

Infestation of a Single Seed of Sapota Fruit by Seed Borer (*Trymalitis Margaritas*) Enhances Viability of Neighboring Seeds

Seshadri Shivashankar*, Manoharan Sumathi

Division of Plant Physiology and Biochemistry, ICAR-Indian Institute of Horticultural Research, Bengaluru, India

Abstract

Sapota fruit, *Manilkara achras* (Mill) Fosberg cv. Cricket ball shows high incidence of the physiological disorder known as Corky tissue under rainfed cultivation due to poor seed viability. Interestingly, however, fruits naturally infested by sapota seed borer (SSB), *Trymalitis margaritas* Meyrick (Tortricidae: Lepidoptera), remain free from the disorder. Hence, this study was carried out to elucidate the mechanism by which SSB infestation prevents corky tissue development in sapota. Comparative biochemical analyses of the uninfested seeds of SSB affected fruits and the seed of H and CT fruits showed that the activities of dehydrogenases, PEP carboxylase, concentrations of key metabolites such as, ATP, pyruvate, NAD and NADH and the contents of the growth hormones, ABA, IAA and GA were significantly higher in SSB seed showing that it was metabolically more active compared to seed from H and CT. The significantly higher rate and percentage of germination of uninfested seeds of SSB affected fruits confirmed higher seed viability and the markedly higher ratio of seed dry weight to fruit dry weight indicated higher sink strength and sink capacity compared to H and CT affected fruits. The study thus, confirmed that seeds from SSB affected fruit were more vigorous and viable compared to seed from CT infested and healthy fruits. The relatively lower levels of ROS and MDA coupled with lower electrical conductivity and higher pH reflected a higher level of tissue integrity of the SSB seed. Taken together, the study unequivocally established that infestation of a single seed by SSB led to enhanced seed vigour and viability in the neighbouring seeds and thus ensured that the infested sapota fruits remained free from corky tissue disorder. This novel finding could pave the way for prevention of incidence of corky tissue disorder in sapota by enhancement of seed viability during fruit growth.

Keywords

Corky Tissue, Dehydrogenases, Free Radicals, Sapota cv. Cricket Ball, Sapota Seed Borer, Seed Viability, TCA Cycle Intermediates

Received: April 27, 2018 / Accepted: May 29, 2018 / Published online: June 14, 2018

© 2018 The Authors. Published by American Institute of Science. This Open Access article is under the CC BY license.

<http://creativecommons.org/licenses/by/4.0/>

1. Introduction

Sapota fruit [*Manilkara achras* (Mill) Fosberg] cv. Cricket ball exhibits a physiological disorder known as corky tissue (CT) during periods of drought stress due to a reduction in seed viability [1]. Interestingly, however, fruits affected by sapota seed borer (SSB), *Trymalitis margaritas* Meyrick (Tortricidae: Lepidoptera), a common insect pest of sapota

fruit in Southern India, were found to be completely free from corky tissue even when the incidence of the disorder was 50% or higher in un-infested fruits. Past studies had revealed that the adult insect of SSB invariably infests a single seed among many present in a sapota fruit [2]. The adult lays eggs following fruit set and the neonate larva bore into the fruit and enters the seed. Subsequently, the larva feeds exclusively on the seed endosperm and emerges

* Corresponding author
E-mail address: drshivaiihr@gmail.com (S. Shivashankar)

tunneling out of the fruit on completion of larval cycle at or before fruit ripening [3]. In view of the fact that SSB feeds exclusively on the seed endosperm for its growth, it was apparent that there was a possible link between seed infestation and absence of CT. Incidentally, infestation by seed-feeding insects was reported to influence viability of neighboring hard seeds in some species [4, 5, 6]. It was therefore, likely that the absence of CT incidence in *Trymalitis margaritas* infested sapota fruits was possibly related to increased viability of un-infested seeds. In this backdrop, we studied the viability of seeds from healthy, CT and SSB infested fruits of sapota using biochemical indices and the results are discussed in relation to the formation of corky tissue disorder.

2. Materials and Methods

2.1. Fruit Sample

Studies were conducted on Twenty-year-old sapota (*Manilkara achras*) trees of c v 'Cricket Ball' planted at a spacing of 5 m × 5 m and maintained under standard agronomic practices at Bengaluru, India. The site had a mild tropical climate, with a mean annual rainfall of 800 mm from July - November. The soil was red loam with a pH of 6.7. The water retention capacity of the soil, assessed gravimetrically, was 28% (w/w). During the experiment, maximum and minimum temperatures ranged from 28.2°-33.4°C and 16.4°-19.5°C, respectively. The average relative humidity (RH) at 13.00 h was 45%.

Fully mature fruits of sapota cv. 'Cricket ball' were harvested from trees and ripened under ambient conditions at a mean maximum temperature of 29 - 31°C and relative humidity of 55 - 67%. At optimum ripeness, fruits were weighed and cut open for recording observations on seed parameters and CT incidence. CT affected fruits were identified based on the presence of a hard lump in the pulp under the skin [1]. Infestation of fruits by SSB was identified based on the presence of a tiny hole on the skin (Figure 1).

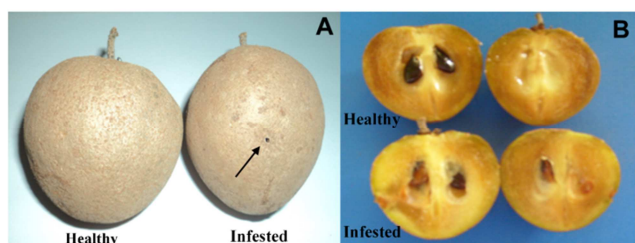


Figure 1. Picture shows (A) healthy and SSB infested sapota fruit. Arrow indicates the hole through which the neonate larva bore into the fruit (B) seed development in healthy and infested fruits

Fruits were cut into two equal halves and examined for the presence of CT in the pulp. Seeds in SSB affected fruits were

extracted and the infested seed was separated out from the rest and data on seed parameters of healthy seeds from the infested fruits were recorded.

2.2. Moisture Content, Seed Germination and TDH Activity

Pulp and seed moisture were determined gravimetrically following ISTA [7]. Germination test was performed on seeds sown on moist filter paper in closed Petri dishes and placed in an incubator in darkness at 28°C. Seeds were considered as germinated when the radicle length was more than 2 mm [8]. The number of germinated seeds was recorded daily up to 120 days. Seed viability was measured by measuring total dehydrogenase (TDH) activity using triphenyl tetrazolium chloride (TTC) assay [7]. After removing the seed coat, the embryo was soaked overnight in water. Three ml of 0.1% (w/v) TTC reagent was added to 100 mg of embryo, and incubated for 24 h at 37°C in a water bath. The tissue was homogenized in 10 ml 70% (v/v) methanol, centrifuged at 2500 × g for 10 min, and the absorbance of the clear supernatant was read in a spectrophotometer at 485 nm. A standard curve prepared from known concentrations of triphenyl formazan was used to express TDH activity as µg formazan produced g⁻¹ FW.

2.2.1. Amylase

α-Amylase was extracted by homogenising 1.0 g of seed in 10 ml of 16 mM sodium acetate buffer, pH 4.8, containing 0.5 M NaCl and centrifuged at 10,000 × g for 10 min at 4°C. Five-hundred µl of the supernatant was added to a reaction mixture containing 0.5 ml of 1% (w/v) starch dissolved in the same extraction buffer and incubated for 30 min at 20°C. The reaction was terminated by adding 0.5 ml DNS reagent and heated for 5 min in a boiling water bath. The mixture was made up to a final volume of 20 ml, and its absorbance was read at 540 nm [9]. α-Amylase activity was expressed as mg glucose formed mg⁻¹ protein min⁻¹.

2.2.2. Protein

Protein content was determined colorimetrically using the Folin-phenol reagent [10]. One gram of tissue was homogenised in 5 ml of 0.1 M Na-phosphate buffer, pH 7.6 and centrifuged at 2,500 × g for 10 min at room temperature. One-hundred µl of the supernatant was mixed with 900 µl water and mixed with 5 ml alkaline copper reagent [50 ml Reagent A plus 1 ml Reagent B; where Reagent A = 2% (w/v) sodium carbonate in 0.1 M NaOH; and Reagent B = 0.5% (w/v) copper sulfate in 1% (w/v) potassium sodium tartrate]. The reaction mixture was allowed to stand at room temperature for 10 min. Then, 0.5 ml of Folin-phenol reagent was added, mixed, and incubated for 30 min in the dark. The absorbance of the solution was measured at 660

nm. The amount of protein in each sample was calculated from a standard curve and expressed in mg g^{-1} FW.

