Indian Journal of Experimental Biology
Vol. 60, March 2022, pp. 184-191

Impact of mannitol and poly ethylene glycol 6000 induced water deficit on plant biomass and major secondary metabolites in *Centella asiatica* (L.) Urb. *in vitro*

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Received 20 April 2020; *revised 21 November 2021*

Low water potential related stresses are regulated by modifying water uptake and loss to avoid low water potential, accumulating solutes which in turn enhance active principles and its gene expressions. Present study examined effect of *in vitro* induced absorption of mannitol and PEG (poly ethelene glycol) 6000 in Indian pennywort, *Centella asiatica* (L.) Urb., neutraceutical plant, evidenced by phenotypic, molecular and phytochemical analyses. Both mannitol and PEG 6000 induce water deficit conditions in plants and retarded normal plant biomass in terms of fresh and dry weights. These effects were significantly less severe in plants subjected to mannitol, compared to PEG. PEG and mannitol imposed water deficit, resulted in decline in major active compound, asiaticoside evidenced by HPTLC of asiaticoside content. Differential expression of some selected key genes in the asiaticoside pathway including squalene synthase and β amyrin synthase by qPCR, confirmed decrease in transcript level expression of asiaticoside, whereas upregulated transcript level expression was observed in cycloartenol synthase for synthesis of phytosterols. Estimation of total flavonoids and phenolics under different water deficit conditions were found declined. In conclusion, water deficit by mannitol and PEG 6000 can significantly affects processes associated with biomass growth and ability to synthesize secondary metabolites in *C. asiatica.*

Keywords: Abiotic stress, β Amyrin synthase, Flavonoids, *Gotu kola*, Indian pennywort, *Kodavan*, Phenols, Squalene synthase

Abiotic stress is considered as serious issue which receives increasing attention because of the potential impacts of climate change on rainfall patterns and temperature extremes, etc.[1]. Inorganic salts, polyethylene glycol (PEG), mannitol and glycerol are considered as major osmo-regulators in plants[2]. In spite of providing salt and osmotic stress as a compatible solute, mannitol is reported for its possible role in plant responses to pathogen attack[3]. In the present study, PEG-6000 was also used to induce water deficit conditions *in vitro* along with mannitol. PEG molecules with a molecular weight greater than 3000 are apparently not absorbed[4] and is having no toxicity[5]. In a recent study Meher et al. (2018), PEG 6000 with concentrations of 5, 10, 15 and 20% along with controls was used to impose drought stress in peanut leaves and roots for a period of 24 h[6]. The objective of this research was to obtain the effect of mannitol and PEG 6000 induced water deficit in *C. asiatica* and its related variations in the plant biomass, asiaticoside content (Transcript level expression by q PCR and final asiaticoside content by HPTLC), total flavonoids and phenolics.

Centella asiatica (L.) Urb. 'major brain tonic' is considered as one of the major therapeutic agents in many parts of the world. *Centella* is represented as at least 33 species inhabiting tropical and subtropical regions[7]. Various ancient cultures and tribe groups has been using this ethnomedicinal plant[8]. Whole plant extracts are used prevalently in memory improving tonics and for mental and stress related disorders[9]. Utilization of *C. asiatica* has been known for several years in considering different diseases like gastro-intestinal disease, wound healing and eczema[10]. Antipyretic, diuretic, wound-healing and anti-inflammatory properties of plant have been reported earlier[11]. Bioactives responsible for these neutral-ceutical potentials are the secondary metabolites from *C. asiatica*. Different works are available on the enhancement of its constituents, such as triterpenoids

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[*Abbreviations:* CYS, Cyclo artenol synthase; βAS, β Amyrin synthase; OSβ AC, 2,3-Oxido squalene β amyrin cyclase; 2,3-Oxido squalene cycloartenol cyclase (OSCC); PEG 6000, Poly ethelene glycol 6000; SQS, Squalene synthase]

