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Metabolic pathway of solasodine synthesis in *Solanum mammosum* l. fruits

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Abstract:
Distribution of metabolites in the developing fruits of Solanum mammosum was investigated by analysing and estimating the dry matter, primary metabolites such as sugars, starch, protein, amino acids and secondary metabolite solasodine. Fruits of different growth phases sampled at 5 comparable stages- starting from post anthesis to ripened stages were analysed by separating the fruit parts such as outer/ inner flesh and seeds. During fruit development III stage showed colour change of fruit wall, mucilaginous and chlorophyllous inner flesh and significant qualitative changes of metabolites. The role of primary metabolites in general and free amino acids and sugars in particular in the synthesis of solasodine in the developing fruits is discussed.

Keywords: *Solanum mammosum, fruit, metabolites, solasodine*

1. Introduction

Plants belonging to the genus *Solanum* (Solanaceae) have been intensively investigated in terms of their chemical and biological properties. The genus *Solanum* is rich in steroidal glycoalkaloid which is an important group of secondary metabolites. In majority of the *Solanum* species, solasodine occurs as a glycone part of glycoalkaloids and it is a potential moity to be used as a substitute of diosgenine in the production of steroidal hormones and pharmaceuticals. Therefore, steroidal glycoalkaloids from *Solanum* plants have become increasingly important as a starting material for the production of hormones (Solouski *et al*., 2011). More than hundred different types of glycoalkaloids have been isolated from *Solanum* species (Sun *et al*., 2010). A concise report on the medicinal/pharmacological aspects solasodine by Patel *et al*. (2013) comprises updated information regarding pharmacological activity and analytical techniques.

Solasodine is a nitrogen containing steroidal glycoalkaloid obtained from different parts of the *Solanum* species. According to Grabbe and Fryer (1982) and Weissenberg (2001), even though the steroidal glycoalkaloid- solasodine is obtained from different species of the genus *Solanum*, *S. xanthocarpum* and *S. khasianum* are the main plant sources of solasodine. *S. mammosum* also is known to produce solasodine and fruits and seeds are the principal source (Perez-Medina *et al*., 1964; Saini *et al*., 1965; Seelkopf, 1968; Telek, 1977; Telek *et al*., 1977). Phytochemical/ pharmacological properties of *S. mammosum* include antioxidant activity (Kuo *et al*., 2000) and many traditional ethnomedicinal uses (Munoz *et al*., 2000; Wiart, 2000; DeFilipps *et al*., 2004; Chai, 2006; Stuart, 2010; Crommett, 2011).

Even though *Solanum* species are known to produce a number of secondary metabolites, steroid alkaloids are not participate directly in growth and development of the plant. Plants biosynthesise these secondary metabolites from simple precursors using unique enzymes (James, 1950; Robinson, 1981). Precursor feeding experiments have shown that in most cases aromatic amino acids tryptophan, phenylalanine, lysine *etc*. are the precursor of almost all alkaloids. Fruits and seeds of *S. mammosum* are reported to contain 0.2 to 2% solasodine content (Telek *et al*., 1977) But the distribution of metabolites during fruit development and ripening and their role in the synthesis of solasodine is yet to be elucidated. The present communication describes the involvement of primary metabolites in general and amino acids and sugars in particular in the biosynthesis of solasodine in *S. mammosum* fruits.

2. Materials and Methods

2.1. Plant Material

Seeds were collected from the plants cultivated in the Botanical garden of Calicut University where the germplasm has been maintained. The plants were raised from the seeds obtained from Venezuela as far back as 1972 by Prof. B.K. Nayar, Head of the Department of Botany. Seeds were germinated in seedling trays filled with soil, sand and farmyard manure. Seedlings were transplanted after one month to the field prepared by mixing sand, soil and manure and watered properly.

2.2. Collection of Fruit

Flowers were tagged on the day of anthesis and fruit growth was observed. Fruits were harvested at 5 developmental stages. Stage I was collected two weeks after anthesis and subsequent samples of fruits were collected at 2 week interval until fully ripe. The fruits attained full maturity and commenced ripening as indicated by localised patches of diffused yellow colour in stage III and in stage IV the fruits were uniformly pale yellow and in stage V they were orange yellow in colour.

