BIOCHEMICAL ANALYSIS CONCERNING SEED LONGEVITY IN SOYBEAN [*Glycine max* (L.) Merill]

By

Miss. PAWAR PRIYANKA VISHWNATH
(Reg. No. R/013/162)

A Thesis submitted to the
MAHATMA PHULE KRISHI VIDYAPEETH,
RAHURI - 413 722, DIST. AHMEDNAGAR,
MAHARASHTRA, INDIA

In partial fulfilment of the requirements for the degree

of

MASTER OF SCIENCE (AGRICULTURE)
in
BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY
POST GRADUATE INSTITUTE
MAHATMA PHULE KRISHI VIDYAPEETH,
RAHURI-413 722, DIST. AHMEDNAGAR

2015

CONTENTS

CANDIDATES DECLARATION			iii
CERTIFICATES			
1.	Chairman and Research Guide		iv
2.	Associate Dean (PGI)		v
ACKNOWLEDGEMENTS			vi
LIST OF TABLES			xi
LIST OF PLATES			xii
ABBREVIATIONS			xiii
ABSTRACT			xiv
1.	**INTRODUCTION**		**1**
2.	**REVIEW OF LITERATURE**		**9**
	2.1	Physical characteristics of seed	9
	2.2	Oil content	13
	2.3	Tocopherol (Vitamin E content)	14
	2.4	Lignin content	15
	2.5	Calcium content	15
	2.6	Antioxidative enzymes	16
	2.6.1	Ascorbate peroxidase	17
	2.6.2	Superoxide dismutase	19
	2.6.3	Catalase	20
	2.6.4	Glutathione reductase	22
	2.7	Lipid peroxidation	22
	2.8	Molecular identification of candidate markers for seed longevity in soybean	24
3.	**MATERIAL AND METHODS**		**26**
	3.1	Physical characteristics of seed	27
	3.2	Determination of oil content	29
	3.3	Determination of tocopherol content (Vit. E)	31
	3.4	Estimation of lignin	32

	3.5	Calcium content	34
	3.6	Determination of antioxidative enzymes	35
	3.6.1	Ascorbate peroxidase	35
	3.6.2	Superoxide dismutase	37
	3.6.3	Catalase	38
	3.6.4	Glutathione reductase	39
	3.7	Lipid peroxidation	40
	3.8	Isolation of genomic DNA from soybean	41
	3.8.1	Purification of genomic DNA	43
	3.8.2	DNA amplification by SSR primer	44
4.	**RESULTS AND DISCUSSION**		**48**
	4.1	Physical characteristics of seed	48
	4.2	Oil content	54
	4.3	Vitamin E content	54
	4.4	Lignin content	56
	4.5	Calcium content	58
	4.6	Antioxidative enzymes	61
	4.6.1	Ascorbate peroxidase	61
	4.6.2	Superoxide dismutase	63
	4.6.3	Catalase	65
	4.6.4	Glutathione reductase	68
	4.7	Lipid peroxidation	70
	4.8	Identification and validation of candidate markers for seed longevity in soybean	73
5.	**SUMMARY AND CONCLUSIONS**		**76**
	5.1	Physical characteristics of seed	76

	5.2	Oil content	78
	5.3	Vitamin E content	79
	5.4	Lignin content	79
	5.5	Calcium content	80
	5.6	Antioxidative enzymes	80
	5.6.1	Ascorbate peroxidase	80
	5.6.2	Superoxide dismutase	81
	5.6.3	Catalase	81
	5.6.4	Glutathione reductase	82
	5.7	Lipid peroxidation	82
	5.8	Identification and validation of candidate markers for seed longevity in soybean	83
6.	**LITERATURE CITED**		**84**
7.	**VITA**		**103**

LIST OF TABLES

No.	Title	Page
1.	Physical characteristics of seed 1.Proportion of seed coat 2.Seed volume 3.Mechanical strenght	51

2.	Physical characteristics of seed 4.Electrical conductivity 5.Seed permeability	53
3.	Oil and vitamin E content in soybean seed	55
4.	Lignin content in soybean seed coat	57
5.	Calcium content in soybean seed coat	60
6.	Ascorbate peroxidase activity in cotyledon of soybean	62
7.	Superoxide dismutase activity in cotyledon of soybean	64
8.	Catalase activity in cotyledon of soybean	67
9.	Glutathione reductase activity in cotyledon of soybean	69
10.	Lipid peroxidation activity in cotyledon of soybean	72

LIST OF PLATES

NO.	Title	Between pages
1	Amplification pattern of the soybean genotypes obtained using the SSR marker Satt371 and Satt453.	75-76
2	Amplification pattern of soybean genotypes obtained using SSR markers Satt523 and Satt162.	75-76

CANDIDATE'S DECLARATION

I hereby declare that this thesis or part there of has not been submitted by me or other person to any other University or Institution for a Degree or Diploma.

Place: M.P.K.V., Rahuri. (Priyanka. V. Pawar)
Date: / /2015.

Dr. R. M. Naik
Professor and Head,
Department of Biochemistry,
Mahatma Phule Krishi Vidyapeeth,
Rahuri, 413 722, Dist. Ahmednagar,
Maharashtra State (INDIA)

CERTIFICATE

This is to certify that the thesis entitled,**"**Biochemical analysis concerning seed longevity in soybean [*Glycine max* (L.) Merill]**",** submitted to the Faculty of Agriculture Mahatma Phule Krishi Vidyapeeth, Rahuri, Dist. Ahmednagar, Maharashtra, India in partial fulfilment of requirement for the award of **MASTER OF SCIENCE (AGRICULTURE)** in **BIOCHEMISTRY**, embodies the results of a *bona fide* research work carried out by **MISS. PAWAR PRIYANKA VISHWNATH,** under my guidance and supervision and that no part of the thesis has been submitted for any other Degree or Diploma in any other form.

