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FUNGAL DECAY OF CUCURBITACEOUS VEGETABLES IN KOSI DIVISION, BIHAR STATE

SHEO PRASAD SINGH, ANIL KUMAR SINGH[1], ALOK KUMAR[2], PUSPAA SINHA[3], PREM RAJ SINGH[4], MANOJ KUMAR[5], B. K. PRASAD[5]

Post-Graduate Dept. of Botany, V.K.S. University, Ara - 802301, India.
Department of Botany, T. P. College, Madhepura-852113, India.
1.C/o Prof. Sahdeo Lal, Pethia Bazar, Opp. Shri Durga Asthan, Phulwari Sharif, Patna-801505, India.
2. Department of Botany, S.G.G.S. College, Patna City -800008, India.
3. Department of Botany, B.D. College, Patna- 800001, India.
4. Department of Botany, College of Commerce, Patna-800020, India.
5. 106, Mahavir Enclave, Ambedkar Path, Bailey Road, Khajpura, Patna- 800014, India.

On surveying the storage fungi of popular cucurbitaceous vegetables in Kosi Division, Bihar State, 19 spp. were isolated of which 7 spp belonged to *Fusarium.* On biochemical analysis of green parwal fruit due to *Fusarium moniliforme* as incitant of decay during storage, starch and protein contents were observed decreased with concomitant increase in the level of sugar and amino acid. Amylase, invertase, exo- and end-β-1, 4-glucanase and protease activity were found stimulated. Of the nine amino acids detected alanine, valine and phenylalanine were meagre in concentration. Their decarboxylase, non-oxidative deaminase and oxidase were excited in their activity. Oxidase and decarboxylase of these amino acids were recorded in inoculated fruit only. The activity of starch phosphorylase and dehydrogenase of pyruvic acids, α-Ketoglutaric acid and succinic acid was recorded excited due to *F. moniliforme* besides uptake of more O_2 on addition of glucose, fructose and pyruvic acid in the extract of inoculated fruit.

Keywords: Biochemical Changes; Cucurbitaceous Vegetables; Fungal Decay in storage.

Introduction

It is established that the vegetables being perishables in nature are grievously destroyed in storage by the fungi[1-5]. Though the work of this nature has been extensively conducted in Allahabad University nearly five decades back besides some stray reports but no detailed report on vegetables is available. Therefore, there appears need of meticulous observation of biochemical change in course of rotting of the vegetables due to storage fungi.

In this paper, the rot inducing fungi of cucurbitaceous vegetables during storage in Kosi Division has been reported besides investigating biochemical changes in them more elaborately than communicated earlier[1-5]. The weather conditions of this division appear more conductive to the rotting of the noted vegetables as a result of high relative humidity prevailing due to a number of tributaries of the river Kosi in the region and the warm temperature.

Material and Methods

Stale and near rotting pumpkin *(Cucurbita pepo),* bottle gourd *(Laginaria siceraria),* Parwal *(Trichosanthes dioica),* bitter gourd *(Momordica charantia)* and Snake gourd *(Momordica anguina)*, stored in the godown and with retailers of the market of Kosi Division that includes Madhepura, Saharsa and Supaul Districts, were collected in sterilized polyethylene pockets in three different seasons of the year 2009 and 2010. The noted vegetables as such or after cutting in suitable size, were incubated at room temperature over distilled water in big sealed glass jars for 7 to 10 days and the growth of fungi was observed daily after three days of incubation. The fungi growing over the surface of the vegetables were isolated and cultured in pure form on potato dextrose agar (PDA) medium, and preserved on slants. These were identified with the help of standard texts and the frequency of the isolates was calculated.

Symptoms of the disease: The pathological symptoms were observed during natural and artificial storage. It appears worthwhile to mention that white pressed/ sparse mycelial growth was clearly visible on parwal and bitter gourd in storage after 4 to 5 days. The appearance of the similar symptoms took 6-8 days in case of bottle gourd, snake gourd and pumpkin. It is remarkable that the white mycelium was identified as the spp. of *Fusarium*[6] mainly

F.moniliforme as the pioneer. The colour of the noted fruits around the fungal growth was observed brownish, water soaked and pulpiness of the flesh.