2.3. Sampling

Fruits from stage I and II was cut open longitudinally and the inner part with placenta bearing the tender seed was scooped out 2-3 mm thick from the either half. In this manner the fruit was separated rather arbitrarily into an outer and inner layer. From stage III the inner layer was greenish in colour with mucilage. The seeds hardened in this stage and could be separated from the inner pulp. So three parts- outer pulp, inner pulp and seeds were separately analysed. Fruits of stage IV and V also were analysed similarly. Weight of the entire fruits were taken initially and from the sum of the component parts cut separately, the total weight was also taken. Dry weight content and dry weight percentages of each component and total fruits were estimated. For each sample, 10-15 fruits of each stage was collected and pooled and randomised samples were used for analytical study.

3. Analytical Methods

i. Ethanol soluble sugars: Sugars were extracted in hot ethanol by refluxing. After centrifugation, supernatant was passed through Dowex- 50W-X8 column. Sugars were separated by descending paper chromatography (Bacon and Edelman, 1951; Buchnan and Savage, 1952) and estimated according to Montgomery (1957).
ii. Starch: Starch was analysed according to Pucher *et al*. (1948) as described by Whelan (1955) and estimated (Montgomery, 1957).
iii. Free amino acids: Amino acids were extracted in hot alcohol as described under ethanol soluble sugars and after passing through the Dowex column the eluted sample was used for amino acid estimation following the method of Lee and Takahashi (1966).
iv. Protein: Total protein was estimated according to Lowry *et al*. (1951).
v. Solasodine: Ethanol extraction method was followed for the extraction and isolation of steroid alkaloid. Tissue was homogenised in 95% ethanol and the ethanol was evaporated over a steam bath to concentrate. Fractionation was done with solvent ether and precipitated with dilute ammonia. The final precipitate dissolved in ethanol was separated by Thin layer Chromatography (Stahl, 1966). The spots were visualized by spraying with antimony trichloride and identified as glycoalkaloid. Quantification of solasodine was done following the method of Birner (1969) using solasodine (Sigma) as the standard.
vi. Statistical analyses: All experiment were repeated a minimum of 5 times with randomised fruit samples of many plants and the data were analysed for significance using Fisher's 't' test.

4. Results

Distributions of fresh weight during five developmental stages of fruits show a gradual increase up to stage III followed by significant reduction during further growth (Table I). Dry weight percentage of the outer and inner pulp decreased gradually. But seed dry weight percentage was gradually and significantly increased during development (Table 2).

4.1. Sugars

The sugars were analysed separately in different stages of pulp and seeds. Only 5 sugars were present in the tissues analysed: the trisaccharide raffinose, disaccharide maltose and sucrose and the monosaccharides glucose and fructose (Table 3). During early stages outer pulp contained maltose, glucose and sucrose and IIIrd stage onwards raffinose was present which doubled during stage IV. While stage IV sample contained reduced level of maltose, fructose disappeared. Soluble sugar profile of stage III was very interesting because all the 5 components were at the highest spectrum which coincided the maximum concentration of solasodine (Tables 3, 7). Stage V was characterised by very high content of maltose which was the highest in any part of *S. mammosum* plants at any stage of fruit development.

Sugar concentration of the inner pulp was markedly lower than the outer pulp. Glucose was 6 fold higher than that of sucrose due to reduced sucrose content and the same trend was observed in stage II also. Stage III inner flesh showed only glucose, fructose and sucrose and fructose disappeared during stage IV and V. Maltose was very high in stage IV and raffinose was reduced to trace, fructose disappeared and glucose was abruptly reduced. In stage V, the inner flesh contained only sucrose.

