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Research Article

# BIOCHEMICAL CHANGES OF VARIOUS METABOLITES IN *CITRULLUS COLOCYNTHIS* (LINN) SCHRAD

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**ABSTRACT**

*Citrullus colocynthis* (Bitter apple) is a medicinally important plant. It is also known as colocynth. It belongs to family cucurbitaceae. In the present set of investigations, biochemical changes of various cellular metabolites/enzymes were observed from *in vivo* (leaf, stem, fruit and root) and *in vitro* (callus and differentiating callus) of *Citrullus colocynthis.* Total soluble sugar content was observed maximum in differentiating callus while minimum amount was observed in callus. Maximum amount of reducing sugar was observed in callus while minimum amount was observed in root sample. Starch was observed maximum in root while minimum amount was observed in callus. α-amylase activity was maximum in callus and minimum in root sample. More α-amylase activity could be correlated to less starch contents and vice-versa.

**Keywords:** Primary metabolites, Total soluble sugar, Reducing sugar, Starch, α- amylase, *Citrullus colocynthis.*

## INTRODUCTION

A large proportion of the drugs used in modern medicine are either directly isolated from plants or synthetically modified from a lead compound of natural origin. In addition, in the form of natural products or as functional foods, medicinal plants and their extracts offer an alternative to specifically targeted drugs in the treatment and prevention of many diseases. Primary metabolic routes produce primary metabolites, which are present almost everywhere in nature and are essential for all life forms. These compounds include the common carbohydrates, fats, proteins and nucleic acids that are needed to create and maintain life. Apart from fats, the compounds are polymeric and usually chemically large molecules. In general, primary metabolites obtained from higher plants for commercial use are high-volume, low-value bulk chemicals. Typically they are involved in the energy regulation of organisms and with growth and development of tissues; in short, they are the building blocks of organisms.

As the world, population is increasing day by day, the tissue culturists have tried to obtain biochemical components from the cultured cells. The investigations on the cultures have shown the presence of biochemical products in the cultures. Therefore, the idea of using plant tissue culture for the production of primary metabolites seems to be an important tool to carry out studies on the presence, isolation and quantitative estimation in medicinal herbs.

In the present set of investigations, biochemical changes of various cellular metabolites/enzymes were observed from *in vivo* (leaf, stem, fruit and root) and *in vitro* (callus and differentiating callus) of *Citrullus colocynthis.* The callus obtained by the culture of nodal stem segment of the plant on MS medium supplemented with BAP (2.0 mg/l) and NAA (2.0 mg/l) and differentiating callus achieved on MS medium supplemented with BAP (1.0 mg/l) and Kn (1.0 mg/l) in tissue culture experiment, was used for present biochemical analyses.

Carbohydrates are among the most widely distributed compounds in both plant and animal kingdom. Carbohydrates are products of photosynthesis, the most fundamental process in metabolism. They provide means of storage, energy and are also the building blocks of the cell wall and raw materials for all other synthetic processes. Along with plant growth substances, the quality and quantity of carbohydrates plays a pronounced role in maintaining the growth and metabolic events of the cells and tissues (Maretzki *et al.*, 1974). Carbohydrates have many uses in pharmacy such as the use of sugar in syrups to mask the taste of drug or for suspending certain forms of medication. The carbohydrates, often termed as sugars, are the 'staff of life' for most organisms. On the basis of mass, they are the most abundant class of biomolecules in nature. Carbohydrates are also known as saccharides (Sakcharon[G] = sugar or sweetness). Carbohydrates are aldehyde and ketone derivatives of polyhydroxy alcohols. Each carbohydrate therefore, contains an aldehyde or a ketone group and is known as an aldose or a ketose. Carbohydrates are usually classified as monosaccharides, derived monosaccharides, oligosaccharides and polysaccharides. Hexoses are 6-carbon atom sugars and starch is the reserve substance in plant cells. It is a polymer of D-glucopyranose units linked by α-1-4 glycosidic bond. It consists of a mixture of amylose and amylopectin in the proportion of 1 to 4. Both are high molecular weight compounds.