The assistance and help received during the course of this investigation have been acknowledged.

Place: M.P.K.V.,Rahuri. (R. M. Naik)
Date: / /2015 . Research Guide

Dr. B. R. Ulmek
Associate Dean,
Post Graduate Institute,
Mahatma Phule Krishi Vidyapeeth,
Rahuri, 413 722, Dist. Ahmednagar,
Maharashtra State (INDIA)

CERTIFICATE

This is to certify that the thesis entitled"Biochemical analysis concerning seed longevity in soybean [*Glycine max* (L.) Merill]" submitted to the Faculty of Agriculture, Mahatma Phule Krishi Vidyapeeth, Rahuri, Dist. Ahmednagar, Maharashtra, India in partial fulfilment of the requirement for the award of the degree of **MASTER OF SCIENCE (AGRICULTURE)** in **BIOCHEMISTRY,**embodies the results of a *bona fide* research carried out by **MISS. PAWAR PRIYANKA VISHWNATH ,**under the guidance and supervision of **Dr. R. M. Naik,** Professor and Head, Department of Biochemistry, M.P.K.V., Rahuri and that no part of the thesis has been submitted for any other Degree or Diploma.

Place: M.P.K.V., Rahuri (B. R. Ulmek)
Date: / /2015. Associate Dean

ACKNOWLEDGEMENTS

Gratitude cannot be seen or expressed, it can be felt deep in heart and is beyond description. Although 'Thanks' are poor expression of debt of gratitude one feel, yet there is no way to express it.

A formal statement of acknowledgement will hardly meet the ends of justice in the matter of expression of my deeply felt sincere and heartiest gratitude to my research guide Dr. R. M. Naik, Professor and Head, Department of Biochemistry, Post Graduate Institute, M.P.K.V, Rahuri for erudite guidance, constant encouragement, painstaking efforts in suggesting, designing and improving the quality of study throughout the entire span of research work and consuming this manuscript successfully.

It is my proud privilege to offer sincere and well devoted thanks to Dr. T. A. More, Hon. Vice chanceller, M.P.K.V. Rahuri and Dr. B. R. Ulmek, Dean Faculty of Agriculture and Director of Instruction, and Associate Dean, Post Graduate Institute, M.P.K.V. Rahuri for providing necessary facilities during the experimentation of research work.

I wish to record my profound sense of gratitude towards Dr. R. M. Naik, Head, Department of Biochemistry, Post Graduate Institute, M.P.K.V. Rahuri, for providing facilities, giving courage and making suggestions and corrections wherever necessary.

I wish to convey my heartiest appreciation for round the clock help extended to me by advisory committee, Dr. A. A. Kale, Associate Professor, Department of Biochemistry, Post Graduate Institute, M.P.K.V. Rahuri, Dr. V. R. Shelar, Seed Reserch Officer, Department of Botany, MPKV, Rahuri, Dr. M. P. Deshmukh,

Soybean breeder, Kasbe-Digras, Sangli and Dr. C.A. Nimbalkar, Associate Professor, Department of Statistics, Post Graduate Institute, M.P.K.V. Rahuri for their co-operation constructive criticism and valuable suggestions during the whole course of study.

Special thanks to Miss. S.G. Mohite, SRA., Department of Biochemistry. I feel honoured to extend my grateful thanks to Prof. P. K. Lokhande, Dr. D. P. Kachare, Dr. R. D. Satbhai and Mrs. Jadhav SRA., Department of Biochemistry, for their valuable guidance, help and co- operation and encouragement during my research work.

My sincere thanks are due to Mr. P. S. Pawar and Mr. Abhijit Rokde for their assistance in lab work.

Words are small trophies to express my immense love and affection to my dear friends Namita, Dhanashri, Anushri, Jyoti, Praneti, Supriya, Ankita, Sonam, Komal and helpful seniors Manisha Jadhav, Nutan Manapure, Also thanks to all my youth friends for their enthusiastic, cheerful, immutable and selfless encouragement throughout the bright and dark phase of research work.

I have no words to express my feelings in fathomable depths which springs in the very core of my heart of my father Shri. Vishwnath Kundlik Pawar and my mother Sau. Meena Pawar who always stood like a lighthouse for illuminations, in the pathway of every success, unsuccess and for their sacrifice in moulding me and in building up my educational career. I am grateful to my cute brother Rajesh for their love and care.

I am also deeply obliged to all those authors and scientists, past and present, whose contributions were of immense help to

understand research findings as their literature has been aptly cited to undertake the present investigation.

While travelling on the path of life and education, many hands pushed me forth, learned hearts put me on the right path, enlighten by their knowledge and experience. I ever remain thankful to them all.

I would like to place on record my sincere thanks to Mahatma Phule Krishi Vidyapeeth, Rahuri, Dist. Ahmednagar for providing me an opportunity to undertake the under graduate and post graduate studies in this institute of national repute.

Place: M .P.K.V., Rahuri (Priyanka.V. Pawar)
Date: / / 2015.