saponins[12], polyacetylenes[13], flavones[14], sterols and lipids[15]. The formulation containing *C. asiatica* can be used as a natural antioxidant against aging and oxidative stress[10] Recently, it is reported that terpenoids from *Centella asiatica* can function as a novel inhibitor against RNA-dependent-RNA polymerase activity of NSP12 of the SARS CoV-2[16]. The major secondary metabolite with neutraceutical properties in *C. asiatica* is asiaticoside[17]. Metabolic pathways originating from squalene form an extensive net of compounds including asiaticosides with defined branching points that diversify the end products, including compounds with primary roles in membrane architecture (sterols such as sitosterol, stigmasterol and campesterol) as well as a variety of secondary metabolites specific to each plant species[18]. Cycloarterol, the precursor of phytosterol primary metabolites such as stigmasterol and sitosterol, is formed by action of 2, 3-oxido squalene cycloartenol cyclase (OSCC) whereas huge variety of pentacyclic triterpenoid secondary metabolites seen in plants, are generated by the action of 2,3-oxido squalene β amyrin cyclase (OSβ AC).

Materials and Methods

Collection of Plant material

C. asiatica whole plants were collected from the field Genebank maintained at the Division of Biotechnology and Bioinformatics, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Thiruvananthapuram. The plant was authenticated by a taxonomist and was used to develop *in vitro* cultures under controlled conditions for the present study.

Addition of mannitol and PEG 6000 in *C. asiatica* growing media

Two-weeks old *in vitro* rooted and single leafed *C. asiatica* plantlets were sub-cultured to MS media with different concentrations of PEG 6000 (3% and 6%) or mannitol (6% and 12%) using available protocols with modifications[19,20]. A control group was also maintained along with PEG 6000 and mannitol induced *C. asiatica.*

Differential expression of key genes involved in secondary metabolite pathways

Total RNA was isolated from the leaves of different treatment groups using TRI® reagent (Sigma, USA) by the procedure originally described by Chomczynski and Sacchi (1987) with slight modifications[21]. Total RNA was subjected to DNase treatment using DNase treatment kit (The Spectrum Plant Total RNA Kit, Sigma, USA). RNA was treated with 2 µL DNase I (1.0 µL μg^{-1} RNA) in 20 µL reaction containing 10 X reaction buffer to a final concentration of 1X. The reaction mixture was incubated at RT for 15 min and the reaction stopped by adding 2 µL stop solution provided with the kit. The reaction mixture was incubated at 70°C for 15 min for inactivating the enzyme, and then chilled on ice. cDNA synthesis was carried out using the cDNA synthesis kit (Origin, India) as per the manufacturer's instructions. The potential of cDNA was checked using the constitutively expressing 5.8sRNA. Primers were designed using Primer Express 3.0 software (Life Technologies, ABI, California, USA). The primers used are provided in Table 1. Differential expression of selected genes was experimentally validated by real time PCR (qPCR) using step-one plus real time PCR system (Applied Biosystems, USA). The samples were normalized using internal reference 5.8S rRNA. The relative quantification of RNAs was calculated from CT values and values for $2^{-\Delta\Delta CT}$ were also calculated using the double deltaCT analysis method to get expression fold change. The $2^{-\Delta\Delta CT}$ values greater than 1 indicates upregulation and less than 1 denotes miRNA down-regulation. qPCR was performed using DyNAmo Flash SYBR Green qPCR kit (Thermo, USA) with Step-One Real time PCR system. A non-template control was also kept. Results were analysed using Applied Biosystems Step-One data analysis software package. The comparative CT method uses arithmetic formulas to achieve the results for relative quantification. The amount of target, normalized to an endogenous control and relative to a calibrator is given by Δ^{CT}. There is no need of a standard curve and can increase throughput as standard curve samples are no longer required. This also eliminates dilution errors made in creating standard curve samples.

Preparation of plant extracts and Phytochemical analyses in *C. asiatica*

C. asiatica whole plants after osmo-elicitation were collected from *in vitro* cultured plants along with control untreated plants and washed many times to