Several workers have studied carbohydrate contents of various medicinal plant species viz. *Morus alba* and *Psoralea corylifolia* (Singh, 2004); *Bacopa monnieri* (Mohapatra and Rath, 2005); *Boerhaavia diffusa* (Sharma, 2006); *Balanites aegyptiaca* (Vijayvergiya and Vijay, 2006); *Cassia obtusifolia* and *C. siamea* (Sharma *et al.*, 2006); *Terminalia catappa* (Nagesh *et al.*, 2007); *Araucaria cookii* and *A. bidwilli* (Unnikrishnan *et al.*, 2007); Digera muricata (L.) Mart (Sharma *et al.*, 2011).

Starch is the most important storage carbohydrate of plant cells. Starch is a mixture of two polysaccharides, amylose and amylopectin. Occurrence of starch in the leaves is transitory being deposited during the day and translocated to other regions in the night. A number of reports are available on research carried out on starch (Dinges *et al.*, 2001; Critchley *et al.*, 2001).

Alpha amylase is a universally distributed enzyme in plants, which is involved in the hydrolysis of starch. α-amylase (endo-1,4,-α-D-glucan glucohydrolase, EC 3.2.1.1) is an extracellular enzyme that cause endocleavage of substrate and hydrolyses α-1,4 linkages of linear polysaccharides in a random manner to yield a mixture of glucose and maltose. It plays a key role in starch degradation in chloroplasts (Beck, 1985). α-amylase is the best means for mobilization of carbohydrate reserves in the plant and it finds potential application in a number of industrial processes such as in food, textile and paper industries.

α-amylase inhibitory activity of some Malaysian plants (*Phyllanthus amarus*) have been used to treat diabetes (Hasenah *et al.*, 2006). There are several reports on α-amylase activity in medicinal plants *in vivo* and *in vitro* viz. *Cordia gharaf* and *Jatropha curcas* (Rajore, 2002); Barley (Tull *et al.*, 2003); *Morus alba* and *Psoralea corulifolia* (Singh, 2004) and *Lantana camera* (Nagamani *et al.*, 2007).

In the present investigation carbohydrate contents and α-amylase activity were determined in both *in vivo* (leaf, stem, fruit and root) and *in vitro* (callus and differentiating callus) tissues.

## MATERIALS AND METHODS

### Total soluble sugar

The amount of total soluble sugar was estimated by phenol sulphuric acid reagent method (Dubois *et al*., 1951).

**(i) Reagents**

(a) 80% ethanol

(b) 5% phenol solution

(c) 96% sulphuric acid

(d) Standard glucose solution: - 50 mg of glucose was dissolved in 50 ml of distilled water.

(e) Working standard: - 10 ml of standard stock solution was taken, volume made up to 100 ml with distilled water. A series of volumes 0.1-1.0 ml of this standard gave a concentration range from 10 µg to 100 µg and the same procedure was followed as that of the sample.

**(ii) Procedure**

**(a) Extraction of total soluble sugar**

500 mg of both *in vivo* and *in vitro* tissues were homogenized with 10 ml of 80% ethanol using mortar and pestle. Each sample was centrifuged at 2000 rpm for 20 minutes.

**(b) Estimation of total soluble sugar**

Alcoholic extract was used for estimation of total soluble sugar. To 1.0 ml of alcoholic extract, 1.0 ml of 5% phenol was added and mixed. To this, 5.0 ml of 96% sulphuric acid was rapidly added to each tube. It was gently agitated during addition of sulphuric acid and then allowed to stand in a water bath at 26-30°C for 20 minutes. The optical density of characteristic yellow and orange colour was measured at 490 nm in a spectrophotometer (systronic UV-VIS-118) against blank. Blank was prepared with 1.0 ml of 80% ethanol, 1 ml of 5% phenol solution and 5.0 ml of 96% sulphuric acid. The standard curve was prepared by using known concentrations of glucose. The quantity of sugar was expressed as mg/g fresh weight of sample.