2.2.3. Lipase

Lipase activity was determined as described by Jayaraman [11]. Five hundred milligrams of acetone powder was extracted with 10 ml of 0.05M sodium phosphate buffer, pH 7.0 and left standing overnight at 4°C. The extract was centrifuged at $8,000\times g$ for 10 min and supernatant was used as the enzyme source. The assay medium consisted of 5 ml substrate (a mixture of 1 ml groundnut oil, 1.5 ml of 0.1 M phosphate-citrate buffer, pH 7.0, 0.5 ml of 10% gum Arabica and 5 ml water), 3 ml of 0.01M NaCl and 2 ml of enzyme. The reaction mixture was incubated for 30 min at 37 °C. The reaction was terminated after 30 min by the addition of 10 ml absolute alcohol. For the blank, the reaction was stopped at zero time. The reaction mixture was titrated against N/200 NaOH using phenolphthalein indicator. Lipase activity was expressed as mg free fatty acid (FFA) liberated $\text{g}^{-1} \text{h}^{-1}$ based upon the standard graph constructed with linoleic acid as standard.

2.2.4. Free Amino Acids (FAA)

Free amino acids were extracted and estimated following Lee and Takahashi [12]. 0.5 g tissue was homogenized in 80% (v/v) alcohol and centrifuged at $10,000\times g$ and the supernatant was collected and evaporated to dryness over a boiling water bath. The residue was dissolved in 3.0 ml of 10% isopropyl alcohol. To 0.2 ml of the extract, 3.8 ml of ninhydrin-citrate-glycerol reagent prepared by mixing 1.0 ml 1% ninhydrin solution in 0.5 M citrate buffer (pH 5.5), 2.4 ml glycerol and 0.4 ml of 0.5 M citrate buffer were added and mixed. The mixture was heated in a boiling water bath for 12 minutes, cooled to room temperature and the optical density was measured at 570 nm using spectrophotometer. Glycine was used as the standard.

2.2.5. Total dehydrogenase (TDH)

Seed viability was estimated by measuring TDH activity using the triphenyl tetrazolium chloride (TTC) assay [7]. After removing the seed coat, the embryo was soaked overnight in water. Three ml of 0.1% (w/v) TTC reagent was added to 100 mg of embryos, and incubated for 24 h at 37°C in a water bath. The tissue was then homogenized in 10 ml of 70% (v/v) methanol, centrifuged at $2,500 \times g$ for 10 min, and the absorbance of the clear supernatant was read in a spectrophotometer at 485 nm. A standard curve prepared from known concentrations of triphenyl formazan was used to express TDH activity in μg formazan produced g^{-1} FW.

2.2.6. Glyceraldehyde 3-phosphate Dehydrogenase (GA3PDH)

Enzyme activity was determined by measuring the increase

in absorbance caused by the reduction of NAD in a 3 ml reaction mixture containing 2.5 ml of 100 mM Taps buffer (pH 8.6), 0.1 ml of 20 mM NaH_2PO_4 , 0.1 ml of 1mM NAD, 0.1 ml of 6 mM cysteine and 0.1 ml of 1.5 mM glyceraldehyde 3-phosphate. The contents were mixed after the addition of 0.1 ml of the enzyme extract and increase in absorbance was read at 340 nm for 5 min. One unit was defined as the amount of enzyme needed to cause an initial rate of reduction of one micromole of NAD per minute at 25°C [13].

2.2.7. Glucose 6-phosphate Dehydrogenase (G6PDH)

Two sets of tubes containing 100 μl aliquot of the crude extract, total dehydrogenase assay buffer, 50 mM Tris-HCl, pH 8.1, 1.0 mM MgCl_2 and 0.1 mM NADP were prepared. Into one set, 0.2 mM glucose 6-phosphate was added while 0.2 mM 6-phosphogluconate was added to the second set. The reduction of NADP in both sets was measured as the rate of change of absorbance at 340nm for the initial 6 min. Glucose 6-phosphate dehydrogenase activity was calculated as the difference of activities between the two sets [13].

2.2.8. Malate Dehydrogenase (MDH)

Malate dehydrogenase activity was determined as described by Selvaraj et al. [14]. The assay mixture consisted of 0.4 ml of 0.2 M Tris-HCl buffer, pH 7.5, 0.5 ml of 1 mM NADH, 0.5 ml of 15 mM oxaloacetate, and 0.1 ml enzyme extract. Absorbance at 340 nm was measured at 30°C and malate dehydrogenase activity was expressed as change in absorbance at 340 nm $\text{mg}^{-1} \text{protein min}^{-1}$.

2.2.9. Phosphoenol Pyruvate Carboxylase (PEP Case)

PEP-carboxylase activity was determined spectrophotometrically at 340 nm by coupling the reaction to NADH oxidation mediated by malate dehydrogenase. The reaction mixture contained 1.7 ml of 50 mM HEPES, pH 7.5, 0.1 ml of 5 mM MgCl_2 , 0.1 ml of 1 mM NADH, 0.3 ml of 2.5 mM NaHCO_3 , 0.1 ml of 2.5 units of malate dehydrogenase, 0.1 ml of 1.5 mM phosphoenol pyruvate (PEP), and 0.1 ml of enzyme extract. Control was without PEP. The contents of tubes were quickly mixed by inversion and the decrease in absorbance was recorded at 340 nm for 5 min. One unit of enzyme was defined as the formation of 1.0 μmole of oxalacetate from phosphoenol pyruvate per minute at pH 7.5 [15].

2.3. Estimation of Plant Growth Substances

Seeds (100mg) were ground in liquid nitrogen, mixed with 4 ml of a 99:1 (v/v) mix of isopropanol: glacial acetic acid containing 20 ng each of internal standard [gibberellin (GA_3)],

abscisic acid (ABA), and indole-3-acetic acid (IAA)], and incubated at 4°C on a shaker at 300 rpm for 24 h in the dark. Samples were centrifuged at 300×g for 10 min and each supernatant was transferred to a clean test tube. The pellet was re-suspended in 500 µl of extraction solvent [a 20:80 (v/v) mix of methanol: isopropanol with 1% (v/v) glacial acetic acid] and centrifuged again at 300×g for 10 min at 4°C. Subsequently, the two supernatants were combined and mixed with 4 ml of 100% (v/v) methanol, followed by 4 ml of extraction solvent as shown above. The purified extract was concentrated under a continuous stream of nitrogen and the residue was re-dissolved in 200 µl HPLC-grade methanol, centrifuged at 12,740 ×g for 10 min at 4°C, filtered through a 0.22-µm PTFE filter (Waters, Milford, MA, USA) to remove particulate matter, and transferred to a 200 µl HPLC vial.

High-performance liquid chromatography (HPLC): The HPLC method of Kelen et al. [16] was combined with electro-spray ionisation-tandem mass spectrometry (ESIMS-MS; [17]. The separation of compounds was performed using a Shimadzu LC-10 AD VP liquid chromatograph, equipped with a stainless steel analytical column, (Synergi 4µ Hydro RP 80A; 250 mm × 4.6 mm id; Phenomenex, Torrance, CA, USA). Each sample (10 µl) was injected into the HPLC system and the eluting ions were monitored by Multiple Reaction Monitoring (MRM; [18].

Separation of ABA and GA₃ was carried out by isocratic elution using a 26:74 (v/v) mix of acetonitrile: water, pH 4.0, as the mobile phase, at a flow rate of 0.8 ml min⁻¹. IAA was separated using a 30:70 (v/v) mix of acetonitrile: water, pH 4.0, at a flow rate of 1.0 ml min⁻¹ [16]. The mobile phase was filtered through a 0.22-µm nylon membrane and de-gassed before use. Plant growth regulator (PGR) concentrations were quantified using calibration curves prepared using 10 µl of each standard (Sigma, St. Louis, MO, USA) injected into the HPLC system linked to a tandem mass spectrometer (Quattro Ultima; Micro-Mass, Manchester, UK). The area under the MRM product ion peak was determined for each sample and each internal standard (IS) in a dilution series. The response was measured as: Analyte ion peak area × (IS concentration/IS product ion peak area) where, IS concentration was the amount of the internal standard added. Calibration curves were prepared for each compound by plotting the known concentration of each compound against the calculated response for each standard solution in a dilution series.

Mass spectrometry: Standard solutions (10 µM) were prepared in a 1:1 (v/v) mix of acetonitrile: water, acidified with 5% (v/v) glacial acetic acid to pH 4.3 and infused directly and separately in a quadrupole tandem mass spectrometer (Quattro Ultima) attached to an electrospray ion source (ESI-MS/MS) to select diagnostic precursor-to-

product ion transitions. Electrospray capillary and cone voltages were optimized to produce the requisite precursor molecular ions in negative or positive ionization mode. IAA, ABA, GA₃ and their respective internal standards were analyzed in the negative-ion mode. After determining the characteristic precursor-to-product ion transitions, a mixture of all unlabelled compounds and their internal standards was separated by reverse-phase HPLC and analysed by tandem mass spectrometry, with MRM, to determine the retention times of test compounds.