Biochemical changes: Among the biochemical changes starch (ST), total soluble sugar (TSS), indivisual sugars (IS), total free amino acid (TFAA) and indivisual amino acids (IAA) were estimated besides assaying the activity of the relevant hydrolyzing enzymes *i.e.* amylase, invertase and protease; decarboxylase, oxidase and non-oxidative deaminase of those amino acids which seemed quantitatively diminutive and the activity of respiratory enzymes such as dehydrogenase of pyruvic acids, α-ketoglutaric acid and succinic acid in parwal fruit due to *F. moniliforme.* O_2 uptake by the extract of the flesh on addition of glucose, fructose and pyruvic acid was also recorded. For these purposes, the fungus was grown on PDA medium for 7 days at 28±1°C and the inoculums was applied to the fruit after its surface sterilization with 0.1% $HgCl_2$ for 1 min and thorough washing with autoclaved distilled water. The sterilized absorbant cotton was inserted in sterilized cork borer of 1.5 cm diameter. The fluffed cotton was cut with the help of scissors. Now, the plane surface of cotton of the borer was touched to the culture of the said fungus in petri dish and the swabe was touched to the surface sterilized fruit. The control fruit was maintained uninoculated. Both the fruits were incubated over 80% RH (Saturated solution of $(NH_4)_2SO_4$) at 30±1°C[7] for total period of 10 days and ST, TSS, protein and TFAA were estimated on, 0, 2nd, 6th, and 10th day after inoculation. IS and IAA were semiquantitatively graded on the 10th day only.

Estimation of ST: The flesh from both types of fruit i.e. inoculated and the control was taken and frozen at -10°C for 30 min and 1.0 g was extracted with 5 ml of 80% warm ethanol five times using 5 ml each time. It was centrifuged at 10,000 rpm for 15 min. The supernatant was preserved at -10°C for the estimation of TSS, IS, TFAA and IAA while the residue was extracted with 2 ml of 52% perchloric acid and the amount of ST was estimated after dividing with a factor 0.9 in the resulting sugar[8] which was measured spectrophotometrically at 620 nm using Anthrone reagent (Table 2).

Estimation of protein: Protein was estimated in the flesh after its extraction with warm ethanol for the estimation of sugar and amino acids, and treatment with perchloric acid to remove starch. The resulting residue after washing with water and centrifugation at 10,000 rpm for 10 min, was dissolved in 5 ml of 0.1 M NaOH solution and read at 750 nm with Folin- Ciocalteau reagent[9]. Standard curve was prepared with 0.1% casein for quantitative estimation (Table 2).

Estimation of TSS: The ethanol in the extract noted earlier was removed by warming on sand bath and by reduced pressure. TSS was estimated as noted for ST (Table 2).

Semiquantitative grading of IS. The ethanol free extract was concertrated by warming and IS was separated adopiting paper chromatography[10] using Whatman No. 1 paper and n-Butanol: Ethanol: Water (5:1:4 v/v/v) as solvent[11]. After completion of ascending run for 40hr, the paper was spray with detecting reagent consisting of 4% ethanolic aniline, 5 volume, 4% ethanolic diphenylamine, 5 volume and syrupy orthophosporic acid 1 volume[12]. IS were identified with the help of co-chromatogram prepared simultaneously and semiquantitatiely graded by + sign based on the area and concentration of sugar spots[10] (Table 4).

Estimation of TFAA and IAA: The ethanol free extract was used for the estimation of TFAA spectrophotometrically at 570 nm by Ninhydrin method[13] (Table 2). IAA was semiqutitatively graded like is after chromatographic Separation on Whatman No. 1 paper adopting double dimension technique[14] (Table 4).

Assaying the activity of amylase: One g of the flesh was extracted with 5 ml of 0.5 N Nacl at 5°C using chilled mortar and pestle. The extract was centrifuged at 10,000 rpm for 10 min. The supernatant was used as enzyme extract (EE). The substrate was 1.0 % water soluble starch. 2 ml of EE and 5 ml of ST were permitted to react. After 30, 60 and 90 min, the unreacted ST was read at 620 nm using iodine solution[15]. The activity of amylase was calculated as mg ST digested/ ml EE (Table 3).

Assaying the activity of invertase: Two g of the flesh was extracted with 10ml of 20% of glycerol 5ml of this was reacted with 5ml of 2.5% sucrose and 10ml of 1M acetic acid acetated buffer, pH5. After reaction for 30, 60 and 90 min, 1ml of the reaction mixture was read with 1ml of dinitrosalycylic acid reagent at 575nm[16]. The result was calculated as mg glucose released / ml EE using standard glucose solution for calibration curve (Table 3).