Seeds of *S. mammosum* fruit was analysed only stage III onwards and at this stage seed contained more sugar component than in the following stages. Maltose and sucrose was nearly in equal quantities as also were glucose and fructose. Only in this stage could the fructose be quantified in the seeds. It may be recalled that it was only in stage III that the inner pulp contained fructose and that at a level nearly equal to that of glucose. Total sugar concentration was maximum in the seeds of stage III. Maltose, sucrose and glucose were continued to be present in stage IV and V. But total sugar content reduced less than half than stage III. Seeds of stage V showed increased sucrose while and sugar profile remained unchanged.

4.2. Starch

Starch concentration was reduced during growth to stage II and 2 fold increase was observed in the outer flesh at stage III and stage IV showed a sharp decline followed by a significant increase. Inner pulp contained considerably higher starch content than in the outer pulp. Starch content of III stage was 17 fold compared to the outer pulp (Table 4) which coincided maximum solasodine content and commencement of mucilage production (Table 7). Starch content of inner pulp showed a significant reduction and increase in stage IV and V respectively. Seeds contained maximum starch in III stage and reduced to one fourth in the stage IV and a further reduction was observed in the last stage.

4.3. Amino Acids

Total free amino acid was comparatively very low in the outer pulp of stage I and the amount was doubled in stage II which was the peak volume, the highest of any other stage (Table 5). It was reduced to less than one half in stage III and further reduced to the lowest quantity in stage V. Inner pulp contained low quantity of amino acid than in the outer pulp. Stage III, IV and V also showed more amino acid content in comparison with the outer pulp.

Seeds contained maximum amino acid content in stage III and it was reduced drastically in the successive stages retaining only one eighth in these stages.

4.4. Total Protein

Protein content of the outer pulp was maximum in stage I reflecting the high metabolic activity (Table 6). It decreased to less than one half in stage II, again decreased further during stage III and remained unchanged afterwards. Protein content of the inner pulp fluctuated without a definite pattern. However, protein concentration was higher in the inner pulp than the outer in all stages of growth. Seeds in stage III contained comparatively low and nearly the same magnitude as in the fruit pulp. In stage I there occurred a 3 fold increase of protein and this value remained unaltered in stage V signifying accumulation of reserve protein.

4.5. Solasodine

Solasodine was present in the outer pulp from the earliest stage of fruit development, but in much lower levels than in the inner pulp (Table 7). Stage II showed a significant increase of the solasodine and in stage III there was 2 fold increase. In the last stages solasodine content reduced significantly compared to stage III.

The general pattern of changes in solasodine concentration in the inner pulp paralleled that in the outer pulp (Table 7). A two fold increase in stage II was followed by a 6 fold increase in stage III to yield a peak concentration. The alkaloid content decreased significantly in IV and V . Seeds contained solasodine in a very low concentration, when the alkaloids in the inner flesh was maximum. In stages IV and V solasodine content increased slightly compared to that of stage III.

Tissues			**Fresh weight, g/fruit part in developmental stages**				
			I	II	III	IV	V
Fruit	Pulp	Outer	4.45±0.31	14.6±1.31	23.29±1.72	26.03±1.39	28.81±1.65
		Inner	1.09±0.04	3.43±0.17	3.42±0.30	4.49±0.23	5.14±0.19
	Seeds		.....	.....	3.95±0.24	4.38±0.26	5.48±0.41
Whole fruit			5.55±0.32	18.03±1.36	30.67±1.75	34.86±1.94	39.43±1.9

Table 1: Fresh weight distribution of S. mammosum L. fruits during different stages of development

Tissues			**Dry weight percentage, g/fruit part in developmental stages**				
			I	II	III	IV	V
Fruit	Pulp	Outer	7.73±0.23	6.56±0.19	10.46±0.18	13.14±0.26	14.5±0.46
		Inner	11.22±0.35	9.32±0.6	10.73±0.55	7.91±0.42	5.92±0.16
	Seeds		.....	.....	31.35±0.21	62.75±0.21	78.64±0.44
Whole fruit			9.45±0.26	7.94±0.36	17.51±0.29	27.94±0.51	33.02±0.48

Table 2: Dry weight distribution percentage of S. mammosum L. fruits during different stages of development