2.3.1. Polyamines (PA)

Polyamines were extracted with perchloric acid and analyzed by the benzylation method as reported by Serrano et al. [19]. Extracts for polyamine analysis were prepared by homogenizing 2g of sapota seed sample in 5 ml of 5% HClO₄ using a mortar and pestle. The homogenate was centrifuged for 30 min at 20,000×g and 2 ml of supernatant was mixed with 2 ml of 4M NaOH and 0.2 ml of benzoyl chloride in a glass tube. After vortexing the contents for 15s, the mixture was incubated for 20 min at room temperature. Four ml each of saturated NaCl and cold diethyl ether were then added. The tube contents were vortexed for 15s and incubated for 30 min at -18°C. Two ml of the ether phase containing benzoyl-polyamines was evaporated under nitrogen and re-dissolved in 1 ml of HPLC grade methanol. The benzoyl- polyamines mixed with hexanediamine as the internal standard were separated using Shimadzu LC-10A system (Shimadzu, Kyoto, Japan) on a reverse-phase column (Synergi, 250 × 4.6 mm, 5µm Hydro-RP, C18, Phenomenex, Torrance, CA, USA) using a mixture of ethanol: water (64:36, v/v), run isocratically at a flow rate of 0.8 ml min⁻¹ and detected by absorbance at 254 nm. A relative calibration procedure was used to determine the amounts of polyamines in samples using standard curves of putrescine, spermidine and spermine (Sigma, CA, USA) and the results were expressed as mg g⁻¹ FW.

2.3.2. HPLC Separation of Sugars

The identification and quantification of sugars was performed by HPLC according to the method described by Galdón et al. [20]. One g of the frozen sapota seed was directly transferred to polypropylene tubes and mixed with 2 ml of 4:1 ethanol: water. The tubes were placed in an ultrasound bath for 5 min and centrifuged for 5 min at 1090 × g. The supernatant was carefully collected so as to prevent contamination. Two ml of 4:1 ethanol: water was added to the pellet and placed in the ultrasound bath and centrifuged again. The two supernatants were mixed and concentrated under a continuous stream of nitrogen until the ethanol was completely removed and the residue was dissolved in 5 ml of 50% acetonitrile and passed through a 0.45µm filter GHP

(Waters, Millford, MA, USA) prior to HPLC analysis.

HPLC analysis was performed by Shimadzu LC-10 AD VP high performance liquid chromatography system under isocratic conditions at $25 \pm 1^\circ\text{C}$. The mobile phase consisted of (A) acetonitrile and (B) water (70:30). The flow rate was set at 1 ml min^{-1} and injection volume was $10\text{ }\mu\text{l}$. The analytical column Alltech-NH₂ (4.6 mm \times 250 mm \times 5 μm) was used for separation and detected by refractive index detector (RID-10A). Total time of analysis was up to 30 min. The identification of sugars in samples was done by comparison of retention times of individual sugars with reference standards. The quantification of sugars was done based on comparison of peak areas obtained from the reference standards. Concentrations were expressed as $\text{g } 100\text{ g}^{-1}\text{ FW}$.

2.3.3. GCMS Separation of Fatty Acids

Seeds were homogenized in a mixture of chloroform-methanol (2:1 v/v) and filtered through Whatman no. 1 filter paper. The chloroform phase containing lipids was separated, dried in a rotary vacuum evaporator at 40°C and stored at -20°C until further used [21]. The extracted lipids were methylated by dissolving in methanol and refluxed for 10 min at 70°C , followed by addition of 14% BF₃ in methanol and further refluxed for 30 min at 70°C . Methyl esters of fatty acids were subsequently extracted in heptane and dried on anhydrous sodium sulfate and filtered through $0.2\text{ }\mu\text{m}$ nylon membrane.

GC-FID analysis of fatty acid methyl esters was carried out with Varian-3800 Gas chromatograph system equipped with flame ionization detector (FID) on a fused silica capillary column (VF-5 Factor Four, Lake Forest, CA, USA), $30\text{ m} \times 0.25\text{ mm i.d.}$ and $0.25\text{ }\mu\text{m}$ film thickness. The temperature program for the column was as follows: The initial oven temperature was 100°C for 4 min, increased by 3°C per min up to 220°C , held for 4 min, temperature increased further at the rate of 5°C per min up to 260°C and held for 10 min. Injector and detector temperatures were maintained at 250°C and 260°C respectively. Helium was used as the carrier gas at a flow rate of 1 ml/min . Flow rates of H₂ and air were maintained at 20 ml/min and 250 ml/min respectively. The initial injection mode was split-less followed by split mode (1:30) after 1.5 min.

Gas chromatography-mass spectrometry analysis was performed on Varian-3800 gas chromatograph coupled with Varian 4000 GC-MS-MS ion trap mass selective detector. Fatty acids were separated on VF-5MS fused silica capillary column (Varian, USA) ($30\text{ m} \times 0.25\text{ mm i.d.}$ with $0.25\text{ }\mu\text{m}$ film thickness) by applying the same temperature program as described above for GC-FID analysis. The carrier gas was helium at a flow rate of 1 ml/min ; injector temperature, 260°C ; ion source-temperature, 220°C ; trap temperature,

200°C and transfer line temperature, 260°C . Mass detector conditions were: EI-mode at 70 eV with full scan range, $50\text{--}450\text{ amu}$. Fatty acids were identified by comparing the relative retention times of FAME peaks with those of reference standards (Sigma-Aldrich, USA) and the spectra with those available in Wiley and NIST-2007 spectral libraries [22]. The total quantity of FAME was estimated as the sum of all GC-FID peak areas in the chromatogram and individual compounds were quantified by comparing the known individual FAME procured as standard. All the analyses were performed on three samples run separately.

2.4. Free Radicals (ROS)

Superoxide anion (O_2^-) levels were estimated following Doke [23]. The levels of hydroxyl radicals ($\bullet\text{OH}$) were determined as described by Von Tiedemann [24], using 2-deoxyribose as the scavenger molecule. Hydrogen peroxide content was measured according to Schopfer et al. [25] and expressed as $\text{ng H}_2\text{O}_2$ generated $\text{g}^{-1}\text{ FW}$ of tissue.

2.5. Malondialdehyde (MDA)

Lipid peroxidation was monitored by measuring the conversion of lipids to malondialdehyde (MDA) using the thiobarbituric acid reactive substances (TBARS) assay, as described by Draper and Hadley [26]. TBARS reagent (1 ml) was added to a 0.5 ml aliquot of tissue homogenate and heated for 20 min at 100°C . The antioxidant, butylated hydroxytoluene, was added before heating the samples. After cooling on ice, samples were centrifuged at $840\times g$ for 15 min and absorbance of the supernatant was read at 532 nm . Blanks for each sample were prepared and assessed in the same way to correct for the contribution of A_{532} to the sample. TBARS results were expressed as MDA equivalents using 1,1,3,3-tetraethoxypropane as standard.

2.6. NAD

NAD was determined as described by Tezuka et al. [27]. Sapota seed sample was homogenised in 0.1 M HCl at 95°C , cooled in an ice bath and then the pH was adjusted to 6.5 with 0.1 N NaOH . 0.5 ml of 0.2 M glycylglycine (pH 6.5) was added to the coenzyme fractions, and centrifuged at $10,000 \times g$ for 20 min at 4°C . The supernatant was immediately used for the assay of NAD. 0.1 ml of the extract was added to the reaction mixture containing 50 mM glycylglycine (pH 6.5), 20 mM nicotinamide, 1 mM phenazine methosulfate (PMS), 1 mM thiazolyl blue (MTT) and 3,3'-diaminobenzidine (ADB) to a final concentration of 40 mg ml^{-1} . After placing the cuvette containing the reaction mixture in a UV-visible spectrophotometer for measurement at 570 nm , 40 ml of 80% ethanol was added to start the reaction. Results were expressed as $\text{ng g}^{-1}\text{ FW}$.

2.6.1. NADH

Measurement of NADH was performed as described by Tezuka et al. [27]. Seed sample was homogenised with 0.1M NaOH at 95° C, cooled in an ice bath, and then the pH was adjusted to 7.5 with 0.1 N HCl. 0.5 ml of 0.2M glycylglycine (pH 7.5) was added to the coenzyme fractions and centrifuged at $10,000 \times g$ for 20 min at 4°C for 10 min, and the resulting supernatants were immediately used for the measurement of NADH. For measurements, 0.1 ml of the extract was added to the reaction mixture containing 50 mM glycylglycine (pH 7.5), 20 mM nicotinamide, 1 mM phenazine methosulfate (PMS), 1 mM thiazolyl blue (MTT) and ADB to a final concentration of 40 mg ml⁻¹. 40 ml of 80% ethanol was added to start the reaction. The absorbance was read at 570 nm and expressed as ng g⁻¹ FW.