### Reducing sugar

Estimation of reducing sugar was done by the method of Miller (1972).

**(i) Materials**

(a) 80% ethanol

(b) 0.1% DNSA (Dinitro salicylic acid)

(c) 40% Rochelle salt (Potassium, sodium tartarate)

(d) Standard glucose solution

**(ii) Procedure**

**(a)Extraction of reducing sugar**

500 mg both *in vivo* and *in vitro* tissues were homogenised with 10.0 ml of 80% ethanol. Each sample was centrifuged at 2000 rpm for 20 minutes and the supernatant was collected separately.

**(b) Estimation of reducing sugar**

Alcoholic extract was used for estimation of reducing sugars. To 3.0 ml of alcoholic extract, 3.0 ml of 0.1% DNSA reagent was added. The mixture was heated for 5 minutes in a boiling water bath. After colour had developed, 1.0 ml of 40% Rochelle salt was added when the contents of the tubes were still warm. The tubes were cooled under running tap water. Absorbance was recorded using spectrophotometer at 575 nm against blank. Blank was prepared by adding all reagents used in sample preparation except plant material. The amount of reducing sugar was calculated using standard curve prepared from glucose. The quantity of reducing sugar was expressed as mg/g fresh weight of tissue.

### Starch

Estimation of starch was carried out by the method of McCready *et al.* (1950).

**(i) Reagents**

(a) 80% ethyl alcohol

(b) 52% perchloric acid

(c) Standard glucose solution

**(ii) Procedure**

**(a) Extraction of starch**

500 mg of both *in vivo* and *in vitro* tissues were homogenized with 10.0 ml of 80% ethanol. Each sample was centrifuged at 2000 rpm for 20 minutes. After centrifugation the supernatant was discarded and residue was collected.

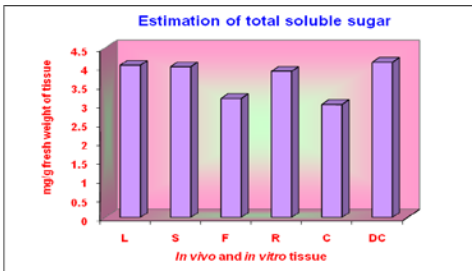


**Fig. 1:**

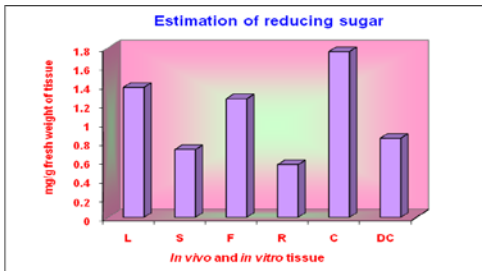


**Fig. 2:**

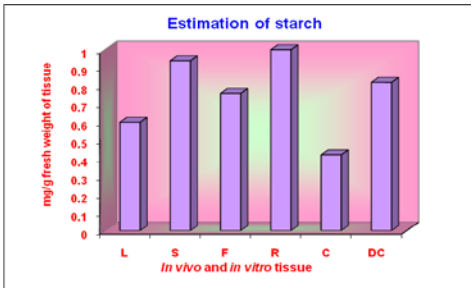


**Fig. 3:**

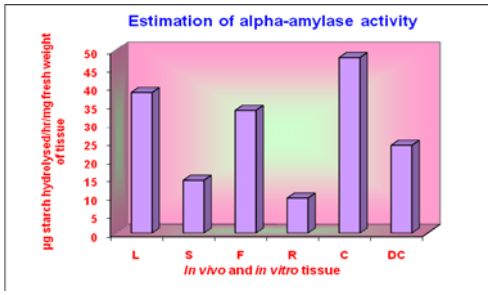


**Fig. 4:**

**(b) Estimation of starch**

Residue was used for estimation of starch. It was suspended in 5.0 ml of water and subsequently 6.5 ml of 52% perchloric acid was added to the residue. After stirring the mixture, the contents were centrifuged for 20 minutes at 2000 rpm. The supernatant was decanted and collected and the whole procedure was repeated thrice. Supernatant of each step were then pooled and the total volume was made up to 100 ml with distilled water. The mixture was then filtered through Whatman filter paper (No. 42). 1.0 ml of aliquot of this filtrate was analysed for starch content following the same procedure as that of total soluble sugars. The quantity of starch was calculated in terms of glucose equivalent and factor 0.9 was used to convert the values of sugar to starch. The quantity of starch was expressed in terms of mg/g fresh weight of tissue.