Assaying the activity of starch phosphorylase: The enzyme activity was estimated[17]. 2g of the flesh was extracted with 10 ml of water at 2°C and centrifuged at 10,000 rpm for 20 min. In the reaction mixture 0.5 ml of 0.6 M citrate buffer, pH6, 0.2ml of water and 2ml of EE in combination was incubated at 30°C for 15 min. 0.5 ml of 5% ST solution and 0.4 ml of 0.26 M glucose phosphate were added and mixed. The control was maintained using trichloroacetic acid to make its 5% strength before adding ST. After completion of reaction for 30, 60 and 90 min 1ml of the reaction mixture was taken out from each lot and the

reaction was stopped using 5% trichloroacetic acid. The phosphate released was estimated[18], using 1-Amino-2 naphthol 4- Sulphonic acid, $NaHSO_3$ and Na_2SO_3 (Eikonogen). The amount of phosphate released was calculated by reference curve prepared from serial dilution of 01% Na_2HPO_4 after reading at 660 nm (Table 3).

Assaying the activity of exo-β-1, 4-glucanase: Two g of the flesh was extracted with 10 ml of chilled 1M acetic acid acetate buffer, pH5 and centrifuged of at 10,000 rpm for 10 min. The activity was recorded at 485 nm using 1.0% of Avicel (Trade name of cellwlose) and 3.0 ml of 1.0% orcinol in 67% H_2SO_4[14]. The release of glucose was determined by calibration curve using serial dilution of 1.0% glucose solution.

Assaying the activity of endo-β-1, 4-glucanase: The activity of this enzyme was estimated by extracting 2g of the flesh with 5ml of chilled 1 M acetic acid acetate buffer pH 5.2 and 0.5% carboxymethylcellulose as substrate[14]. The amount of released reducing sugar was estimated[19]. The enzyme activity was calculated as glucose released / ml EE/30 min (Table 3).

Enzyme involved in release of amino acids and their degradation: More of the concentration of amino acid in the rotting parwal is expected due to excited protease activity which was confirmed here. For the decrease of alanine, valine and α-phenylalanine in concentration, the activity of their decarboxylase, oxidase and non-oxidative deaminase was suspected and was worked for verification.

Assaying the activity of protease: Two g of the flesh was extracted with 20 ml of chilled 0.1 M phosphate buffer, pH7. The extract was centrifuged at 10,000 rpm for 10 min and protease was estimated[20] using 5 ml of 1% casein as substrate and 2.5 ml of EE. The released amino acid was read at 570 nm using Ninhydrin reagent and the amount of amino acid released from casein / 30 min/ml extract was calculated using serial dilution of 0.1% glutamic acid as standard (Table 3).

Assaying decarboxylase of alanine and β-phenylalanine: Two g of the flesh was extracted in 10 ml of 0.1 M phosphate buffer, pH7 and centrifuged at 10,000 rpm for 10 min. The activity was measured by absorption of released CO_2 in alkaline phenolphthalein indicator[21]. 0.2% of alanine, Valine and β-phenylalanine were used as substrate. The fading in pink colour of alkaline phenolphthalein due to release of CO_2 was recorded at 550 nm as O.D (Table 5).

Assaying oxidase of alanine, valine and β-phenylalanine: Two g of the flesh was extracted with 10 ml of 0.067 M phosphate buffer, pH 7 and the oxidase activity was recorded with Warburg's manomets[14] using 0.2 ml of 0.25% substrate. µl O_2 uptake/ml enzyme was recorded (Table 5).

Assaying non-oxidative deaminase of alanine and β-phenylalanine: Two g of the flesh was extracted with 5 ml of 0.05 M phosphate buffer, pH 6.8, 2.5 ml of the extract was taken in two separate tubes. In one of them trichloroacetic acid was added to make its concentration 5% while in another tube the same volume of water was added. 5ml of 0.002M solutions maintained at pH 5 of the said amino acids were used separately as substrate and incubated at 37°C of 30 min. The reaction was stopped using trichloroacetic acid to make its 5% concentration. The reaction mixture of both the tubes was centrifuged separately at 10,000 rpm for 10 min and 2 ml of the supernatant was read with 0.1 ml of Nessler's solution at 420nm[22]. The result as O.D. was recorded in Table 5.

