metabolite is due to loss of gene or mutation of gene involved in particular steps of biosynthetic pathway of the metabolite or unavailability of substrate and enzymes or lack of specialized cells involved in metabolite formation (Bohm, 1982).

Bohnert *et al.* (1995) reported that biosynthetic pathways are altered by abiotic stresses. During the present investigations saline and high pH stress showed negligible effect on hypericin production, however remarkable change was observed in pseudohypericin production (Table 2). Thus the findings are in agreement with the previous findings of Cushman *et al.* (1990) who revealed that salt and dehydration stress shows biochemical effects along with morphological, physiological and molecular effect.

LC/MS analysis revealed that all the three strains varied from one another in their protein content as was evident from the variation in the number as well as in molecular weight of proteins. Changes

in gene expression at transcriptional and post-transcriptional level are initially demonstrated by analysis of protein profiles elicited in plant using salt treatment. These studies revealed both qualitative and quantitative changes in pattern of polypeptides synthesized following salt treatment (Ericson *et al.*, 1984; Chen *et al.*, 1991). In the present study, high pH reduced the number of proteins perhaps due to creation of free amino acid pool due to inhibition of peptide bond formation; while salinity increased the number of proteins due to accumulation of large number of low molecular weight proteins. This coincides with Lopez *et al.* (1994) who reported accumulation of protein in leaves of *Raphanus sativus* in response to salt stress or water deficiency. The qualitative change in proteins was also indicated by change in polarity and difference in retention time. Saline stress in present study altered the protein profile by formation of proteins at different retention times. The change in protein profile in response to stress is also reported by Ramgopal *et al.* (1991) and Sairam *et al.* (2004).

We conclude from our results that among the three strains used in this study HP-2 strain showed best potential for accumulating hypericins and pseudohypericins (antidepressant in function). Hypericin production did not show any change in response to stress, however pseudohypericin production was highly influenced by both kinds of stress. High pH stress showed more drastic effect on both morphogenetic as well as biosynthetic potential of the plant as compared to saline stress. Change in protein profile was observed in response to both kinds of stress however this change could not be correlated with the change in accumulation of secondary metabolites due to stress.

References

- Bohm, H. 1982. The inability of plant cell cultures to produce secondary metabolites. In: *Plant tissue culture*, Fujiwara, A. (Ed.) Japanese Assoc. for Plant tissue culture, Tokyo. 325-326.
- Bohnert, H.J., D.W. Nebson and R.G. Jonsen, 1995. Adaptation to environmental stress. *Plant Cell*, 7: 1099-1111.
- Cambell, M.H. and C.E. May, 1992. Variation and varietal determination in *H. perforatum*. *Plant Protection Quarterly*, 2: 43-45.
- Chen, R.D. and Z. Tabaeizadeh, 1991. Alteration of gene expression in tomato plants by drought and salt stress. *Genome*, 35: 385-391.
- Cushman, J.C., E.J. De Rocher and H.J. Bohnert, 1990. Gene expression during adaptation to salt stress. *Environmental inquiry of plants*. Ed. F. Kalterman, Academic Press, Bandigo; 177-203.
- Ericson, M.C. and S.H. Alfinito, 1984. Protein produced during salt stress in tobacco cell culture. *Plant Physiology*, 74: 506-509.
- Fritz, Z. and A. Schleicher, 1993. Effect of substituting milfoil, St. Johns Wort and lovage for antibiotics on chicken performance and meat quality. *J. Anim. Free Sci.*, 2(4): 189-195.
- Karting, T., I. Goebel and B. Heydel, 1996. Production of hypercin and pseudohypercin and flavanoids in cell cultures of various *Hypericum* species and their chemotypes. *Planta Medica*, 62 (1): 51-53.
- Lavie, G., F. Valentine, B. Levin, Y. Mazur, G. Gallo and D. Weiner, 1989. Studies of microorganism of action of antiretroviral agents hypercin and pseudohypercin. *Proc.Natl. Acad. Sci*, 86: 5963-5967.
- Lopez, B.I. and J.B. Hudson, 1991. Antiviral activity of the photoactive plant pigment hypercin. *Photochem and Photobio.*, 54 (1): 95-98.
- Lopez, F., G. Yansuyt, P. Fourcroy and F. Cassedelbart, 1994. Accumulation of a 22 Kda protein and its mRNA in the leaves of *Raphanus sativus* in response to salt stress or water deficit. *Plant Physiol.*, 91: 605-614.
- Mosen, B.V., K. Zdunik, H.J. Woerdenbag, N. Pras and A.W. Alfermann, 1993. Production of natural products by shoots in cultures cultivated SW *in vitro*. *J. Pharma. World*, 15: 6.
- Ramgopal, S. and J.B. Carr, 1991. Sugarcane proteins and messenger RNA regulated by salt in suspension cells. *Plant Cell Environ.*, 14: 46-47.
- Sairam R.K. and T. Aruna, 2004. Physiology and molecular biology of salinity stress in plants. *Curr. Sci.*, 86: 407-422.
- Wentworth J.M. and M. Agostini, 2000. St. John's Wort a herbal antidepressant activates the steroid X receptor. *J. Endocrino.*, 166(3): R11-R16.