Table 1 — Primers used in the study

Primer	Forward primer	Reverse primer
BAS RT	TCCCTCAGCAGGAAACAAC	GGTACTCTCCAAGTGCCCATA
SQSRT	CAAATTTTCCGTGGC	GGGTTTATTTCTCCAGAAGAC
CYSRT	ATGCCTGGTTTGGTTATCACT	AACCCCACCCACCATCTCTAT
5.8S rRNA RT	TCGATGGTTCACGGGATTC	TGAAGAACGGTAGCGAAATG

remove the media. Plants were further dried in an incubator at 37ºC and powdered. Samples were prepared in same way as that for the phytochemical analyses. The concentrated extracts were stored at −20ºC freezer (Elanpro, India) for further experiments. *C. asiatica* plants under different stress conditions were initially dried at 40ºC in hot air oven and the dried samples were powdered using mortar and pestle. Methanolic extracts were prepared from all samples using cold extraction method for 72 h. The extracts were filtered using Whatman No. 1 filter and concentrated in rotary evaporator (Roteva, India)[22]. Phytochemical characterization was carried out using HPTLC. Mobile phase used was n-butanol: ethyl acetate: water in the ratio 4:1:5. One µL of each sample was spotted on the pre-coated silica gel plate and detection was done at 287 nm.

Phenotypic characterization of *C. asiatica*

Control as well as stress-induced *C. asiatica* were analysed at initial and final stages of water deficit induction. The quantitative traits used include length, number and diameter of shoots, total number of roots and fresh and dry weights of whole plants. Plants were washed to remove media components and agar and dried with towels, before proceeding with its fresh weight. Both the fresh and dry weights of the whole plant samples were measured. Dry weights were measured after oven drying of samples at 40°C till it became completely dried.

Analysis of flavonoids by Aluminium chloride colorimetric assay

Total flavonoids content was measured by the aluminium chloride colorimetric assay. The reaction mixture consists of 1mg of extract and 4 mL of distilled water and was taken in a 10 mL volumetric flask. To the flask, 0.30 mL of 5 % sodium nitrite was treated and kept for 5 min and then 0.3 mL of 10 % aluminium chloride was added. After 5 min, 2 mL of 1M sodium hydroxide was added and diluted to 10 mL with distilled water. A set of reference standard solutions of morin (20, 40, 60, 80 and 100 µg/mL) were also prepared. The absorbance for test and standard solutions were determined against the reagent blank at 510 nm with an UV/Visible spectrophotometer. The total flavonoid content was expressed as µg of Gallic acid equivalent (GAE) /mg of extract.

Phenolic analysis

The amount of total phenols in the plant tissues was estimated[23]. Different aliquots were pipetted out and the volume in each tube was made up to 3.0 mL with distilled water. Folin-Ciocalteau reagents (0.5 mL) was added followed by 2 mL of sodium carbonate solution and incubated for 1 min. The tubes were cooled and the absorbance was read at 650 nm in a spectrophotometer against a reagent blank. Gallic acid was used as the standards. All determinations were done in triplicates and the total phenolics content was expressed as mg g^{-1} gallic acid equivalent.

Statistical analysis

For each experiment, three *C. asiatica* plants (for phenotypic traits and molecular analyses) were analysed and experiments were repeated thrice. For analysis of growth parameters, in each experiment, three *C. asiatica* plants were analysed and the experiments were repeated twice. A total of 15 treated plants were studied in comparison to untreated control *C. asiatica* plants. One plant each was maintained as a control for each growth parameter studied. Mean values were compared and analysis of data was carried out using Graphpad Instat version 3.6 (Graphpad Software Inc., USA).

Results

The total plant biomass declines with mannitol and PEG 6000 induced water deficit conditions in *C. asiatica*.

Single node and single leafed plants (Fig. 1; Panel 1) in solid media were selected initially for inducing stress conditions. 4 weeks old plants were