LIST OF ABBREVIATIONS

@	:	At the rate of
^{0}C	:	Degree Celsius
%	:	Per cent
Cm	:	Centimeter (s)
dSm^{-1}	:	Deci seimens per meter
E.C.	:	Electrical conductivity
et al.,	:	And others (et alli)
Fig.	:	Figure
i.e.	:	That is
G	:	Gram (s)
Ha	:	Hectare (s)
Hrs	:	Hour (s)
Kg	:	Kilogram (s)
kg ha^{-1}	:	Kilogram per hectare
M	:	Meter (s)
N.S.	:	Non-significant
CD	:	Critical difference
S.Em.	:	Standard error of mean
Temp.	:	Temperature
viz.,	:	Videlicet (Namely)
Mg	:	Milligram
Min	:	Minute
µg	:	Microgram
µl	:	Microlitre
µmoles	:	Micromoles
Nmoles	:	Nanomoles
N	:	Newton
pH	:	Potenz (-log $[H^+]$)
SOD	:	Superoxide dismutase
APX	:	Ascorbate peroxidase
CAT	:	Catalase
GR	:	Glutathione reductase
ROS	:	Reactive oxygen species
DHA	:	Dehydroascorbate
GSH	:	Glutathione
GSSG	:	Glutathione disulfide
ASH	:	Reduced ascorbate

ABSTRACT

BIOCHEMICAL ANALYSIS CONCERNING SEED LONGEVITY IN SOYBEAN [*Glycine max* (L.) Merill]

By

Miss. Pawar Priyanka Vishwnath

A candidate for the degree
of
MASTER OF SCIENCE (AGRICULTURE)
in
Biochemistry
Mahatma Phule Krishi Vidyapeeth
Rahuri – 413 722
2015

Research Guide	**:**	**Dr.R.M.Naik**
Discipline	**:**	**Biochemistry**

The present investigation entitled "Biochemical analysis concerning seed longevity in soybean [*Glycine max* (L.) Merill]" seven genotypes of soybean comprising of two (Birsa soya 1, Kalitur) with higher seed longevity; two genotypes (MAUS 71, DS 228) with low seed longevity; one rust resistant genotype (EC 241780); one recently released variety Phule Agrani (KDS 344) and one variety JS 335 a national check were used. Eleven crosses were effected using Birsa soya 1 and EC 241780 (10 crosses) and one cross effected JS 9305 × EC 241780 were used in the present investigation. The F_3 progenies of these crosses exhibiting variable seed coat colour *viz.*, yellow (5), brown (4) and black (2) were also analyzed for different biochemical parameters which are related to better seed longevity. The genomic DNAs of soybean genotypes with higher seed longevity (Birsa soya 1, Kalitur) and low seed longevity (DS 228) and crosses exhibiting better morpho

-physiological parameters, strength and biochemical composition were amplified using 9 SSR markers which have been reported to be linked with the seed longevity or seed coat colour.

The level of physical characteristics, oil, vitamin E, lignin and calcium contents were higher in black seed coat genotypes and crosses except electrical conductivity which was less in black seed coat colour genotypes.

The significantly higher antioxidative enzyme activities (SOD,catalase, APX, GR) were recorded in black seed coat genotypes as compared to yellow colour genotypes. The ascorbate peroxidase and catalase activity was higher in KDS 1038, and superoxide dismutase activity was higher in KDS 1025, glutathione reductase activity was higher in KDS 1032. The antioxidative enzyme activity was higher in some the crosses effected using Birsa soya 1 with EC 241780.

The significantly more lipid peroxidation rate was recorded in yellow testa colour genotypes which are poor storer, while the less lipid peroxidation rate was recorded in black testa colour genotypes and also in F_3 progenies exhibiting black or brown seed coat. The lipid peroxidation rate which is inversely related to membrane injury caused by ROS (Reactive oxygen species) was the highest in DS 228 which is a poor storer and lowest in JS 335 which is better storer genotype.

Based on mechanical strength, vitamin. E content the F_3 progeny of the cross KDS 1034 with black seed coat colour,

appears to be promising. This progeny also recorded the lowest seed permeability value with highest lignin content. The KDS 1034, also reported an allelic size band of 180 bp, which is also reported in black seed coat colour genotype *i.e.* Birsa soya 1 with Satt162 primer and also with Satt523. The calcium content and catalase activity were found the highest in KDS 1038 genotype. The KDS 1038 cross marked second in respect of seed coat proportion, ascorbate peroxidase, glutathione reductase and ranked sixth in lignin content.

It thus appears that the black seed testa colour genotypes to be better storer and this character can be introgressed through breeding. The biochemical parameters *viz.*, physical characteristics, oil content, vitamin E content, lignin content, calcium content, antioxidative enzyme activities and lipid peroxidation rate appears to be positively correlated with seed longevity and the SSR primer *viz.*, Satt371, Satt453, Satt523, Satt162 can aid in screening as they produced specific allelic size fragment in soybean genotypes and crosses with better seed longevity.

1.Introduction

Soybean [*Glycine max* (L.) Merill] is one of the most important protein and oil seed crops throughout the world. Its oil is the largest component of the world's edible oils. Soybean seed contains 20 per cent oil and 40 per cent protein. The world production of edible oils consists of 30 per cent soybean. It is an ingredient of more than 50 per cent of the world's high protein meal. The United States of America has largest area under soybean cultivation with the highest yield and production (Anon., 1996).

The native of soybean is Eastern Asia. Soybean was introduced to India during 1880. Soybean is globally grown over an area of 91.40 m ha with a production of 204.00 mt and productivity of 2233 kg/ha. In India soybean is grown over an area of 8.87 m ha with a production of 9.46 mt and productivity of 1069 kg/ha which is much below the average productivity of the world (2233 kg/ha). The major soybean producing states in India are Madhya Pradesh, Maharashtra, Rajasthan, Karnataka, Uttar Pradesh and Gujarat.

In Maharashtra state also soybean has become popular as an oil seed crop. The increase in area was from 2.74 m ha during 1991-92 to 3.32 m ha during 2013-14. Maharashtra ranks fourth in area and production. The state soybean productivity increased from 712 kg/ha in 1991-92 to 1221 kg/ha during 2013-14. Latur, Aurangabad, Amaravati and

Kolhapur are the major soybean growing districts of Maharashtra.