Estimation of the respiration: The respiration the flesh was measured after extracting 2g of the flesh with 5ml of 0.1M phosphate buffer pH, 6.8 and using 2ml of clear extract after centrifugation at 10,000 rpm for 10 min as enzyme source uptake of O_2 in µl was recorded at an interval of 10 min using 0.1 ml of 0.1 M glucose and the same volume of 0.1 M pyruvic acid solution with the help of Warburg's manomets using Brodie's fluid[14]. Besides that the activity of duly dropenase of pyruric acid, β-ketoglutaric acid and succinic acid in volved in Krebs cycle was also measurd and recorded (Table 6).

Estimation of the activity of dehydrogenase of pyruvic acid, α-Ketoglutaric acid and succinic acid: The activity of the noted enzymes involved in krebs cycle was estimated extracting 2 g of the flesh in 10 ml of chilled 0.1M phosphate buffer, pH7. As substrate 1 ml of 1.0% sodium salt of these acids 1 ml of buffer, 1ml of 0.4% picric acid as reagent and 1 ml of the enzyme source were taken and read at 550 nm in a spectrophotometer after extraction with 5ml of acctone[21]. The result was recorded (Table 7) as O.D. due to reduced picramic acid produced from picric acid.

Results and Discussion

Altogether 19 spp of fungi were isolated from stored *cucurbitaceous* vegetables (Table1), of which 7 spp belong to *Fusarium.* Remarkably *F.moniliforime* occurred with 100% frequency on the noted vegetables .Other notable fungi were *F. solani, F. Sporotrichloides, Aspergillus flavus, Botryodiploida theoleromae* and *Myrothecium roridum.* Other spp of *Fusarium* occurred on all the stored vegetables with low frequency. Other notable fungi were *Aspergillus flavus, Botryodiplodia theobromae, Myrothecium roridum* and *Paecilomyces varioti.*

Starch and protein content of the rotting

Table 1. Frequency of the fungus isolates of the rotting cucurbitaceous vegetables in storage.

Fungus isolates	Vegetables*				
	Pr	Ni	Bg	Pk	Sg
Fusarium moniliforme Sheldon	100	100	100	100	100
F. Solani Appel & Wollen weber	39	49	25	28	18
F. sporotrichioides sherbokff	49	-	18	36	26
F. roseum schwabe	12	6	10	8	12
F. lateritium snyder & Hansen	10	8	5	14	16
F. equisetti Sacc	16	10	10	15	19
F. culmorum Sacc	7	6	6	8	7
Rhizopus mgricans Ehren	11	4	-	3	6
Aspergillus flavus Link ex Fries	32	25	29	21	27
Penicillium digitaticum Sacc	21	-	-	25	09
Phomopsis sp.	17	-	-	-	05
Phytophthora sp.	-	7	-	-	-
Botryodiplodia theopromae Pat	12	42	20	15	23
Verticillium terrestre Lindau	11	31	-	-	12
A. sydowi Thom & Church	09	45	-	11	12
Ceplcalosporium roseo, grislum Saxena	-	15	-	07	-
Paecilomyces varioti Bainer	-	12	18	11	13
Myrothecium roridum Tode exFries	08	31	22	16	20
Trichothecium roseum Link	-	-	12	-	10

*Pr= Parwal, Ni= Ninua, Bg= Bitter guard, Pk= Pumkin, Sg= Snake gourd

Table 2. Periodic quantitative change in starch, total soluble sugar and total free amino acid in parwal fruit due to *F. moniliforme.*

Biochemicals	*I/C	Day of observation			
		0	2	6	10
Starch (expressed as mg/g fresh weight)	I	43.32	40.63	28.74	8.46
	C	43.32	41.94	36.87	28.49
Total soluble sugar (expressed as mg/g fresh weight)	I	28.88	30.27	38.12	37.08
	C	28.88	27.67	24.13	20.57
Protein(expressed as mg/g fresh weight)	I	12.62	11.46	6.54	2.17
	C	12.62	12.56	10.87	9.08
Total free amino acid(expressed as mg/g fresh weight)	I	6.97	8.28	17.81	29.37
	C	6.97	6.73	5.08	3.85

* I = Rotting, C = Control

Table 3. Rate of the activity of amylase, protease, invertase, starch phosphorylase, exo- and endo-β-1, 4-glucenase in parwal fruit due to *F. moniliforme.*