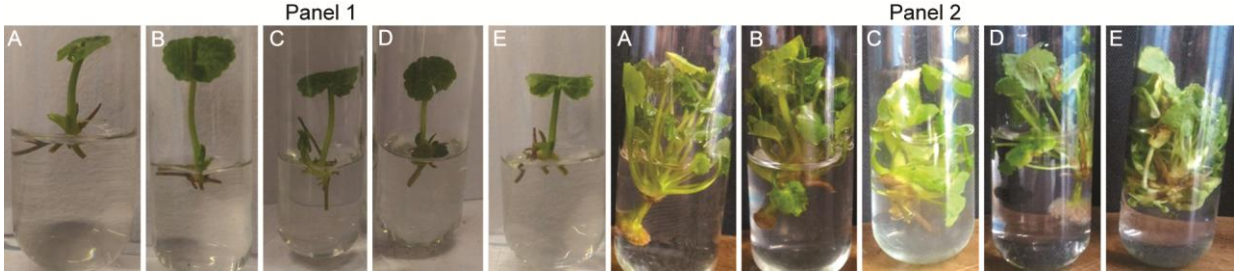

Fig. 1 — Comparative analysis of water stress. Panel 1 shows single noded *C. asiatica* for initiation of stress conditions. (A) Control; (B) in 3% PEG 6000; (C) in 6% PEG 6000; D in 6% Mannitol; and (E) in 12% Mannitol. Panel 2 shows plants exposed to stress had changes in phenotypic appearance. (A) Control; (B) in 3% PEG 6000; (C) in 6% PEG 6000; D in 6% Mannitol; and (E) in 12% Mannitol.

again transferred to hydroponic MS containing 3 and 6% PEG 6000, 6 and 12% mannitol along with control *C. asiatica.* Variation in phenotypic characters was observed among four different treatments along with control (Fig. 1; Panel 2). The total plant biomass (fresh and dry weights) were found declined under mannitol and PEG 6000 induced water deficit conditions (Fig. 2). The maximum deterioration in the biomass was observed with *C. asiatica* growth in the presence of 12 % mannitol. Numbers of leaves in all treatments were compared (Fig. 3) and significantly important (P <0.001) maximum number of leaves were observed in treatment with 6% mannitol and minimum number of leaves observed in 6% PEG 6000. On increasing concentration of mannitol from 6 to 12%, number of leaves had greatly reduced. Number of roots was observed maximum in increased concentration of mannitol (12%; P <0.001) and minimum in 6 % mannitol. Maximum number of roots observed was around 14 in 12 % and minimum observed was 5. Maximum length of roots was observed in increased concentration of mannitol (12%; P <0.001), which is around 10 cm and minimum length of root, 4.05 cm was seen in 6% mannitol. All elicitation treatments showed augmented thickness of petioles in comparison with control *C. asiatica* (P <0.001) and maximum was observed in 3% PEG 6000 and minimum thickness was observed in control. Diameter of leaf was higher for control, 6% mannitol and 12% mannitol. Lowest was observed in 3 and 6% mannitol. When total plant biomass was considered in fresh and dry form, control *C. asiatica* was with maximum fresh and dry weights than other treatments given under *in vitro* conditions. 3% PEG-6000 and 6% mannitol treated plants showed comparatively less reduction in its total plant biomass, whereas 12% mannitol was with lowest total plant biomass. Maximum number of shoots was observed in *C. asiatica* treated with 6% mannitol, though the total biomass was lower in comparison with control *C. asiatica*. Level of significance in each sample was done using Graphpad Instat 3 is given in Supplementary Table S1. (*All supplementary data are available only online along with the respective paper at NOPR repository at http://nopr.res.in*)

Asiaticoside synthesis declines in *C. asiatica* under mannitol and PEG 6000 induced water deficit conditions

Asiaticoside content was determined using HPTLC and maximum asiaticoside content was found in control samples (2.95%) and decreased amount of asiaticosides was observed in osmo-elicitated treatments of 3% PEG 6000 (1.65%), 6% PEG 6000 (2.35%), 6% mannitol (1.05%) and 12% mannitol (2.76%) from all repeated experiments (Fig. 4). The

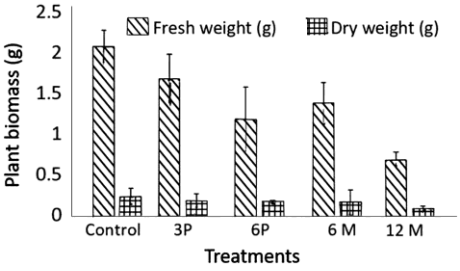

Fig. 2 — Analysis of plant biomass in *C. asiatica*. Fresh and Dry weights in g in Control; *C. asiatica* treated with 3% PEG 6000 (3P); *C. asiatica* treated with 6% PEG 6000 (6P); *C. asiatica* treated with in 6% Mannitol (6M); *C. asiatica* treated with 12% mannitol (12M).