Soybean has many commercial applications and its processing form large agro-industrial complex because of its high protein content. There has been an increasing demand for soybean seeds to the demands of an expanding producer and consumer market with very promising future perspectives. Despite the high yielding potential and various advantages of soybean, the yield per unit area of the crop is low in India. Poor germination and low seed viability are among the serious problems limiting the production of soybean.

Soybean seed is classified as “poor storer”, when compared to other grain crops. Germination is often reduced below the minimum standards prior to planting time under warm and humid or hot climate. Loss of germination potential is more acute in tropical and sub tropical regions compared to temperate environments.

The permeability property of a seed coat should be related to its structure. A typical legume seed coat contains several specialized areas, *i.e.* hilium micropyle and raphe and the rest of seed coat commonly known as the extrahilier region. Many legumes are well known for hard seededness, this feature can be beneficial for long -term seed survival, but is undesirable for the food processing industry. Permeable seed coats may be susceptible to mechanical damage during pre-processing of the seeds, leading to losses. Seed coat permeability is important to both scientific and industrial communities.

According to the results of the study and a previous paper (Ma *et al.,* 2004), the difference between hard and soft soybean seeds is the continuity of the outermost seed cuticle in the former and the presence of small cracks in the cuticles of the latter. In addition, Ma *et al.,* (2004) reported that isolated pieces of cuticle from soft seeds are more delicate and prone to breakage than those from hard seeds. Thus, the strength of the cutin polymer may be critical to forming one or the other type of seed.

The seed coat is one of the main determinants of seed germination, vigour and longevity potentials. Seed coat thickness and its mechanical strength is another most commonly considered parameter for longevity (Fraczek *et al*. 2005). An intact seed coat is capable of regulating the speed of water absorption protecting the embryo from injuries, which might otherwise result from rapid imbibition, but this condition does not always suffice. However, the legume cultivars with seed coat capable of delaying imbibition, instead of impeding it, were suggested as better alternatives (Chachalis and Smith, 2000).

Seed coat resistance for mechanical damage is a genetic character that varies among soybean cultivars. The soybean breeders are always in search of lines having good longevity for developing improved cultivars. The present investigation was aimed to study seed longevity as the function of variety, seed size, soil emergence, rupture force of seed coat and water imbibition kinetics.

The soybean seed is highly susceptible to field weathering and mechanical damages which adversely affect its longevity. Mechanical injury can occur at any time during harvesting, drying and conditioning of seeds and includes cracks or breaks in the seed coat or cotyledon, injury or breakage of hypocotyls-radicle axis and complete breakage of seed to the point where it would no longer be classified as part of pure seed fraction. Lignin the complex phenolic polymers found in the cell wall are believed to contribute to the compressive strength, resistance to degradation by microbial attack and water permeability to the polysaccharide protein matrix of the cell wall. Lignin in the seed coat of soybean reportedly imparts resistance to mechanical damage (Alvarez *et al.* 1997). The higher the lignin content in the seed coat, the greater is expected resistance to mechanical damage. The resistance is a genetic characteristic that varies among soybean cultivars (Carbonell and Kryzanowski, 1995). Seed coat surface properties, particularly the presence of pits (pores) and deposits also influence the water permeability, fungal invasion and hence the seed longevity in soybean.

The mechanism by which the cell protects its integrity is by mopping-up (the excessive) free radicals by the activity of an active oxygen-scavenging system (AOSS), *viz.*, superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT). Activity of such enzymes lowers active oxygen/hydroxyl radical formation. Loss of seed viability is reported to be associated with a reduction in the activities of antioxidant enzymes. However,

inconsistent results have been reported with respect to the storage patterns and antioxidant activities in the fresh seeds of different genotypes. Lipid oxidation has been proposed to be a significant factor in seed longevity, with some studies reporting an inverse correlation of various lipid oxidation products and seed longevity during natural and accelerated aging.

Superoxide radical and hydrogen peroxide can reduce seed vigour (Bailly *et al.,* 2008) and the accumulation of reactive oxygen species (ROS) is the main contributor to seed deterioration (Oracz *et al.,* 2009; Bailly and Kranner, 2011; Bellani *et al.,* 2012; Yao *et al.,* 2012). The antioxidative system prevents ROS accumulation in seed ageing (Bailly *et al.,* 2002; Pukcacka and Ratajczak, 2005, 2006; Yao *et al.,* 2012) and several studies have revealed a close relationship between seed deterioration and a reduction in the activity of various antioxidative system in soybean (Sung, 1996), cotton (Goel *et al.,* 2003), sunflower (Kibinza *et al.,* 2006), pea (Yao *et al.*, 2012).

Tocopherols (vitamin E) are lipophilic antioxidants synthesized by all plants and are particularly abundant in seeds. A primary function of tocopherols in plants is to limit non-enzymatic lipid oxidation during seed storage, germination, and early seedling development.

Although most of soybean is grown in temperate region, there is tremendous potential to expand its production in the tropics. However, one of the major problems encountered in soybean production in tropical and sub tropical region is rapid deterioration of seed quality during storage, leading to poor

germination and sub optimal plant stand. Accelerated ageing is a quick test based on increased seed deterioration under hot and humid condition of storage. Use of seed vigour test in addition to viability test for estimating seed deterioration during long term seed storage has also been made by Hampton and Tekrony (1995). The Accelerated ageing tests were useful for identifying lines with inferior field emergence but were not reliable enough to replace field tests for identifying the best emerging lines (Francisco *et al*. 2001).

Most frequently cited cause of seed deterioration is lipid peroxidation. Lipid peroxidation begins with the generation of a free radicals (an atom or a molecule) with an unpaired electron either by autoxidation or enzymaticlly by oxidative enzymes such as lipoxygenase present in many seeds. Various forms of free radicals have been observed or detected in living tissue, each with a differing capability for cell damage (Gille and Joneje, 1991; Larson, 1997).