2.6.2. ATP

ATP was assayed as described by Stitt et al. [28] with some modifications. The assay medium in a final volume of 1 ml contained 100 µl of extract, 100 mM Tris-HCl, pH 8.1, 5mM MgCl₂, 0.25 mM NADP, 1 mM glucose, 0.7 Units ml⁻¹ G6PDH and 0.7 Units ml⁻¹ Phosphoglucosomerase (PGI). After 2 min incubation, 0.6 Units ml⁻¹ hexokinase was added. The absorbance was measured at 340 nm and expressed as ng g⁻¹ FW.

2.6.3. Pyruvate

Pyruvate was assayed as described by Chen et al. [29] with some modifications. To the reaction mixture containing 200 mM Tris-HCl, 3.0 mM EDTA-NaOH pH 7.0, 0.15 mM NADH and an aliquot of extract, 2.0 Units ml⁻¹ Lactate dehydrogenase (LDH) was added. Pyruvate content was

determined as the difference in absorbance at 340 nm before and after addition of LDH and expressed as ng g⁻¹ FW.

2.7. Conductivity and pH

One gram of pulp tissue was suspended in 10 ml of distilled water and electrolyte leakage was measured as conductance using a conductivity bridge (ELICO model CM-180) and expressed as dSm⁻¹. pH was recorded using a combination electrode.

2.8. Statistical Analysis

The experiment was laid out in a completely random block design with three replications. Seed germination and viability tests were performed on ten seeds, each replicated ten times. Data were subjected to analysis of variance (ANOVA) using the MSTAT statistical program (Michigan State University, East Lansing, MI, USA) and are presented as means \pm standard error (SE) [30]. Differences at $P \leq 0.01$ were considered significant. Principal component analysis (PCA) was performed after mean-centering the variables and dividing each variable by its standard deviation (auto scaling).

3. Results

An examination of 790 ripe fruits of sapota cv. 'Cricket ball' showed SSB infestation in 275 fruits. Among the remaining 515 non-infested fruits, 125 fruits showed corky tissue symptoms while SSB infested fruits were absolutely free from corky tissue. The seed number was higher in SSB affected fruits (4.17) than H (3.65) or CT (2.84) fruits (Table 1).

Table 1. Fruit growth and seed germination parameters. The different letters between H, CT and SSB columns indicated significant difference at ($P \leq 0.05$).

Parameters	H	CT	SSB
Seed moisture (%)	43.90 \pm 1.67 ^a	40.60 \pm 0.45 ^b	50.81 \pm 0.82 ^c
Pulp moisture (%)	72.37 \pm 0.91 ^a	68.09 \pm 0.64 ^b	71.91 \pm 0.78 ^a
Germination rate (days)	36.73 \pm 1.55 ^a	60.66 \pm 1.18 ^b	20.61 \pm 1.48 ^c
Germination (%)	58.54 \pm 0.98 ^a	34.88 \pm 1.47 ^b	64.15 \pm 1.06 ^c
Total dehydrogenase activity (μ g g ⁻¹ FW)	18.36 \pm 1.02 ^a	14.09 \pm 0.90 ^b	25.38 \pm 1.33 ^c
Fruit weight (g)	69.98 \pm 2.10 ^a	72.20 \pm 2.31 ^b	51.53 \pm 1.82 ^c
Individual Seed weight (g)	0.538 \pm 0.02 ^a	0.540 \pm 0.015 ^a	0.688 \pm 0.022 ^b
Total seed weight (g)	1.96 \pm 0.35 ^a	1.95 \pm 0.27 ^b	2.251 \pm 0.30 ^c
Seed weight/Fruit weight	0.109 \pm 0.003 ^a	0.088 \pm 0.002 ^b	0.137 \pm 0.006 ^c
Mean Seed number (no.)	3.65 \pm 0.31 ^a	2.84 \pm 0.20 ^b	4.168 \pm 0.40 ^c
Amylase activity (mg glucose formed mg ⁻¹ protein min ⁻¹)	0.358 \pm 0.035 ^a	0.339 \pm 0.029 ^a	0.524 \pm 0.013 ^b
Lipase activity (mg free fatty acid liberated g ⁻¹ h ⁻¹)	58.21 \pm 1.15 ^a	36.58 \pm 0.976 ^b	62.31 \pm 1.34 ^a

Total seed weight was higher in SSB infested fruits (2.88g) compared to H (1.96g) and CT affected fruits (1.53g). On the contrary, fruit weight was lower in SSB infested fruits (51.53g) compared to H (69.98g) or CT (72.20g) and as a result, the ratio of the weight of seed to that of fruit was the

highest in SSB fruits (0.056) followed by H (0.028) and CT (0.021). The seed moisture content was higher at 50.81% in SSB infested fruits while seeds from healthy and CT fruits had 43.90% and 40.60% respectively. The activity of hydrolytic enzymes, amylase and lipase and rate of seed

germination were higher in un-infested seeds from SSB-infested fruits (64.2%) than healthy (58.5%) or CT affected (34.9%) fruits. Data provided in Table 1 on the seed characteristics of SSB- infested, healthy and CT-affected fruits revealed that the number of days required for seed germination of un-infested seeds from SSB-infested fruits was significantly lower (20.6 days) compared to seeds from healthy (36.7 days) and CT affected fruits (60.7 days). TDH activity was significantly higher in un-infested seeds from

SSB fruits compared to H and CT fruits.

SSB seed had the highest concentration of sucrose followed by H and CT while the levels of glucose, fructose and galactose were very low. The free amino acid content was higher in SSB followed by H and CT. The ratio of ABA/GA was higher in SSB followed by H and CT (Table 2). The concentrations of the growth hormones, ABA, IAA and GA were significantly higher in SSB seed while CT had

Table 2. Sugars, free amino acids and plant growth substances in seed. Data are expressed as the mean (\pm SE) of three replicates. The different letters between H, CT and SSB columns indicated significant difference at ($P \leq 0.05$).

Sugars	H	CT	SSB
Glucose (g 100 g ⁻¹ FW)	0.011 \pm 0.003 ^a	0.009 \pm 0.001 ^b	0.018 \pm 0.002 ^a
Sucrose (g 100 g ⁻¹ FW)	4.052 \pm 0.087 ^a	3.65 \pm 0.109 ^b	4.356 \pm 0.124 ^a
Fructose (g 100 g ⁻¹ FW)	0.053 \pm 0.006 ^a	0.024 \pm 0.004 ^b	0.075 \pm 0.005 ^c
Galactose (g 100 g ⁻¹ FW)	0.023 \pm 0.005 ^a	0.157 \pm 0.02 ^b	0.018 \pm 0.002 ^a
Free amino acids (g 100 g ⁻¹ FW)	72.31 \pm 1.80 ^a	34.56 \pm 1.43 ^b	86.54 \pm 1.31 ^c
ABA/GA	0.227 \pm 0.02 ^a	0.173 \pm 0.03 ^b	0.242 \pm 0.01 ^a

The total free fatty acid content in SSB seed was markedly lower compared to H or CT seed. However, it was noteworthy that the linolenic acid content was more than eight times higher in SSB compared to H or CT seed. Among the major fatty acids present, the contents of palmitic acid and myristic acids were the lowest in SSB while it was six times higher in CT. (Table 3).

Table 3. Fatty acid profile of H, CT and SSB seeds. Data are expressed as the mean (\pm SE) of three replicates. The different letters between H, CT and SSB columns indicated significant difference at ($P \leq 0.05$).

Fatty acids	Seed (mg 100 g ⁻¹ FW)		
	H	CT	SSB
Caprylic	0.43 \pm 0.04 ^a	0.28 \pm 0.019 ^b	0.013 \pm 0.004 ^c
Capric	0.02 \pm 0.003 ^a	0.01 \pm 0.002 ^a	0.038 \pm 0.0025 ^b
Tridecanoic	0.41 \pm 0.038 ^a	0.70 \pm 0.061 ^b	0.51 \pm 0.048 ^a
Myristic	0.72 \pm 0.04 ^a	2.99 \pm 0.081 ^b	0.50 \pm 0.07 ^c
Pentadecanoic	0.26 \pm 0.032 ^a	0.28 \pm 0.031 ^a	0.19 \pm 0.041 ^a
Palmitic	145.80 \pm 1.27 ^a	132.80 \pm 1.16 ^b	115.18 \pm 1.90 ^c
Heptadecanoic	1.80 \pm 0.118 ^a	2.58 \pm 0.098 ^b	0.80 \pm 0.06 ^c
Linoleic	62.28 \pm 0.75 ^a	37.06 \pm 0.89 ^b	45.75 \pm 0.72 ^c
Oleic	32.38 \pm 0.42 ^a	24.03 \pm 0.73 ^b	25.86 \pm 1.07 ^b
Linolenic	2.38 \pm 0.116 ^a	2.35 \pm 0.073 ^a	19.69 \pm 0.64 ^b
Stearic	49.18 \pm 0.66 ^a	49.93 \pm 1.23 ^a	41.88 \pm 1.01 ^b
Eicosenoic	2.47 \pm 0.116 ^a	2.35 \pm 0.120 ^a	2.62 \pm 0.31 ^a
Arachidic	4.82 \pm 0.119 ^a	3.86 \pm 0.185 ^b	3.36 \pm 0.136 ^b
Behenic	2.47 \pm 0.159 ^a	0.79 \pm 0.118 ^b	0.93 \pm 0.09 ^b
Triicosenoic	1.13 \pm 0.103 ^a	0.91 \pm 0.078 ^a	1.59 \pm 0.121 ^b
Hexaeicosenoic	0.29 \pm 0.051 ^a	0.73 \pm 0.091 ^b	0.42 \pm 0.043 ^a

There was an increase in the total polyamine content of SSB and the ratio of putrescine to spermidine in SSB was also higher (Figure 2).