**Alpha-amylase**

Alpha amylase activity was determined by measuring the production of maltose and other reducing sugars from amylopectin by amylase using the 3,5-dinitro salicylic acid (DNSA) colorimetric procedure of Bernfeld (1955).

**(i)Reagents**

(a) 0.02 M phosphate buffer (pH = 6.9): 45 ml of 0.02 M $KH_2PO_4$ and 55 ml of 0.02 M $K_2HPO_4$ were used for preparation of phosphate buffer (pH = 6.9).

(b) DNSA (3,5-dinitrosalicylic acid) reagent.

(c) Substrate solution (1.0 g soluble starch dissolved in 100 ml of 0.02 M phosphate buffer, pH 6.9, containing 0.0067 M NaCl).

**(ii)Procedure**

**(a) Extraction of enzyme**

200 mg fresh weight of both *in vivo* and *in vitro* tissues were crushed with 5.0 ml of 0.02 M chilled phosphate buffer (pH 6.9). The homogenate was centrifuged at 2500 rpm for 20 minutes. The supernatant was used to determine the enzyme activity.

**(b) Estimation of alpha-amylase activity**

Reaction mixture consisted of 1.0 ml of enzyme extract and 1.0 ml of substrate solution. This mixture was incubated at 30°C for 45 minutes and the reaction was stopped by adding 1 ml of DNSA reagent. The tubes were heated in boiling water bath for 15 minutes and then cooled. 20 ml of distilled water was added to the cooled mixture. O. D. of the yellow colour was read at 560 nm against a zero hour blank. The activity was expressed in terms of µg starch hydrolysed per hour per mg fresh weight of tissue.

**RESULTS**

**Total soluble sugar**

Results are presented in Fig.1.

There were marginal variations in total soluble content in all the samples tested (*in vivo* and *in vitro*). However, total soluble sugar was maximum in differentiating callus as compared to *in vivo* leaf, stem, root, fruit and callus. Minimum amount of total soluble sugar was observed in callus.

**Reducing sugar**

Results are presented in Fig.2.

Among *in vivo* plant parts, leaf showed maximum reducing sugar level followed by fruit, stem and root. In *in vitro* samples, callus had more reducing sugar content as compared to differentiating callus.

**Starch**

Results are presented in Fig.3.

In *in vivo* plant parts root had higher starch level as compared to stem, fruit and leaf. *In vitro* differentiating callus showed more starch content as compared to callus. Maximum level of starch was noticed in root and minimum level of starch was observed in callus.

**α- -amylase**

Results are presented in Fig.4.

Maximum α- amylase activity was observed in callus as compared to leaf, fruit, stem, root and differentiating callus. In *in vivo* plant parts, leaf had maximum α- amylase activity as compared to fruit, stem and root while *in vitro* samples callus showed more α- amylase activity as compared to differentiating callus. Lowest activity of α- amylase was observed in root.

**DISCUSSION**

In the present studies maximum level of total sugar was noticed in differentiating callus as compared to leaf, stem, root, fruit and callus. Organ initiation is an energy requiring process so the accumulation and utilization of sugars becomes a primary event of organogenesis *in vitro*. Thorpe and Meir (1974) determined that carbohydrates and enzymes involved in carbohydrate metabolism were in higher levels in shoot forming culture of tobacco. In contrast to above results, Sharma (2006) observed highest sugar contents in leaf as compared to other *in vivo* and *in vitro* tissues of *Boerhaavia diffusa.*