Enzymes	*I/C	Time in min. 30	60	90
Amylase (mg starch digested/ ml extract)	I	4.162	5.816	6.755
	C	1.113	1.762	2.014
Protease (mg amino acid released/ ml extract)	I	1.052	1.862	2.645
	C	0.463	0.827	1.007
Investase (mg glucose released/ ml extract)	I	2.262	2.931	3.471
	C	0.624	0.962	1.627
Strach phospharylase (mg phosphate released/ ml extract)	I	0.167	0.208	0.253
	C	0.063	0.098	0.146
Exo β-1, 4-glucanase (mg glucose released/ml extract)	I	1.965	2.761	3.635
	C	0.683	1.108	1.198
Endo β-1, 4-glucanase (mg glucose released/ml extract)	I	1.467	2.123	2.912
	C	0.612	1.208	1.872

* I = Inoculated, C = Control

Table 4. Semiquantitative grading of individual sugars and individual amino acid.

	Sugars					
*I/C	Gl	Fr	Su	Ma	Os_1	Os_2
I	4+	4+	+	2+	t	t
C	2+	+	2+	t	-	-

	Amino acids										
*I/C	GA	Gl	AA	Al	IL	Va	PA	AR	HI	U_1	U_2
I	5+	4+	4+	2+	3+	+	2+	3+	3+	+	+
C	2+	+	+	3+	0+	2+	3+	+	+	-	-

* I=Inoculated, C= Control, Gl= Glucose, Fr= Fructose, Su=Sucrose, Ma= Maltose, Os_1 = Oligosaccharide 1, Os1= Oligosaccharide 2, GA= Glutamic acid, Gl= Glycine, AA= Aspartic acid, Al= Alanina, IL= Iso-leucine, Va = Valine, PA = Phenylalanine, AR = Arginine, HI = Histidine, U_1 = Unidentified 1, U_2 = Unidentified 2, - = not detected.

vegetables due to *F. moniliforme* decreased considerably with rapid rate as the period of storage was extended .The rate of decrease of these two biochemicals was appreciably low in the control (Table 2). TSS and TFAA increased with rapid rate due to the fungus and decreased slowly in the control (Table 2). The activity of amylase, invertase, starch phosphorylasem exo- and endo-β-1, 4- glucarase and protease was considerably augmented in inoculated fruit of Parwal (Table 3). Glucose, fructose and maltose were higher in concentration on 10th of storage with the fungus while sucrose appeared with less concentration and there was *de novo* appearance of two oligosaccharides. In

Table 5. Activity of amino acid decarboxylase, oxidase and non-oxidative deaminase of alanine, valine and β-*phenylalanine* in parwal fruit due to *F. moniliforme.*

Enzymes	*I/C	Time in min. 15	30	45
Decarboxylase (expressed as O.D.)				
Alanine	I	0.010	0.005	0.002
	C	0.020	0.020	0.020
Valin	I	0.018	0.009	0.002
	C	0.020	0.020	0.020
β-Phenylalanine	I	0.015	0.004	0.001
	C	0.020	0.020	0.020
Non-oxidative deaminase (expressed as O.D)				
Alanine	I	0.021	0.028	0.037
	C	0.009	0.014	0.019
Valine	I	0.016	0.022	0.030
	C	0.007	0.010	0.014
β-Phenylalanine	I	0.027	0.036	0.042
	C	0.008	0.016	0.022
Oxidase (expressed as/ µl O_2 uptake / ml enzyme				
Alanine	I	0.1	0.2	0.3
	C	-	-	-
Valine	I	0.2	0.3	0.4
	C	-	-	-
β-Phenylalanine	I	0.2	0.3	0.4
	C	-	-	-

* I = Inoculated, C = Control.

Table 6. O_2 uptake by the extract of the flesh on addition of glucose, fructose and pyruvic acid as substrate (expressed as µl O_2 uptake /10 min).

Substrate	*I/C	Time in min. 10	20	30
Glucose	I	0.4	0.7	0.9
	C	0.2	0.3	0.4
Fructose	I	0.5	0.8	1.0
	C	0.2	0.3	0.4
Pyruviv acid	I	0.6	0.9	1.2
	C	0.2	0.3	0.4

* I = Inoculated, C = Control

Table 7. Activity of dehydrogenase of pyruvic acid, α-ketoglutaric acid and succinic acid on incubation of the fruit on 0, 2nd, 6th and 10th days period (expressed as O.D.)