Tissues				Sugars, mg/g dw in developmental stages				
				I	II	III	IV	V
Fruit	Pulp	Outer	R	ND	ND	1.80±0.21	3.25±0.40	ND
			M	5.9±0.56	T	1.84±0.04	T	6.15±0.6
			S	21.67±0.26	5.52±0.54	3.45±0.41	11.23±0.12	13.66±0.89
			G	23.67±0.13	23.97±0.24	11.26±0.92	15.97±0.35	2.93±0.35
			F	ND	11.23±0.45	8.16±0.67	ND	ND
			Total Sugars	51.24±3.36	40.72±3.95	26.51±0.61	30.45±0.37	22.74±1.42
		Inner	R	ND	ND	ND	T	ND
			M	ND	T	T	4.22±0.04	T
			S	3.94±0.27	14.56±0.16	7.32±0.38	7.35±0.09	21.99±0.9
			G	23.94±0.23	3.59±0.17	9.72±0.11	T	ND
			F	ND	ND	7.77±0.24	ND	ND
			Total Sugars	27.88±1.12	18.15±0.86	24.81±1.42	11.57±0.73	21.99±0.9
Seeds			R			ND	ND	ND
			M			1.31±0.08	1.18±0.07	1.69±0.04
			S			1.36±0.2	0.95±0.05	2.56±0.02
			G			4.92±0.42	2.73±0.03	1.83±0.02
			F			4.89±0.4	ND	ND
			Total Sugars			12.48±0.76	4.86±0.24	6.08±0.27

Table 3: Ethanol soluble sugar concentration in developing fruits of S. mammosum L.
R-Raffinose M- Maltose S-Sucrose G-Glucose F- Fructose T- Traces ND- Not detected.

Tissues			Starch mg/g dry tissue in developmental stages				
			I	II	III	IV	V
Fruit	Pulp	Outer	12.8±0.38	8.79±0.6	21.89±0.66	3.88±0.15	22.96±0.62
		Inner	45.27±0.49	40.34±2.47	364.58±4.38	54.61±5.3	149.32±5.41
		Total	23.34	16.7	66.75	8.65	31.5
	Seeds		.....	.....	23.82±0.57	6.21±0.99	3.59±0.15
Whole fruit			23.34	16.7	53.6	7.62	17.87

Table 4: Starch concentration in developing fruit of S. mammosum

Tissues			Total free amino acid concentration mg/g dry tissue in developmental stages				
			I	II	III	IV	V
Fruit	Pulp	Outer	22.77±5.82	41.16±5.03	15.67±1.91	6.47±1.6	15.59±2.62
		Inner	21.66±2.05	32.19±3.65	26.75±4.19	12.9±2.65	21.62±0.84
	Seeds		.....	.....	17.61±1.28	2.45±0.29	2.63±0.43

Table 5: Amino acid concentration in developing fruit of S. mammosum

Tissues			Protein mg/g dry tissue in developmental stages				
			I	II	III	IV	V
Fruit	Pulp	Outer	146.83±4.53	61.74±2.94	51.05±3.35	66.06±0.84	50.62±3.24
		Inner	118.81±2.59	125.43±2.25	54.89±3.36	99.49±3.29	68.41±5.74
	Seeds		.....	.....	57.42±0.51	172.3±1.22	170.08±4.37

Table 6: Protein concentration in developing fruit and subtending leaf of S. mammosum

Tissues			Solasodine mg/g dry tissue in developmental stages				
			I	II	III	IV	V
Fruit	Pulp	Outer	1.04±0.2	1.61±0.05	3.33±0.3	1.66±0.02	1.02±0.03
		Inner	4.3±0.13	10.76±0.23	64.81±1.94	45.97±1.5	24.69±1.52
	Seeds		.....	.....	0.25±0.05	0.33±0.01	0.39±0.03

Table 7: Solasodine concentration in developing fruit and subtending leaf of S. mammosum L