Two common storage methods to limit seed deterioration are to reduce the moisture content and storage temperature (Walters *et al.,* 2004; Lu *et al.,* 2005). Even under optimal storage conditions, however seed suffer a variety of biochemical and metabolic alterations, including lipid peroxidation, enzyme inactivation, disruption of cellular membranes (Rajjou *et al.,* 2008; Sveinsdottir *et al.,* 2009; Kaewnaree *et al.,* 2011; Hu *et al.,* 2012). Understanding the mechanism of seed ageing will lead to new methods for seed conservation and longevity.

A number of seed characters such as seed size percent hard seedness, seed coat thickness and permeability hull percentage, oil content etc., are associated with seed quality in soybean and were shown to be under genetic control. These traits are being utilized in breeding programs to improve seed quality and seed longevity in soybean. Verma and Ram (1986) reported that two to four genes probably governs seed longevity in soybean.

Based on the construction of soybean genetic maps, quantitative trait loci (QTL) for number of agronomic traits soybean have been mapped. Singh *et al.,* (2008) reported that the four SSR markers (Satt434, Satt538, Satt281 and Satt598) are associated with seed coat permeability and electrolyte leaching in soybean. Another set of four SSR markers (Satt538, Satt285, Satt600 and satt434) has been reported to be significantly associated with seed longevity in an F2:3 soybean population in a cross involving good and poor storer genotypes.

The present study " Biochemical analysis concerning seed longevity in soybean [*Glycine max* (L.) Merill] " is therefore proposed with the objective of morpho physiological, biochemical and molecular basis of seed longevity.

Objectives

1. To evaluate the colour, mechanical strength, seed permeability, EC of seed leachate, lignin, vit. E, oil per cent and calcium content of soybean genotypes and crosses which are resistant and susceptible to mechanical damage and which are poor storer and better storer.

2. To analyze lipid oxidation products, malondialdehyde content and leachate conductivity.
3. To evaluate the activities of ROS detoxifying enzymes (superoxide dismutase, glutathione reductase, and ascorbate peroxidase).
4. To screen the seed longevity resistant (Birsa soya 1, Kalitur) susceptiable (DS 228, EC 241780) and crosses using specific SSR primers.

2. Review of Literature

The soybean seed is highly susceptible to field weathering and mechanical damage which adversely affect its longevity. In general the black seed coat colour is associated with better seed longevity in soybean. The analysis has also revealed that the black seed coat colour has better strength and better biochemical composition which protect them from mechanical damage and can retain better seed viability. The literature pertaining to physical characteristic, enzymatic and non enzymatic antioxidants, lignin, calcium has been reviewed under appropriate headings. In soybean some molecular markers have been reported to be associated with seed longevity on different linkage groups. The information concerning the molecular identification of candidate markers has also been reviewed.

2.1 Physical characteristics

2.1.1 Proportion of seed coat

Soybean seed *testa* consists of an epidermis of one cell layer of highly cutinized palisade cells, a hypodermis of single layer of large cells with thick anticlinal walls having intercellular spaces and an inner parenchyma composed of 6–8 layers of flattened thin-walled cells (Carlson 1973).

The seed coat characteristics of soybean seeds were examined via scanning electron microscopy and the hourglass cells lying in between the internal palisade and parenchyma cell

layers become distorted at the seed coat wrinkle site resulting in seed coat damage. Pereira and Andrew (1985) reported that these hourglass cells, with a cushioning effect, help in preventing the wrinkle caused by the hydration and dehydration. However, varietal assessment for this character has not yet been reported.

It has been reported that black-seeded lines of soybean are more resistant to field weathering because of thicker seed coat than that of yellow-seeded lines (Dassou and Kueneman, 1984). Higher proportion of the seed coat was not correlated with higher seed weight. The proportion of the seed coat is of primary importance in providing protection against mechanical damage during harvesting and processing, which cause significant loss in viability of a seed lot. The black-seeded varieties, 'Kalitur' and 'Birsa Soya 1', had highest seed coat to seed proportion, *i.e.,* 11.25% and 10.21%, respectively 'NRC 7' (7.07%), 'PK 472' (7.58%), 'NRC 2' (8.12%), 'NRC 55' (8.25%), and 'PK 416' (8.15%) recorded significantly lower proportion of the seed coat as compared to other varieties.

2.1.2 Mechanical strength

Lignin is known to impart mechanical strength to the seed coat. Black-seeded varieties 'Birsa Soya 1' (1.0131kg) and 'Kalitur' (0.9689kg) had significantly higher amount of lignin than other varieties. Among yellow-seeded varieties, 'PS 1029' (0.8054 kg), 'NRC 7' (0.7725kg), and 'JS 335' (0.7691kg) had significantly higher amount of lignin in seed coat, whereas variety 'PK 472' (0.7288kg), which is highly susceptible to

mechanical injury, had significantly lower amount of lignin content among all varieties (Alvarez *et al.*, 1997).

Scanning electron micrographs revealed that the hour-glass cells, provide cushioning effect to the seed coat and impart protection against mechanical damage and field weathering (Pereira and Andrew 1985).

2.1.3 Electrical conductivity of seed leachate

Parrish and Leopold, (1978) observed that if membrane damage is a primary effect of ageing and a primary cause of vigour loss, it should be expressed immediately upon hydration. Leakage of electrolytes is increased by ageing during the initial burst of leakage before membranes reorganize to their hydrated orientation as well as during the subsequent period of linear H_20 uptake and leakage.

Srivastav (1976) observed that the reduction in mobilization efficiency, DNA and RNA levels and RNAase activity in the seeds are some of the metabolic aspects related with seed deterioration. Higher electric conductivity of seed leachate is also associated with high magnitude of seed deterioration.