Among all *in vivo* plant parts root had higher starch level as compared to stem, fruit and leaf. In *in vitro* differentiating callus showed more starch content as compared to callus. Maximum level of starch was noticed in root and minimum level of starch was observed in callus. In contrast to above result Singh (2004) found high amount of reducing sugar in callus as compared to differentiating callus in *Psoralea corylifolia*. A declination in level of reducing sugar during shoot bud initiation from differentiating callus, is accompanied by low invertase activity in the present study is in confirmation with the results by Russel and Morris (1982). This decline in the level of reducing sugar in differentiating callus indicated the probable utilization of sugars in the organ initiation process. Role of sugars in differentiation has been well documented (Patel and Berlyn, 1982; Chatrath *et al.,* 1996).

Among of starch was maximum in root as compared to stem, leaf, fruit, callus and differentiating callus. According to Sharma *et al*. (2006), the presence of higher levels of starch in intact plant part might be due to more storing capacity to escape the drought conditions.

In the present studies, it was found that differentiating callus showed more starch than callus. Starch is known to accumulate during shoot formation in callus cultures of *Solanum surattense* (Swarnkar *et al.,* 1986). The accumulation of starch may be due to the presence of sucrose in the medium (Sjolund and Weier, 1971) or due to the presence of continuous light (Vierskar and Andersen 1982). Maximum level of starch observed during shoot bud formation from callus was supported by the observation of Jasrai *et al.* (1987) as they observed accumulation of starch at specific points from which shoot primordia arose.

α-amylase is the enzyme that is responsible for hydrolysis of starch and represent the best means for mobilization of carbohydrate reserves in the plant. Reduction in α-amylase activity corresponds to the active accumulation of starch in the tissues, as α-amylase acts on α-1-4 glycosidic linkage between starch molecules. In the present investigation maximum α-amylase activity was observed in callus as compared to leaf, fruit, stem, root and differentiating callus. In *in vivo* plant parts, leaf had maximum α-amylase activity as compared to fruit, stem and root while *in vitro* sample callus showed more α-amylase activity as compared to differentiating callus.

Similarly Sharma (2007) reported minimum α-amylase activity in differentiating callus as compared to callus and *in vivo* plant parts. Maximum α-amylase activity in callus tissues of *Citrullus colocynthis* corresponds to the minimum accumulation of starch in callus tissue.