5. Discussion

In developmental stages I and II, *S. mammosum* fruit contained developing seeds and hence the assimilates imported from the subtending leaves get utilized predominantly for respiration and structural development and comparatively small amount only get stored. A distinctive feature of *S. mammosum* fruits in stage III was the greening of inner pulp which is mucilaginous in texture and the greening of inner pulp is preceded by the progressive loss of green colour of the fruit exterior. It is possible that the greening of the inner pulp of *S. mammosum* in stage III was due to the translocation of dis-assembled chloroplasts from the fruit wall. Occurrence of chlorophyll and synthesis of alkaloids have been reported to be associated each other as observed in *Catheranthus roseus* in which vindalin as the predominant alkaloid is synthesized in light (Robinson, 1981). According to Ramaswamy *et al*. (1976), greening due to chlorophyll synthesis and solanin formation takes place simultaneously in potato. More or less a similar association is found to occur in *S. mammosum* since the greening of inner flesh and abundant occurrence of solasodine are observed in the fruits of stage III (Table 7).

Another aspect of the metabolite distribution pattern of stage III fruit of *S. mammosum* is the production of mucilage and solasodine content increase (Table 7). Similarly, fruit pulp in general and inner pulp in particular of *S. mammosum* contains maximum amount of sugars, starch and solasodine (Tables 3, 4, 7) indicating high metabolic activity of the fruit and the alkaloids tend to accumulate in active tissues as opined by Robinson (1981) and Robinson *et al*., (1987). Even though starch is the storage form of polysaccharide in *S. mammosum* fruits an estimate of total physiologically available carbohydrate can be had by adding together the hexose equivalence and the free sugars and this content is maximum in stage III when the ripening is started. It appears that *S. mammosum* fruits the accelerated accumulation of solasodine, the secondary metabolite and the commencement of mucilage production events are hand in hand with transient starch accumulation. Fruit at stage III, the inner pulp in particular, stood out prominently both physiologically and biochemically, since tissue softening is commenced in stage III presumably due to polygalacturonase activity as reported in fruits during softening of pulp (Coombe, 1976; Friend and Rhodes, 1981; Brady, 1987; and Giovannoni, 2001).

Total amino acid content showed maximum quantity in stage III of fruits. As pointed out earlier, active metabolism and incipient ripening coincide at this stage. A decisive role of amino acids in general and aspartate in particular is played during the starting phase of fruit ripening (Seymour *et al*., 2013), according to whom in fleshy fruits, level of indole acetic acid (IAA), the abundant auxin get reduced and forms a conjugant with aspartic acid during the initiation of fruit ripening. Similar profile of IAA- amino acid conjugant has been reported in pepper, banana, musk melon and strawberry (Bottcher *et al*., 2010). An interesting observation is the reduction of free amino acids after stage III. The reduction of free amino acid in stage IV and V is indicative of the utilization of free amino acids for the synthesis of LEA (Late Embryogenesis Abundant) proteins during the maturation of fruit and desiccation of seeds (Mayer and Poljakoff-Mayber, 1989; Bewley and Black, 1994). Similarly increase in the amount of solasodine at these developmental stages also is coincided with reduced amino acid content. Amino acid, tryptophan, phenyl alanine, tyrosine and arginine are known to participate in the synthesis of alkaloid as the precursors in plants (Coruzzi and Last, 2000) and hence the involvement of these amino acids in the synthesis of solasodine in *S. mammosum* cannot be ruled out.

Maximum alkaloid concentration was present in the inner pulp inclusive of the placentation in stage III and it was during the growth of the fruit between stage III and IV that the inner pulp turned green. Mucilage formation in appreciable amounts also commenced in stage III which is coincided with an abrupt increase in starch content. On the basis of the distribution pattern of metabolites, in the developing fruits of *S. mammosum* stage III of fruit development is characterised by several distinctive features inclusive of fruit colour change from green to slight pale yellow, occurrence of chlorophyllous and mucilaginous inner flesh and placentum, abundant

occurrence of dry matter metabolisable carbohydrates, total protein and total free amino acids super imposed with an accelerated synthesis of steroidal alkaloid-solasodine.

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