The EC values of soybean seeds are also influenced by degree of hardseedness of the genotype. Verma and Ram (1986) observed that higher the hardseedness percentages the lower the value of soybean seed EC.

Loeffler *et al.,* (1988) reported that physical injury to seed coat and seed size are reported to adversely affect electrical conductivity. Good storer soybean genotypes with lower electrolyte leakage were characterised by smaller seed size, black

testa colour, and better germination, as compared to poor storer genotypes, which recorded higher electrolyte leakage, larger seed size, and yellow testa colour. An increase in electrolyte leakage from seeds was associated with a decrease in germination, with the change being more pronounced in large seed, (Vyas *et al.,* 1990).

Agrawal and Dadlani (1992) have given the procedure of the extent of membrane permeability measured as electrical conductance of seed leachate. The variations in soybean seed leachate EC observed among different genotypes may be related to the variation in the lignin content in the seed coat. Differences in seed coat lignin content among soybean genotypes were observed Caballero Avarez (1994), and were a correlated soybean seed resistance to mechanical damage. This suggest that a higher lignifications of seed coat cell wall makes it difficult for seed imbibitions process thus affecting the loss of leached substances.

2.1.4 Seed permeability

To understand hard seededness, a comparison of hard and normal seeds is necessary. In impermeable seeds, the hilum, micropyle and raphe must be closed to water; while in permeable seeds these regions may be the initial sites of water entry, but published result on this point in *Vigna unguiculata* are variable (Lush and Evans1980; Arechavalete-Medina and Snyder,1981).

Rolston,(1978); Tran and Cavanagh,(1984) reported that it is biological beneficial for long term survival and can be

important for wild plants Soybean (*Glycine max)* and many other legumes have hard seeds.

Bewley and Black, (1994) described that seed coat can be described as being permeable or impermeable. A permeable

(normal) seed imbibes water readily when available, while impermeable one does not take up water for days or longer. Impermeable seeds are commonly refered to as hard. Tyler (1997) observed that hard seededness in soybeans can protect against seed decay and improve agronomic qualities under certain conditions but hard seededness is undesirable for the food processing industry. Ideally, seeds should take up water quickly and synchronously. This trait is particularly critical when whole seeds are processed, such as for the production of soya milk, soya sauce, tofu and miso. Hard seededness is the reason for seed coat -imposed dormancy, or physical dormancy in a number of families (Baskin *et al.,* 2000).

2.2 Oil content

Leffel and Rhodes, (1993); Liu *et al.,* (1995) reported that protein and oil contents varied significantly between locations; cultivars grown at Manfredi showed the highest values. There was no a negative correlation between protein and oil contents as indicated previously.

Liu *et al.,* (1995) suggested that during selection of soybeans for a particular food application or a particular seed

breeding program, it is important to know whether relationships exist among these quality attributes. Unfortunately, there seems to be little published information on relationships between soybean seed size, protein and oil contents, and fatty acid composition. This investigation was conducted with 18 soybean cultivars grown in Argentina to determine the nature of these associations. Borher *et al.*, (2002) reported that deodorization is a step of soybean oil refine, that removes volatile compounds responsible for undesirable taste of oil producing Soybean Oil Deodorizer Distillate (SODD) like a byproduct. However, this process also removes tocopherols, making SODD a precious byproduct and its price depends on their tocopherol contents. (De Greyt and Kellens, 2000) Neutralization step is enough to use SODD like as a tocopherol supplement. SODD has gained increased attention due to exportation, mainly to countries that obtain tocopherol concentrates to use in food and pharmaceuticals industries as natural antioxidants. Wilson *et al.,* (1980) observed that current biochemical evidence indicates that in soybeans and in several other plant species the polyunsaturated fatty acids (18:2 and 18:3) are produced by the consecutive desaturation of 18:1.

2.3 Tocopherol

Seneratna *et al.,* (1988) Kalpana and Madhava Rao, (1994) suggested that soybean seeds have a lower tocopherol content following ageing, suggesting that tocopherol is consumed and protects the seed against free radical damage. An increase in

Superoxide dismutase (SOD) activity with accelerated ageing has been reported in pigeon pea seed.

Pretreatment of seeds with compounds such as dikegulac-sodium, ascorbic acid, cinnamic acid, and α-tocopherol prior to accelerated and natural ageing improved seed vigour and seed storage of rice. Benites *et al.,* (2005) observed that these concentrates require many techniques to be obtained, making the product expensive. Tocopherols are compounds with vitamin E activity and are important antioxidants, protecting unsaturated lipids in cellular membranes against oxidation.

2.4 Lignin

It has been reported that the soybean seed coat is very thin and low in lignin content, and provides little protection to the fragile radicle that lies in a vulnerable position directly beneath the seed coat (Gupta *et al.,* 1973, Agrawal and Menon 1974, França Neto and Henning 1984).

The stability of seed coat lignin even in storage can be explained by the insolubility and complexity of the lignin polymer. These characteristics make lignin resistant to degradation by most microorganisms (Campbell and Sederoff, 1996).

In soybean, seed coat lignin plays an important role in physical and physiological seed qualities. Seeds of soybean cultivars with seed coat lignin content above 5.0 per cent are less prone to mechanical damage (Alvarez *et al.* 1997).

Breeding soybean for high seed quality is an important approach for developing cultivars for tropical regions, and the lignin content in the seed coat was found to be one of the screening parameters for this trait (Krzyzanowski, 1998).

2.5 Calcium

Calcium content in the seed coat was found to be positively correlated with water absorption (Saio *et al.*, 1973; Saio 1976).

Application of asparaginase to hydrolyzed soybean protein has been reported to improved its calcium binding capability (Hitomi and Yoshiharu 1998).