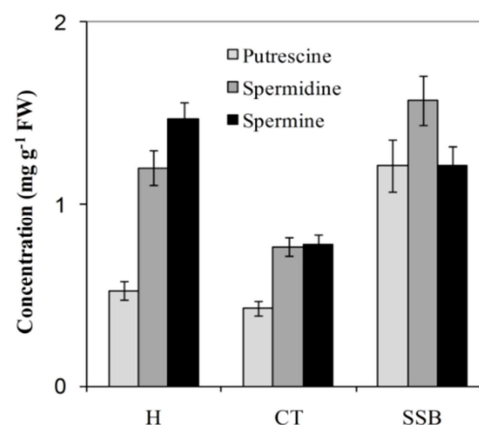


Figure 2. Polyamine levels in H, CT and SSB seeds of sapota. Data are expressed as the mean (\pm SE) of three replicates.

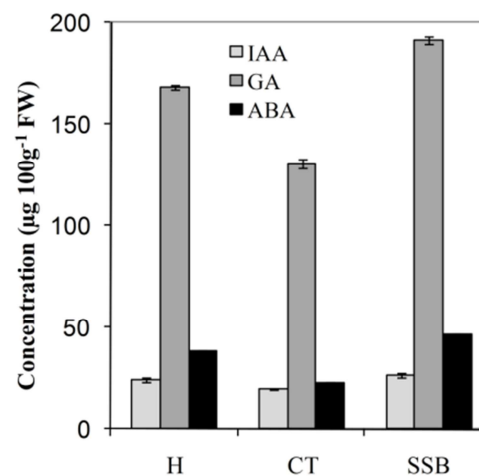


Figure 3. Plant growth substances in H, CT and SSB seeds of sapota. Data are expressed as the mean (\pm SE) of three replicates.

The lowest levels (Figure 3).

The activities of glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, malate dehydrogenase and PEP carboxylase were significantly higher in SSB than H and CT (Figure 4) while the levels of ATP, pyruvate, NAD and NADH (Figure 5) were higher in SSB.

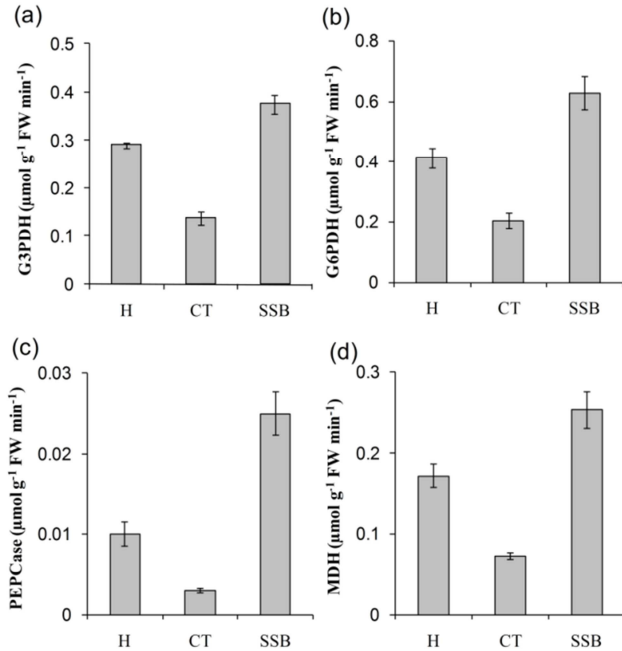


Figure 4. Activities of dehydrogenases and PEPCase in imbibed sapota seeds. Data are expressed as the mean (\pm SE) of three replicates.

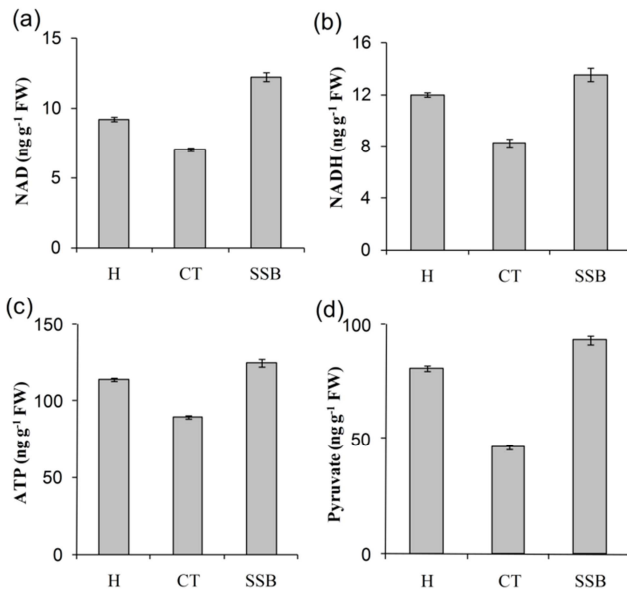


Figure 5. TCA cycle intermediates in the seed of sapota. Data are expressed as the mean (\pm SE) of three replicates.

The generation of reaction oxygen species (ROS) levels including H_2O_2 , O_2^- and $\bullet\text{OH}$ were higher in CT followed by H and SSB. CT showed higher MDA content, conductivity and pH followed by H and SSB (Figure 6).

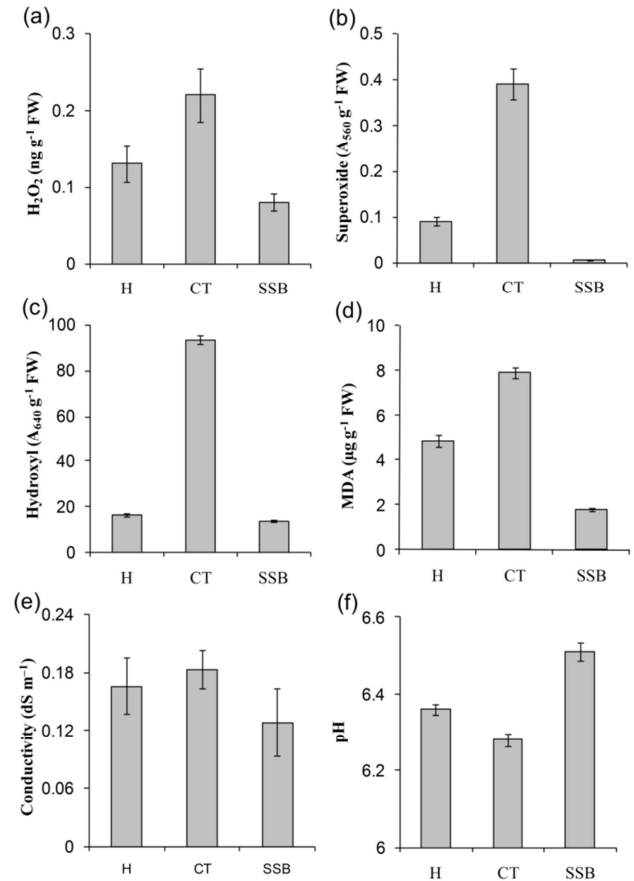


Figure 6. Production of ROS, MDA and its influence on cell permeability of mature fruits. Data are expressed as the mean (\pm SE) of three replicates.

4. Discussion

The study was aimed at investigating the reason for lack of corky tissue disorder in fruits infested by SSB. The body of biochemical evidence presented in this paper has confirmed that the enhancement of seed viability of uninfested seeds in SSB infested fruits was the primary reason for the absence of the disorder in such fruits. The possible biochemical mechanism by which the seed viability increased in the neighbouring seeds after infestation of single seed by SSB is explained in the discussion below.

4.1. Effect of Single Seed Infestation on Growth and Viability of Neighboring un-infested Seeds

Data presented in Table 1 revealed that the number of seeds in fruits affected by SSB was significantly higher compared to H or CT fruits. Similar results were reported by Seifert et al. [31] in spruce cones infested by seed-feeding insects. Total seed weight was higher while the fruit weight was lower in SSB infested fruits compared to H or CT fruits. Consequently, the ratio of the weight of seed to fruit was higher in SSB fruits. A higher ratio of seed dry weight to fruit dry weight is reported to contribute to higher sink

strength in sweet pepper [32] and a higher rate of movement of photosynthates into the fruit as auxin production and export by the fruit is predominantly confined to seed [33, 34]. The significantly higher seed moisture content, increased rates of activities of amylase and lipase enzymes and higher total dehydrogenase activity coupled with faster rate of germination and emergence of radicle confirmed that uninfested seeds in SSB affected fruits exhibited higher sink capacity and seed viability than H and CT seeds.