**Abbreviations**

L=Leaf, S=Stem, F=Fruit, R=Root, C=Callus, DC=Differentiating Callus

**REFERENCES**

1. Maretzki AM Thom, Nickell LG. Tissue Culture and Plant Science (Ed. Street, H. E.), Academic Press: New York; 1974. p. 329.
2. Singh S. Studies on morphogenesis in tissue culture of some economically important plants. Thesis, University of Rajasthan, Jaipur; 2004.
3. Mohapatra HP, Rath SP. *In vitro* studies of *Bacopa monnieri*–an important medicinal plant with reference to its biochemical variations. Indian J Exp Biol 2005;43(4):373-6.
4. Sharma A. *In vitro* morphogenetic regulation of *Boerhaavia diffusa*. Thesis, University of Rajasthan, Jaipur; 2006.
5. Vijayavergiya R, Vijay P. Quantitative estimation of primary metabolites of *Balanites aegyptiaca* L. In: Abstract Vol. of XXIX. All India Botanical Conference, Udaipur; 2006. p. 16.
6. Sharma RA, Meena RC, Jain SC, Saxena MK. Physio-chemical analysis of two species of *Cassia: C obtusifolia* Linn and *C siamea* Lam In: Proc of International Conference, Bot Expo March, Jaipur; 2006. p. 274-6.
7. Nagesh D, Swarna R, Srinivas P, Sriram S, Sundara Rajan S, Nagamani TS. Biochemical studies in leaves of *Terminalia catappa.* In: Proc 94th Indian Science Congress Part II (Abstracts). Annamalainagar 2007. p. 129.
8. Unnikrishnan V, Khaja M, Reddy S, Kumar KS, Rajan SS, Sharon FM. Biochemical studies in leaves of *Araucaria* species. In: Proc 94th Indian Science Congress Part II (Abstracts). Annamalainagar 2007. p. 149-50.
9. Sharma N, Tanwar BS, Vijayvergia R. Study of primary metabolites and antimicrobial activities of *Digera muricata* (L). Mart J Chem Pharm Sci 2011;3(2):424-31.
10. Dinges JR, Colleoni C, Myers AM, James MG. Molecular structure of three mutations at the maize sugary locus and their allele specific phenotype effects. Plant Physiol 2001;125:1406-18.
11. Critchley JH, Zeeman SC, Takaha T, Smith AM, Smith SM. A critical role for disproportionating enzyme in starch breakdown but not in starch is revealed by a knock out mutation in *Arabidopsis.* Plant J 2001;26:89-100.
12. Hasenah A, Houghtonard PJ, Soumyanath A. α-amylase inhibitory activity of some malaysian plants used to treat diabetes; with particular reference to *Phyllanthus amarus.* J Ethnopharmacol 2006;107(3):449-55.
13. Rajore S. Conservation through micropropagation of medicinal plant species. Thesis, University of Rajasthan, Jaipur; 2002.
14. Tull D, Phillipson BA, Kramhoft B, Kundeson S, Otsen O, Svensson B. Enhanced amylolytic activity in germinating barley through synthesis of a bacterial alpha amylase. J Cereal Sci 2003;37(1):71-80.
15. Singh S. Studies on morphogenesis in tissue culture of some economically important plants. Thesis, University of Rajasthan, Jaipur; 2004.
16. Nagamani TS, Rajan SS, Jeromi AL, Sharon FM, Eswaraiah G, Sriram S, *et al*. Biochemical studies in *Lantana camara*. In: Proc 94th Indian Science Congress Part II (Abstracts). Annamalainagar 2007;3-7:157.
17. Dubois, MK, Gilles JK, Robers PA, Smith F. Colorimetric determination of sugar and related substance. Analyt Chem 1952;26:351-6.
18. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 1972;31:426-8.
19. McCready RM, Guggolz J, Silviera V, Owens HS. Determination of starch and amylase in vegetables. Anal Chem 1950;22:1156.
20. Bernfeld P. α and β-amylases. Methods Enzymol 1955;1:149.
21. Thorpe TA, Meir DD. Starch metabolism in shoot forming tobacco callus. J Exp Bot 1974;25:288-94.
22. Russel CR, Moris DA. Invertase activity, soluble carbohydrates and inflorescence development in the tomato (*Lycopersicon esculentum* Mill.). Ann Bot 1982;49:89-98.
23. Patel KR, Berlyn GP. Phytochemical investigation on multiple bud formation in tissue culture of *Pinus coulteri.* Canad J Bot 1982;61:675-85.
24. Chatrath A, Chandra R, Khetrapal S, Polisetty R. Changes in nitrate, amino acid and sugar content during growth and differentiation of chickpea explants (*Cicer arietinum* L.). Indian J Plant Physiol 1996;1(2):80-3.
25. Swarnkar PL, Bohra SP, Chandra N. Biochemical changes during growth and differentiation of the callus of *Solanum surattense.* J Plant Physiol 1986;126:75-81.
26. Sjolund RD, Weier TE. An ultrastructural study of chloroplast structure and dedifferentiation in tissue cultures of *Streptanthus tortuosus* (Cruciferae). Amer J Bot 1971;58:172-81.
27. Veirskar B, Andersen AS. Dynamics of extractable carbohydrates in *Pisum sativum* III. The effect of IAA and temperature on content and translocation of carbohydrates in pea cuttings during rooting. Physiol Plant 1982;55:179-82.
28. Jasrai YT, Bhatt PN, Mehta AR. Changes in contents of macromolecules during epiphyllous bud regeneration in *Kalanchol mortagei*. Cell Chrom Res 1987;10:2-3.
29. Sharma N. Morphogenetic and biochemical studies in *Cissus quadrangularis* (L.): In important medicinal plant. Ph. D. Thesis, University of Rajasthan, Jaipur India; 2007.