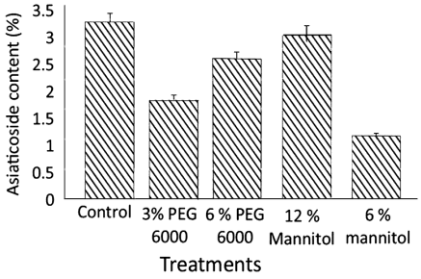

Fig. 4 — Quantitative analysis of asiaticoside content by HPTLC: Asiaticoside content was analysed in *in vitro* grown plants elicited with 3 and 6% PEG 6000 and 6 and 12% mannitol along with control. Number of replications (n)=3.

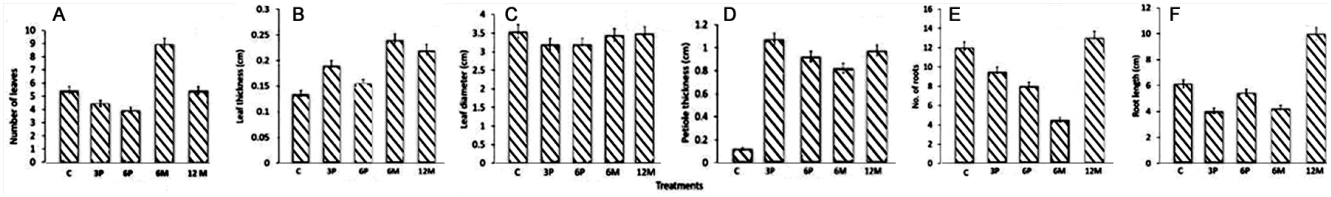

Fig. 3 — Analysis of phenotypic traits in *C. asiatica* on induction of water deficit. Number of leaves, number of roots, petiole thickness (cm), Root length (cm), leaf thickness (cm) and fresh/dry weights under different stress conditions in comparison with control *C. asiatica.* (A) Control; (B) in 3% PEG 6000; (C) in 6% PEG 6000; (D) in 6% Mannitol; and (E) in 12% Mannitol.

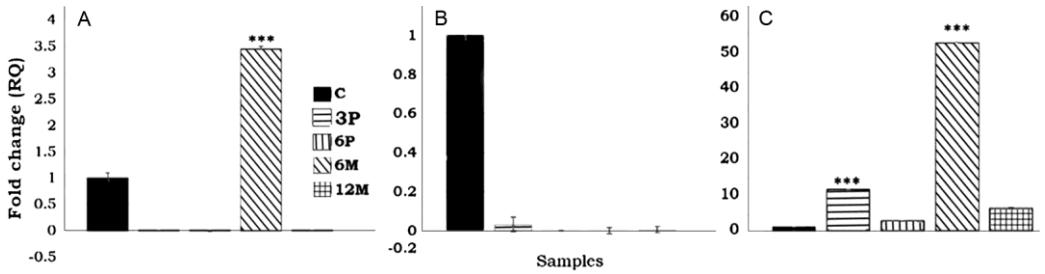

Fig. 5 — Real-time RT-PCR analysis of(A) SQS; (B) BAS; (C) and CYS expressed as fold upregulation in leaves of *Centella asiatica* under water deficit conditions. Analysis was performed in leaves of control (C) and stress induced by 3% PEG (3P), 6% PEG (6P), 6% mannitol (6M) and 12% mannitol (12 M). Data were analysed in comparison to tissues of control plants maintained for 45 days in culture. Number of replications (n)=3.

results were analyzed by comparing peak area (Suppl. Fig. S1). Three dimensional densitometric chromatogram is provided as Suppl. Fig. S2.

Water deficit by mannitol and PEG 6000 leads to downregulation of Squalene synthase and β amyrin synthase.