Jin and Yizhen (2000) demonstrated that deamidation of soybean protein hydrolysate enhances the inhibition of calcium carbonate crystallization. These results suggest that deamidation increases the calcium binding of soluble hydrolysates, further indicating that the carboxyl group in soluble soybean protein hydrolysate plays an important role in calcium.

Chen *et al.,* (1993), (2001) reported that high calcium content appears to be the main cause of stone seeds. In texture studies, calcium content in soybean seed was found positively correlated with seed hardness, but this correlation was not always consistent because of environmental effects such as soil type and temperature variations.

2.6 Antioxidative enzymes

Plants have also developed complex antioxidant defense systems that respond to various stresses and mitigate the deleterious effects of reactive oxygen species (ROS). Higher plants have active oxygen-scavenging systems involving several antioxidant enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and Ascorbate peroxidase (APX; EC 1.11.1.1).

Mitochondrial antioxidant enzymes activities including superoxide dismutase, ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase, and dehydroascorbate reductase were significantly reduced in aged seeds. A decrease in total ascorbic acid (ASC) and glutathione (GSH) content as well as the reduced/oxidized ratio of ASC and GSH in mitochondria with prolonged ageing showed that artificial ageing reduced ASC-GSH cycle activity.

2.6.1 Ascorbate Peroxidase (APX)

Ascorbate peroxidase (E.C.1.11.1.1) plays the most essential role in scavenging ROS and protecting cells in higher plants, algae, and other organisms. APX is involved in scavenging of H_2O_2 in water-water and ASH-GSH cycle and utilizes ASH as electron doner. APX family consist of at least five different isoforms including thylakoid and glyoxisome membrane from as well as chloroplast stomatal soluble form, cytosolic form

$$H_2O_2 + AA \longrightarrow 2H_2O + DHA$$

The increase in APX activity induced by Hg was reported in seedlings of *Phaseolus aureus* (Shaw, 1995).

The APX stimulation has also been verified in several plants subjected to Cd, Zn, Cu, Pb and Fe treatment. Increased activities of SOD and APX and other antioxidative enzymes under heavy metals treatments may be considered as circumstantial evidence for tolerance mechanism evolved by the plant species (Patra and Panda, 1998; Prasad *et al.*,1999). Asada (1992, 1999) and Shigeoka *et al.*, 2002) reported that ascorbate peroxidase (APX) is an enzymatic antioxidant present in practically all sub cellular compartments. The main hydrogen peroxide detoxification system in plant chloroplast is ascorbate glutathione cycle, in which APX is a key enzyme. APX utilizes AsA as specific electron donor to reduce H_2O_2 to water. The APX and ascorbate glutathione cycle also plays a role in ROS scavenging in cytosol, mitochondria and peroxisomes.

Asada, (1999); Shigeoka *et al.,* (2002) reported that the expression of APX genes is modulated by several environmental stimuli known to increase ROS production, such as drought, high-intensity light, high temperature, salt stress, as well as pathogen attack. Previous reports indicate that APX plays an important role in scavenging ROS that are produced when plants are growing under stressful conditions.

Teixeira *et al.,* (2006) observed that APX are encoded by a multigene family in angiosperms; APX isoforms have a distinct sub-cellular localization enabling them to regulate ROS levels in different cellular compartments for either

signaling or defense purposes. Soluble isoforms can be found in the cytosol, mitochondria and chloroplast stroma, while membrane-bound isoforms are found in the peroxisomes and chloroplast thylakoids. In rice the APX gene family comprises eight members, *viz.,* two cytosolic, two peroxisomal, two chloroplastic and two mitochondrial.

Khanna-Chopra and Selote (2006) reported that increase in ascorbate peroxidase activity in wheat cultivar under water stress. Moti (drought tolerant variety of wheat) had higher ascorbate peroxidase activity than C-306 (drought susceptible variety of wheat).

Passardi *et al.,* (2007) observed that APX are class I heme-peroxidases found in green plants and in chloroplastic protists that catalyze the conversion of H_2O_2 into H_2O using ascorbate as a specific electron donor. Cicek and Cakirlar (2008) reported that salt treatment decreased remarkable ascorbate peroxidase activity in soybean at the 25^0C.

2.6.2 Superoxide dismutase (SOD)

One of the major classes of antioxidant enzymes, which protect the cellular and sub cellular components against harmful reactive oxygen species (ROS), is superoxide dismutase (SOD). SODs play pivotal role in scavenging highly reactive free oxygen radicals and protecting cells from toxic effects.

Superoxide production by rice leaves may be measured by SOD-sensitive oxidation of exogenous epinephrine

on intact leaf surface or in drop diffusates collected from leaves. The reaction was detected in healthy leaves and usually increased within 1-3 days after their inoculation with blast especially in incompatible combinations. The production of H_2O_2 and •OH by rice leaves was also shown. Bowler *et al.,* 1992 reported that, SOD is one of the most important scavenging enzymes and catalyzes the dismutation of superoxide radicals to hydrogen peroxide (H_2O_2).

The toxicity of ferulic acid to *Pyricularia oryzae* spores was diminished by SOD or other antioxidant compounds supposing involvement of AO in the toxic effect. In the mixture peroxidase / H_2O_2 ferulic, caffeic or chlorogenic acid acquired extra-toxicity which was prevented by SOD or hydroxyl radical scavengers (Aver'yanov, Lapikova, 1994).

SOD activity enhances under a variety of stressful conditions including Cu, Al, Mn, Fe and Zn toxicities (Prasad, 1997) for maintenance of overall defense system of plants subjected to oxidative damage (Slooten *et al.*, 1995).

SOD acts as the first line of defences response against ROS, dismutating superoxide to H_2O_2 (Alscher *et al.,* 2002). Following treatment with crude extracts of rice blast fungus, the SOD gene was dramatically induced in suspension-cultured rice cells (Matsumura *et al.,* 2003).