Acids		Period in days			
	*I/C	0	2ND	6TH	10TH
Pyruvic Acid	I	0.15	0.22	0.57	0.38
	C	0.15	0.16	0.16	0.13
α-ketoglutaric Acid	I	0.15	0.20	0.48	0.29
	C	0.15	-	-	-
Succinic Acid	I	0.15	0.21	0.46	0.28
	C	0.15	-	-	-

* I = Inoculated, C = Control

control oligosaccharides were absent. Of the nine amino acids detected except alanine, valine and β-phenylalanine, others were more in concentration in the inoculated fruit (Table 4). Decarboxylase and oxidase of alanine, valine and β-phenylalanine were observed in inoculated parwal only. Non-oxidative deaminase activity of the three amino acids was recorded in the inoculated and control both but in the former the activity appeared faster in rate than the latter (Table 5). In course of estimating the respiratory metabolism, when glucose fructose and pyruvic acid were added as substrate to the enzyme extracted from the flesh the rate of O_2 uptake by the extract of the inoculated fruit was faster than that of control (Table 6). Also the activity of debydrogenase of pyruvic acid, α-ketoglutaric acid and succinic, the enzymes of krebs cycle, was more (Table 7).

The occurrence of the fungi on the stored cucurbitaceous vegetables like others, may depend upon many factors such as the volatiles emitted by them as seen some fruits[23,24], nutrient levels and their quality, anatomical features of the epidermis and enzymic ability of fungi to dissolve cutin layer[25], cell wall and middle lamella, their nutritional requirements and the methods of storage of the vegetables. The vegetables dependant factors my be conceived as an environmental factors[26]. The high frequency of the fungi on these vegetables indicates that these are suitable substrates for their colonization using appropriate levels of extracellular enzymes. The fungi not occurring on some vegetables points out unsuitability of their growth. In the regards the versatility of a *F. moniliforme* seems well established.

Gradual decrease in the level of starch and protein *vis - a - vis* increase in TSS and TFAA indicate the hydrolysis of these polymers by amylase and protease respectively as affirmed by stimulated activity of the two enzymes. This phenomenon has also been reported in stored seed[27] and in coriander gall disease incited by *Protomyces macrospores*[28]. Increase of glucose, fructose and maltose might be due to excited activity of invertase that yields first two sugars and amylase that yields maltose which is expected to produce glucose by the activity of glucosidase. The auqmented activity of exo- and endo-β-1, 4-glucancase that act on cellulose is highly expected to raise the concentration of glucose as the former splits off cellobiose or glucose unit from non-reducing end of cellulose and the latter attacks 1, 4-glucoseidic linkage releasing reducing sugar. Authors[29,30] have reported successive degradation of cellulose by a set of extracellular enzymes secreted by various microorganism with the final result of conversion into glucose.

The deficiency of alanine, valine and β-phenylalanine may be interpreted in term of the preterential utilization of amino acid by the said fungus as reported earlier1. The activity of decarboxylase, non-oxidative deaminase and oxidase which were inflated due to the fungus might be the source of decrease of some of the amino acids. The activity of decarboxylase and oxidase of alanine, valine and β-phenylalanine were observed in inoculated parwal only indicating their fungal origin. After comprehensive account of amino acid metabolism it has been found that *Neurospora crassa, Penicillium chrysogenum, P. notatum, P. roquiforti, Aspergillus niger* and serval bacteria possess glutamine α-ketoacid transaminase and d-amino acid oxidase activity[32]. The concentration of amino acids might be affected by the activity of enzymes alanine - ammonia transferase, glutamic - aspartate transaminase as observed in lablab

bean seed due to *Aspergillus niger*[33]. Free amino released from amino acids might form amide[34] or may be found as such[35]. Augmented respiratory metabolism of parwal fruit can be discerned by more uptake of O_2 by addition of glucose, fructose and pyruvic acid in the enzyme extract of the fungus inoculated flesh, More activity of pyruvic acid, α-Ketoglutaric acid and succinic acid dehydrgenase in the similar condition indicate rapid reactions of Krebs cycle. Exaggerated starch phosphorylase activity that produces more glucose 1-phosphate which due to phosphoglucomutase is expected to forms glucose-6-phosphate and enters the glycolytic pathway.

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