Expression profile of triterpenoid pathway genes was validated by real-time RT-PCR analysis using plant-specific 5.8S rRNA gene served as control for constitutive gene expression in leaves. In each experiment, samples were isolated 10 days post-incubation with mannitol and PEG 6000 and analysed in comparison to tissues of control plants maintained in hydroponic culture. Transcript level expression of Squalene Synthase (SQS) was significantly upregulated up to 3.2-fold on treating with 6% mannitol (Fig. 4; P <0.001). It was apparent by real-time RT-PCR analysis that β Amyrin Synthase (BAS) transcript level expression was down regulated in all elicitor treatments used in study in three repeated experiments (Fig. 5). Maximum downregulation was observed in samples treated with 6 % PEG 6000 (P <0.001) and least transcript level down regulation was observed in *C. asiatica* samples treated with 3% PEG 6000.

Mannitol and PEG 6000 induced upregulation of cycloartenol synthase

Cycloartenol synthase (CYS) is the immediate precursor of phytosterols and its transcript level upregulation was observed maximum in *C. asiatica* treated with 6% mannitol (Fig. 6; P <0.001). All treatments showed significant upregulation of CYS in comparison with the non-treated control.

Impact of mannitol and PEG 6000 induced water deficit on phenolics and flavonoids

The present study used PEG 6000 and mannitol in *C. asiatica* which resulted in reduction in phenolic content. The maximal decline in phenolics was observed in *C. asiatica* supplemented with different

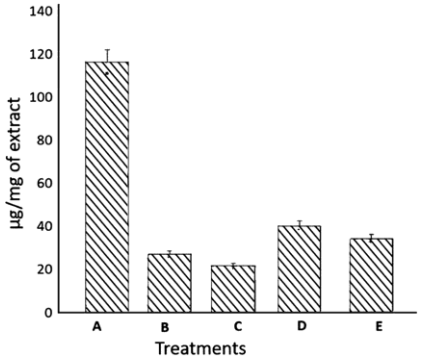

Fig. 6 — Analysis of total phenolics in *C. asiatica* (A) Control; *C. asiatica* grown in media supplemented with (B) 3% PEG 6000; (C) 6% PEG 6000; (D) 6% Mannitol; and (E) 12% Mannitol.

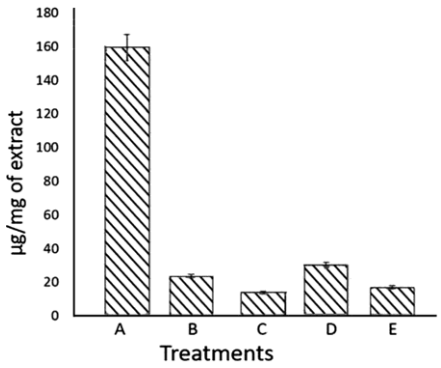

Fig. 7 — Estimation of flavonoid content. Analysis of total flavanoids in *C. asiatica* (A) Control; *C. asiatica* grown in media supplemented with (B) 3% PEG 6000; (C) 6% PEG 6000; (D) 6% Mannitol; and (E) 12% Mannitol.

concentrations of PEG 6000 than with mannitol supplementation. A similar pattern of results was observed in the flavonoid content with the least flavonoid contents in *C. asiatica* with PEG 6000 supplementation.

Discussion

Induction with PEG 6000 and mannitol were tried in a halophyte, *Sesuvium portulacastrum* and its plant

biomass growth was analysed at regular intervals. Reduced growth, decreased leaf number and leaf mean surface area and reduced leaf water and K+ contents were observed which was more severe in PEG treated plants compared to mannitol[24]. PEG 6000 treatment also inhibited seed germination and seedling growth in *Cassia angustifolia*[25]. The plant heights and weights (both fresh and dry weights) were significantly decreased by drought stress conducted in a different study with *Mentha piperita* and *Catharanthus roseus*[26]. Water deficit had significant reducing effect on the biomass and on the dry mass of the plants in *Melissa officinalis* L.[27]. On the contrary, plant dry biomass was found enhanced on osmotic elicitation with both PEG 6000 and mannitol in *Fibigia triquetra*[28].

Abiotic stress of drought is linked by decrease in availability of water and other elements to plant cells. It is quantified as a decline in water potential. Low water potential together with different stresses is controlled by modifying water uptake and loss to avoid low water potential, accumulating solutes etc. For example, secondary metabolites like total phenol, flavonoid, and saponin contents were observed as decreased in response to drought stress in *Mentha piperita* and *Catharanthus roseus* plants[29].