4.2. Seed Maturation, Dormancy and Vigor

Among the sugars present in the seed (Table 2), sucrose was predominant while glucose, galactose and fructose were present in traces. Sucrose has been found to be associated with desiccation tolerance in seeds and pollen [35]. A higher content of sucrose in seed reflected the higher maturation status of seed [36] while its depletion was correlated with a decline in the seed vigor [37]. Similarly, higher FAA content was associated with greater seed vigor [38]. Thus, the higher sucrose and FAA contents in SSB reflected the higher vigor and maturity status of SSB seed.

It was significant that although FFA content was lower in SSB seed, linolenic acid concentration was more than eight times higher in SSB compared to H or CT seed (Table 3). On the contrary, myristic acid was six times higher in CT than SSB. Since linolenic acid is essentially needed for the synthesis of glycolipids, which form the major components of chloroplast membranes [39], a lower concentration of linolenic acid in seeds led to significantly lower seed vigor, germination and field emergence than high linolenic acid seeds [40]. Supporting our observations, Munshi et al. [41] also observed that the fast germinating seeds had higher content of linolenic acid in the embryonic axes compared with slow germinating seeds. From this, it was evident that desaturation and elongation of fatty acids was occurring at a faster pace in the SSB seeds compared to H and CT seeds. Among the other fatty acids, the levels of myristic acid and palmitic acid were significantly lower in SSB. These two fatty acids are known to exert a strong inhibitory action on seed germination [42] by reducing α -amylase activity, ATP content and water uptake by seeds. Hence, the reduction of myristic and palmitic acids in seed would be helpful for germination. Thus, it was clear that the composition and content of FFA favoured higher seed vigour coupled with the ability for fast germination in SSB.

The levels of PAs and the ratio of putrescine to spermidine were higher in SSB seed compared to H and CT (Figure 2). Higher PA levels in maize seeds were positively correlated with seed vigour [43] (Figure 2). The loss of viability and decrease of seed vigour in certain monocot seeds were related to lower endogenous PA levels [44]. Reports have

suggested that the regulation of intracellular levels of free PAs played a role similar to the regulation of plant growth substances influencing cell division and viability [45]. It was noteworthy that SSB seed also recorded a significantly higher concentration of the growth hormones, ABA, IAA and GA₃ while CT and H had lower levels (Figure 3). The higher level of IAA in SSB seed indicated better seed filling, a characteristic of mature seed. On the other hand, the significantly higher levels of GA₃ and ABA in SSB seed compared to H and CT reflected the higher competitive ability of the sink in SSB fruits. In mature seeds, the level of ABA regulates both the accumulation of nutrients in the endosperm, tolerance to desiccation and termination of dormancy while GAs mobilize resources from the endosperm necessary for germination. Since a dynamic balance of synthesis and catabolism of these two antagonistic hormones controls the equilibrium between seed dormancy and germination [46], the ratio of ABA to GA is an important determinant of the progression of seed maturation [47]. Accordingly, the higher ratio of ABA/GA in SSB seed represented relatively higher dormancy, increased viability and germination compared to H and CT [48]. Further, the higher ratio of putrescine to spermidine characterized the higher regeneration capacity of cells in SSB seed [49]. Based on these results, it was clearly apparent that SSB seed with higher levels of PAs and ABA/GA ratio was endowed with higher degree of maturation, dormancy, seed vigour and viability than H or CT.

4.3. Dehydrogenase Activities and Cellular Metabolites in Imbibed Seeds of SSB Reflect Higher Viability

SSB seed after imbibition showed significantly higher activities of TDH, GA3PDH, G6PDH, MDH and PEPCase compared to H and CT seeds (Figure 4). TDH activity represents the sum of the activities of its dehydrogenases and reflects the reductive ability of the tissue [50] and is therefore, considered to be the most important physiological index of seed viability and vigor. Dehydrogenases are primarily involved in the generation of energy from stored seed reserves through citric acid cycle. There was a significant increase in the activity of GA3PDH in the uninfested seeds of sapota similar to the report by Giri et al. [51]. GA3PDH enzyme catalyses the conversion of glyceraldehyde-3-phosphate to 1,3- bisphosphoglycerate which is the central step linking the energy consuming with the energy producing steps of glycolysis through triose phosphate translocators involving plastidic and cytosolic metabolism. Nucleotide cofactors act as co-substrate for GA3PDH leading to production of NADH and NADPH along with cellular intermediates [52]. The activities of G6PDH and MDH were higher in SSB seed compared to H

and CT seeds. G6PDH is known to catalyze the first rate-limiting step in the pentose phosphate pathway which regulates the metabolism of glucose via the PPP [53]. Thus, G6PDH activity plays a critical role in cell growth by providing energy, carbohydrate metabolites and NADPH for redox regulation [54]. MDH, on the other hand, is involved in gluconeogenesis and plays a crucial role in major metabolic pathways including the tricarboxylic acid cycle, glyoxylate bypass, amino acid synthesis, and aids in the exchange of metabolites between cytoplasm and subcellular organelles. Past studies have suggested that changes in the activities of the MDH isoforms may affect the metabolic flux of many organic acids and exert wide-ranging effects on plant growth and development. The increase of PEPCase activity in SSB was also consistent with the changes in dehydrogenase activity. In non-photosynthetic tissues, the activity of PEPCase aids in replenishing the intermediates of the Krebs cycle by providing carbon skeletons to sustain the synthesis of amino acids during NH_4 assimilation [55] and for the regulation of cytoplasmic pH [56]. Consequently, the increased activity of PEPCase alongwith the dehydrogenases could be expected to result in increased production of malate via reduction of oxaloacetate which in turn enters the gluconeogenic pathway via MDH activity. The results shown in Figure 5 highlighting the marked increases in the levels of ATP, pyruvate, NAD and NADH in SSB were thus in tune with the changes in dehydrogenase enzymes. NAD is essential for cellular oxidations and is therefore, crucial for plant growth and development. There is now convincing evidence that NAD, in addition to its redox role, is also a signal molecule controlling vital functions of primary and secondary carbon metabolism. Higher NAD levels in cell are known to directly induce defense-related genes similar to that induced by salicylic acid. Several essential processes such as Ca^{2+} signalling, DNA repair and protein deacetylation [57] are influenced by NAD concentration. Similarly, NADH plays an important role in mitochondrial respiratory metabolism. ATP, on the other hand, is the principal energy currency in plants and plays a vital function in cellular metabolism providing the chemical energy needed for biological activities during seed germination. Studies have indicated that extracellular ATP (eATP) plays a significant role in cell viability and cellular signalling. Pyruvate, a product of glycolysis, is transformed into acetyl CoA before it enters the Krebs cycle and plays the controlling role for

many processes. Considering the above facts, it was evident that the concentrations of the four TCA cycle intermediates and the activities of dehydrogenase enzymes in SSB seed clearly pointed to an enhancement of viability and cell function.

4.4. Seed Moisture Content Influences Seed Integrity

The seed of sapota is recalcitrant characterized by a rapid loss of viability under decreased seed moisture levels. With the decrease of seed moisture content in CT to 40.6% compared to 43.9% in H and 50.8% in SSB, the ROS level in CT was significantly higher especially, with respect to the rapid rise in the $\bullet\text{OH}$ levels. MDA, an indicator of the degree of lipid peroxidation was the lowest in SSB while CT had the highest concentration (Figure 6). Past studies had shown that the loss of viability of desiccation-sensitive seeds of *Quercus robur*, *Aesculus hippocastanum* and *Castanea sativa* coincided with lipid peroxidation and free radical formation in the embryonic axes. Kibinza et al. [58] found that loss of viability in sunflower seeds was associated with the accumulation of MDA. The formation of the highly reactive ROS through lipid peroxidation is thought to influence seed viability through loss of enzymes, degradation of the respiratory system, reduction of ATP levels and membrane deterioration [59]. Taking into account the above facts, it was evident that CT seed characterized by a large excess of the highly damaging $\bullet\text{OH}$ radical would be the most affected while SSB seed with the lowest levels of ROS would be the least affected.

4.5. Biochemical Components of Seed Viability and Seed Germination

As a result of the increased activities of TCA cycle enzymes, higher contents of ATP, NADH and cellular metabolites, higher levels of growth hormones and increased activities of dehydrogenases, the SSB seed showed faster and higher rate of germination compared to H and CT seeds thus confirming higher vigour and viability in SSB infested seeds. These results are in agreement with previous reports.

Principal component analysis (PCA)

Principal component analysis of variables analysed from the three groups, H, CT and SSB showed that 94.61% of the variability observed was explained by PC1 and 4.92% by PC2 (Figure 7).