Drought under field conditions has also resulted in significant molecular changes. Plants biosynthesize diverse triterpenoids and their genomes encode multiple OSC enzymes to form these skeletons. Structural diversity of triterpenes depends on the cyclization of 2,3-oxidosqualene by different OSCs such as cycloartenol synthase (CYS), lupeol synthase and β amyrin synthase (β AS)[30]. *C. asiatica* on elicitation with mannitol and PEG 6000 showed decreased BAS transcript level expression. The expression of SQS, the regulatory gene was maximum in the plant tissues treated with 6% mannitol, which indicated the enhancement of phytosterols instead of asiaticosides. SQS can be considered as the key enzyme in monitoring the overall flux of asiaticoside and phytosterol biosynthesis.

Phytosterols are basically steroidal triterpenes and its role in plant cell membrane in regulating fluidity is important[31]. Biotic and abiotic agents can also be used to enhance secondary metabolites in plants. Drought stress is one of the most significant abiotic stress in plant growth and development[32].

Acetate-malonate and shikimate pathways are responsible for the synthesis of phenols. Significant increase in phenolics was observed which was subjected to salt and drought stress in *Juglans regia* (L.)[33]. Phenylalanine is the precursor for the synthesis of phenolics by the shikimate pathway. Though the amino acid, Phenylalanine was synthesised in plastids[34] its further translation to phenolic compounds occurs outside the plastids. Dai and Mumper (2010) reports many of the phenolic compounds can be simply hydrolysed and oxidized[35]. The oxidation of phenolics can be enhanced under stress conditions which in turn decline the yield of phenolics in the extracts. In a study by Krol *et al*.[36] proved that drought stress caused reduction in total phenolic compounds in grapevine leaves and roots, but with antioxidant properties[36]. Cold stress also resulted in similar results in earlier research on seedlings of *Vitis vinifera*. The total content of phenolics, tannins and phenolic acids in stress sample was lower than in control sample[37]. On the contrary, an augmented phenolics were seen in *Brassica napus* L. during flowering and pod fill under drought stress[38]. In broccoli, water deficit increased not only phenolics but anthocyanin content also[39,40].

A similar pattern of results was observed in the flavonoid content with the least flavonoid contents in *C. asiatica* with PEG 6000 supplementation. In lemon balm (*Melissa officinalis* L. cv. 'Soroksár'), water deficit resulted in reduction in total flavonoids by 14-22%, which is in comparison with the flavonoid content in *C. asiatica*. Decreasing effect of water deficit on flavonoid content was also reported in a study on *Lepidium sativum*[41]. An opposite effect was observed in *Dracocephalum moldavica*, where the severe drought augmented the flavonoid content[42].

Conclusion

Current study indicates decline in plant biomass and variation in other phenotypic traits in response to mannitol and PEG 6000 mediated water deficit stress in *C. asiatica.* Asiaticoside content was found declined in response to mannitol and PEG treatment. Deterioration of BAS, major gene in asiaticoside pathway and upregulation of SQS and CYS transcript level expression indicates diversion of β amyrin precursors which finally channelled for biosynthesis of phytosterols rather than utilising in synthesis of asiaticoside. Plants treated with 6 % mannitol exhibited increased transcript level expression of CYS, key gene in phytosterol pathway and decline in the expressions of β amyrin synthase and squalene synthase. Both PEG

6000 and mannitol has lessened the plant biomass growth with the maximum growth retardation in PEG treated *C. asiatica*. The flavonoid and total phenolics were also observed declining in the PEG and mannitol treated *C. asiatica*. The present study reveals mannitol and PEG induced water deficit concomitantly leads to massive reductions in biomass production as well as in major secondary metabolites with its maximal stress effects in PEG treated *C. asiatica*. In addition, a positive correlation was observed among the biomass growth, transcript level expression of key genes in asiaticoside pathway, the final asiaticoside content, total phenolic and flavonoid contents.

Acknowledgement

One of the authors, SJ, thanks the Indian Council of Medical Research (Grant No. BIC/11(37)/2015), India for research associateship. All the authors thank Dr AS Hemananthakumar, Jawaharlal Nehru Tropical Botanic Garden and Research Institute for help in tissue culture studies.

Conflict of interest

Authors declare no competing interests.

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