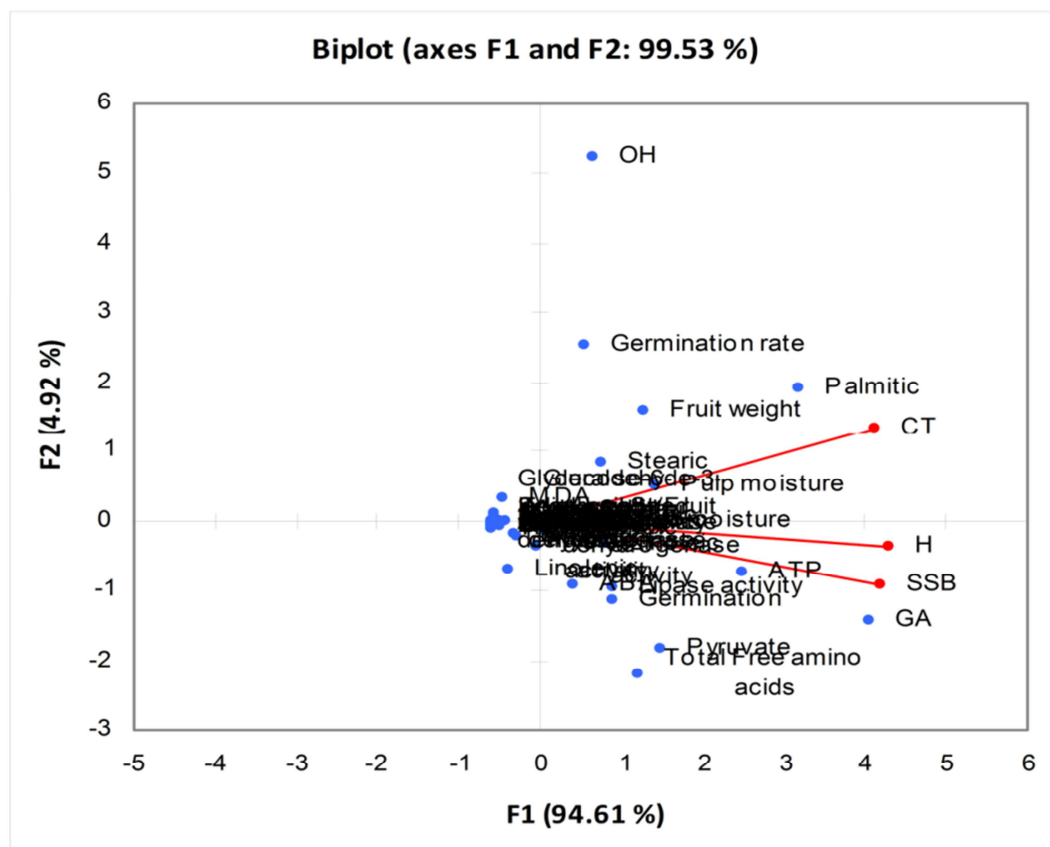


Figure 7. Principal component analysis of H, CT and SSB seeds for biochemical parameters.

The distribution of scores for the three groups along the PC1 axis showed that discrimination of the groups was essentially achieved according to the principal component 1. Accordingly, higher values of linolenic acid, sucrose and dehydrogenase activities and lower values of ROS and MDA were the components found to be strongly correlated with seed viability in SSB. All these components were found in greater proportion in SSB compared to H or CT. Thus, the PCA technique was helpful to establish the variables that contributed most to the differences in viability among groups.

5. Conclusions

The study established that SSB seed was more viable and vigorous than H or CT seeds. It was evident that the faster metabolism of sucrose in SSB seed compared to H and CT led to higher production of TCA cycle intermediates. Consequently, the synthesis of growth hormones and polyamines were up-regulated in SSB seed which resulted in higher sink capacity and improved seed viability in SSB seeds. Further, the relatively lower levels of ROS and MDA in SSB coupled with lower conductivity and higher pH contributed to a higher level of integrity of the SSB seed. These results have clearly established that SSB infestation of a single seed in the developing fruit of sapota by the sapota

seed borer, *Trymalitis margaritas* led to improved seed viability of neighboring seeds and thus ensured that such fruits remained free from corky tissue disorder. This novel finding, reported for the first time in sapota, has revealed that SSB infestation enhanced seed viability of un-infested seeds besides providing direct proof for the central role of seed in the development and regulation of corky tissue disorder in sapota fruit. This chance discovery has clearly established that the incidence of corky tissue disorder in sapota cv. Cricket Ball could be prevented by enhancement of seed viability during early fruit growth.

Acknowledgements

We thank the Director for facilities and the Council of Scientific and Industrial Research, New Delhi for financial grants under the extramural research grants scheme.

References

- [1] Shivashankar S., Jaya Joshi., Sumathi M. 2013. The role of seed viability in development of corky tissue in sapota fruit. J. Hort. Sci. Biotech. 88: 671-677.
- [2] Shukla A. 2009. Seasonal incidence and biology of sapota seed borer, *Trymalitis margaritas* Meyrick. Pakistan. J. Entomol. 2: 31.

- [3] Patel Z. P. 2001. Record of seed borer in sapota (*Manilkara achras* (Mill) Fosberg). *Insect Environ.* 6: 149.
- [4] Nakai Z., Kondo T., Akimoto S. 2011. Parasitoid attack of the seed-feeding beetle *Bruchus loti* enhances the germination success of *Lathyrus japonicus* seeds. *Arth. Plant Int.* 5: 227-234.
- [5] Karban R., Lowenberg G. 1992. Feeding by seed bugs and weevils enhances germination of wild *Gossypium* species. *Oecologia* 92: 196-200.
- [6] Harms K. E., Dalling J. W. 2000. A bruchid beetle and a viable seedling from a single diaspore of *Attalea butyracea*. *J. Trop. Ecol.* 16: 319-325.
- [7] International Rules for Seed Testing. 1985. *Seed Sci. Tech* 1. 3: 299-335.
- [8] International Rules for Seed Testing. 1993. *Seed Sci. Tech.* 21: 1-298.
- [9] Bernfeld P. 1955. Amylases. In: *Methods in Enzymology*. (Colowick, S. P. and Kaplan, N. O., Eds.). Academic Press, New York, NY, USA. 149-158.
- [10] Lowry O. H., Rosenbrough N. J., Farr A. L., Randall R. J. 1952. Protein measurement with Folin- phenol reagent. *J. Biol. Chem.* 193: 265-267.
- [11] Jayaraman J. 1981. *Laboratory manual in biochemistry*. New Delhi, Wiley Eastern Ltd.
- [12] Lee Y. P., Takahashi T. 1966. An improved colorimetric determination of amino acid with the use of ninhydrin. *Anal. Biochem.* 14: 71-77.
- [13] Burrell M., Mooney P., Blundy M., Carter D., Wilson F., Green J. 1994. Genetic manipulation of 6-phosphofructokinase in potato tubers. *Planta* 194: 95-101.
- [14] Selvaraj Y., Pal D. K., Singh R., Roy T. K. 1995. Biochemistry of uneven ripening in Gulabi grape. *J. Food Biochem.* 18: 325-340.
- [15] Sadasivam S., Gowri G. 1981. Activities of phosphoenolpyruvate carboxylase, NAD⁺-malate dehydrogenase and aspartate aminotransferase and chlorophyll a/b ratio in leaves of sorghum cultivars during plant growth. *Photosyn.* 15: 453-456.
- [16] Kelen M., Demiralay E. C., Ozakan S. G. 2004. Separation of abscisic acid, indole-3-acetic acid, gibberellic acid in 99 R (*Vitisberlandieri* × *Vitisrupestris*) and rose oil (*Rosa damascene* Mill.) by reversed-phase liquid chromatography. *Turk. J. Chem.* 28: 603-610.
- [17] Chiwocha S. D. S., Abrams S. R., Ambrose S. J., Cutler A. J., Loewen, M., Ross A. R., Kermode A. R. 2003. A method for profiling classes of plant hormones and their metabolites using liquid chromatography-electrospray ionization tandem mass spectrometry: an analysis of hormone regulation of thermodynamicity of lettuce (*Lactuca sativa* L.) seeds. *The Plant J.* 35: 405-417.
- [18] Segarra G., Jauregui O., Casanova E., Trillas I. 2006. Simultaneous quantitative LC-ESI- MS/MS analyses of salicylic acid and jasmonic acid in crude extracts of *Cucumis sativus* under biotic stress. *Phytochem.* 67: 395-401.
- [19] Serrano M., Romojaro F., Casas J. L., Acosta M. 1991. Ethylene and polyamine metabolism in climacteric and nonclimacteric carnation flowers. *HortSci.* 26: 894-896.
- [20] Galdón B. R., Rodríguez C. T., Rodríguez E. M. R., Romero C. D. 2009. "Fructans and Major Compounds in Onion Cultivars (*Allium cepa*)."
J. Food Com. Anal. 22: 25-32.
- [21] Folch J., Lees M., Stanley G. S. H. 1957. A simple method for the isolation and purification of lipids in animal tissues. *J. Biol. Chem.* 226: 497-509.
- [22] Liu K. 1994. Preparation of fatty acid methyl esters for gas chromatographic analysis of lipids in biological materials. *J. American Oil. Chem. Soci.* 71: 1179-1187.
- [23] Doke N. 1983. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. *Physiol. Plant Pathol.* 23: 345-357.
- [24] Von Tiedemann A. 1997. Evidence for a primary role of active oxygen species in induction of host cell death during infection of bean leaves with *Botrytis cinerea*. *Physiol. Mol. Plant Pathol.* 50: 151-166.
- [25] Schopfer P., Plachy C., Frahy G. 2001. Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberellin, and abscisic acid. *Plant Physiol.* 125: 1591-1602.
- [26] Draper H. H., Hadley M. 1990. Malondialdehyde determination as index of lipid peroxidation. *Methods Enzymol.* 186: 421-431.
- [27] Tezuka T., Yamaguchi F., Ando Y. 1994. Physiological activation in radish plants by UV-A radiation. *J. Photochem. Photobiol. B: Biology* 24: 33-40.
- [28] Stitt M., Lilley R., Gerhardt R., Heldt H. 1989. Metabolite levels in specific cells and subcellular compartments of plant leaves. *Methods in Enzymol.* 174: 518-553.
- [29] Chen L., Lin Q., Nose A. 2002. A comparative study on diurnal changes in metabolite levels in the leaves of three crassulacean acid metabolism (CAM) species, *Ananas comosus*, *Kalanchoe diargemontiana* and *K. pinnata*. *J. Exp. Bot.* 53: 341-350.
- [30] Panse V. G., Sukhatme P. V. 1978. *Statistical Methods for Agricultural Workers*, ICAR, New Delhi, India, pp. 108.
- [31] Seifert M., Wermelinger B., Schneider D. 2000. The effect of spruce cone insects on seed production in Switzerland. *J. App. Entomol.* 124: 269-278.
- [32] Marcelis L. F. M., Hofman-Eijer L. R. 1997. Effects of seed number on competition and dominance among fruits in *Capsicum annuum* L. *Ann. Bot.* 79: 687-693.
- [33] Richard J. P., Fabiana C., Carmen C. 2014. Mechanisms regulating auxin action during fruit development. *Physiol. Plant.* 151: 62-72.
- [34] Sjut V., Bangerth F. 1984. Induced parthenocarpy—a way of manipulating levels of endogenous hormones in tomato fruits (*Lycopersicon esculentum* Mill.) 2. Diffusible hormones. *Plant Growth Reg.* 2: 49-56.
- [35] Hoekstra F. A., Haigh A. M., Tetteroo F. A. A., Van Roekel T. 1994. Changes in soluble sugars in relation to desiccation tolerance in cauliflower seeds. *Seed Sci. Res.* 4: 143-147.

- [36] Ooms J. J. J., Léon-Kloosterziel K. M., Bartels D., Koornneef M., Karssen C. M. 1993. Acquisition of desiccation tolerance and longevity in seeds of *Arabidopsis thaliana*: a comparative study using abscisic acid-insensitive *abi₃* mutants. *Plant Physiol.* 102: 1185–1191.
- [37] Lu W., John W. P., Yong-Ling R. 2018. Live Long and Prosper: Roles of Sugar and Sugar Polymers in Seed Vigor. *Molecular Plant* 11: 1–3.
- [38] Alhadi Fatima A., AL-Asbahi A. A. S., Alhammadi A. S. A., Abdullah Q. A. A. 2012. The effects of free amino acids profiles on seeds germination/dormancy and seedlings development of two genetically different cultivars of Yemeni Pomegranates. *J. Stress Physiol. Biochem.* 8: 114-137.
- [39] Cantisan S., Force E. M., Ortega R. A., Garces R. 1999. Lipid characterization in vegetable tissues of high-saturated fatty acid sunflower mutants. *J. Agri. Food Chem.* 47: 78-82.
- [40] Ghodratollah S. 1998. The effect of seed colour and linolenic acid concentration on germination, seed vigour, seed quality and agronomic characteristics of flax. Doctor of Philosophy in the Department of Crop Science and Plant Ecology University of Saskatchewan Saskatoon, Canada.
- [41] Munshi S. K., Sandhu S., Sharma S. 2007. Lipid composition in fast and slow germination sunflower (*Helianthus annuus* L.) seeds. *Gen. Appl. Plant Physiol.* 33: 235-246.
- [42] Marambe B., Nagaoka T., Ando T. 1993. Identification and biological activity of germination- inhibiting long-chain fatty acids in animal-waste composts. *Plant Cell Physiol.* 34: 605-612.
- [43] Huang Y., Lin C., He F., Li Z., Guan Y., Hu Q., Hu J. 2017. Exogenous spermidine improves seed germination of sweet corn via involvement in phytohormone interactions, H₂O₂ and relevant gene expression. *BMC Plant Biol.* 17: DOI 10.1186/s12870-016-0951-9.
- [44] Mukhopadhyay A., Choudhuri M. M., Sen K., Ghosh B. 1983. Changes in polyamines and related enzymes with loss of viability in rice seeds. *Phytochem.* 22: 1547-1551.
- [45] Handa A. K., Mattoo A. K. 2010. Differential and functional interactions emphasize the multiple roles of polyamines in plants. *Plant Physiol. Biochem.* 48: 540–546.
- [46] Rodríguez-Gacio M. C., Matilla-Vázquez M. A., Matilla A. J. 2009. Seed dormancy and ABA signaling: the breakthrough goes on. *Plant Sig. Behav.* 4: 1035–1048.
- [47] Liu Y., Ye N., Liu R., Chen M., Zhang J. 2010. H₂O₂ mediates the regulation of ABA catabolism and GA biosynthesis in *Arabidopsis* seed dormancy and germination. *J. Exp. Bot.* 61: 2979–2990.
- [48] Finch-Savage W. E., Leubner-Metzger G. 2006. Seed dormancy and the control of germination. *New Phytol.* 171: 501–523.
- [49] Shoeb F., Yadav J. S., Bajaj S., Rajam M. V. 2001. Polyamines as biomarkers for plant regeneration capacity: improvement of regeneration by modulation of polyamine metabolism indifferent genotypes of Indica rice. *Plant Sci.* 160: 1229-1235.
- [50] Zeng X. Y., Chen R. Z., Fu J. R. 1998. The effects of water content during storage on physiological activity of cucumber seeds. *Seed Sci. Res.* 8: 65-68.
- [51] Giri A. P., Winsche H., Mitra S., Zavalal J. A., Muck A., Svatos A., Baldwin I. T. 2006. Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuate*, VII. Changes in the plant's proteome. *Plant Physiol.* 142: 1621-1641.
- [52] Plaxton W. C. 1996. The organization and regulation of plant glycolysis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47: 185–214.
- [53] Kletzien R. F., Harris P. K. W., Foellmi L. A. 1994. Glucose 6 phosphate dehydrogenase: a “housekeeping” enzyme subject to tissue-specific regulation by hormones, nutrients, and oxidant stress. *FASEB J.* 8: 174–181.
- [54] Yang Y., Fu Z., Su Y., Zhang X., Li G., Guo J., Que Y., Xu L. 2014. A cytosolic glucose-6-phosphate dehydrogenase gene, *ScG6PDH*, plays a positive role in response to various abiotic stresses in sugarcane. *Sci. Rep.* 4: 7090: DOI: 10.1038/srep07090.
- [55] Schuller K. A., Plaxton W. C., Turpin D. H. 1990. Regulation of C3 phosphoenolpyruvate carboxylase from the green alga *Selenastrum minutum*. Properties associated with replenishment of TCA cycle intermediates during amino acid biosynthesis. *Plant Physiol.* 93: 1303-1311.
- [56] Latzko E., Kelly G. J. 1983. The many-faceted function of phosphoenolpyruvate carboxylase in C₃ plants. *Physiol. Veg.* 21: 805-815.
- [57] Briggs A. G., Bent A. F. 2011. Poly (ADP-ribosyl) ation in plants. *Trends in Plant Sci.* 16: 372-380.
- [58] Kibinza S., Vinel D., Co`me D., Bailly C., Corbinau F. 2006. Sunflower seed deterioration as related to moisture content during ageing, energy metabolism and active oxygen species scavenging. *Physiol. Planta.* 128: 496–506.
- [59] Oenel A., Fekete A., Krischke M., Faul S. C., Gresser G., Havaux M., Mueller M. J., Berger S. 2017. Enzymatic and Non-Enzymatic Mechanisms Contribute to Lipid Oxidation During Seed Aging. *Plant Cell Physiol.* 58: 925-933.