Interestingly, the same SOD proteins were identified in rice infected with RYMV (Ventelon-Debout *et al.,* 2004).

Monk *et al.,* (1998) reported that Superoxide dismutase (SOD) represents a key element of the enzymatic system that protects the plant cell against deleterious peroxidation reactions. Allen *et al.*, (1997) leaves of *A. esculentus* show an enhancement in SOD activity upon Cd and Hg stress. Increase in SOD activity is often attributed to de-novo synthesis of the enzyme and shown to confer increased protection from oxidative damage in transgenic plants.

2.6.3 Catalase

Catalase (E.C.1.11.1.6) is tetrameric heme containing enzyme with the potential to directly dismutate H_2O_2 into H_2O and O_2 and is indispensable for ROS detoxification during stress conditions. Catalase has one of the highest turnover rate for all enzymes. One molecule of catalase can convert ≈ 6 million molecules of H_2O_2 to O_2 per min. Catalase is important in the removal of H_2O_2 generated in peroxisomes by oxidase involved in β – oxidation of fatty acid.

Catalase activity decreased markedly with respect to the non-treated control at all the stages of growth and in the treatments with both $CdCl_2$ and $HgCl_2$.

This result coincides with experiments in *Phaseolus aureus* (Shaw, 1995); *Pisum sativum* (Dulurzo *et al.,* 1997) and *Amaranthus levidus* (Battacharjee, 1998) following the application of Cd to growth medium.

Decreased CAT activity has also been observed in *Phaseolus vulgaris* (Chaoui *et al.*, 1997; Somashekariah *et al.,* 1992) and pea (Sandalio *et al.,* 2001). *Shah et al.*, (2001) and Vaglio and Landriscina (1999) also described a general reduction of CAT activities upon Cd exposure. The data are also in line with the results obtained by Moussa (2005) where $CdCl_2$ treated faba beans showed a concentration-dependent oxidative stress situation in the leaves, characterized by an accumulation of H_2O_2, as a result of the inhibition of the CAT. It is also regarded as a general response to many stresses and is supposedly due to the inhibition of enzyme synthesis or change in assembly of enzyme subunits (MacRae and Fergusam, 1985).

Fornazier *et al.,* (2002); Shim *et al.,* (2003) observed that catalase is mainly present in peroxisomes and mitochondria, which often decreased following exposure to elevated Cd concentrations. The decrease may also be associated with degradation caused by induced peroxisomal proteases or may be due to photoinactivation of enzyme.

2.6.4 Glutathione reductase

Glutathione reductase (E.C.1.6.4.2) is flavoprotein oxidoreductase found in both prokaryotes and eukaryotes. It is a potential enzyme of the ASH-GSH cycle and plays an essential role in defence against ROS by sustaining the reduced status of GSH. It is localized predominantly in chloroplast but small amount of this enzyme has also been found in mitochondria and cytosol.

$$GSSG + NADP(H) \rightarrow 2\ GSH + NAD(P)^{+}$$

An enhanced activity of glutathione reductase in response to increasing concentrations of $HgCl_2$, a result which can often be exacerbated by the addition of GSSG and ameliorated by NADPH has been reported (Halliwell and Foyer, 1978; Serrano et al., 1984). Activation of the ascorbate glutathione cycle has been found to be essential in stressed plants to combat oxidative stress.

2.7 Lipid Peroxidation

The peroxidation of lipid is considered as the most damaging process known to occur in every living organism. The membrane damage is taken as a single parameter to determine the level of lipid destruction under various stresses. During LPO, products are formed that include small hydrocarbon fragment such as ketones, MDA etc. and compound which react with thiobarbituric acid TBA to form coloured products called Thiobarbituric acid reactive substances (TBARS).

Priestly, (1986), Wilson and McDonald, (1986); Hendry, (1993) suggested that lipid peroxidation and associated free radical oxidative stresses are widely considered to be major contributors to seed deterioration. They affect the structure and function of membranes, including the inactivation of membrane bound proteins and the alteration of membrane permeability.

Rudrapal and Nakamura, (1988) reported that exposure of freshly harvested eggplant and radish seeds to

halogen vapour slowed deterioration under accelerated and natural ageing conditions, presumably because of stabilization of unsaturated fatty acids in membranes making them less prone to peroxidative attack.

Lipid peroxidation has been suggested as the cause of loss of sunflower and soybean seed viability (Bailley, *et al.,* 1996; Sung, 1996).

Dhakal and Pandey (2001) reported that protective mechanisms such as peroxidase activity have been reported in soybean and sunflower to scavenge the free radicals produced within the seeds and minimize the deleterious effects of these compounds. This particular protective mechanism involves several free radical-and peroxide-scavenging enzymes such as catalase, peroxidase, superoxide dismutase and ascorbic acid.

Decrease in SOD, CAT, and glutathione following accelerated ageing of sunflower seed and other enzymes such as glutathione reductase (Bailly, Benarmar, Corbineau, and Come 1997). However, glutathione is an efficient antioxidant and has such a role in aged sunflower (De Paula *et al.,* 1996) and in watermelon (Hsu and Sung, 1997) seeds.

2.8 Molecular identification of candidate markers for seed longevity in soybean

Thirty three genotypes of soybean varying in storability (good and poor) and seed coat colour (black and yellow) were characterized with 53 SSR and 51 RAPD markers.

Polymorphisms detected by SSR and RAPD markers were 62.26 and 68.62%, respectively. Genotypes with black seed coat colour showed better storability (89.85%) than the yellow seed coated genotypes (71.15%) indicating possible association of black seed coat colour with good storability. Genetic similarity coefficients obtained through SSR data analysis grouped the genotypes into two major clusters representing black and yellow seeded genotypes. SSR markers Satt371, Satt453 and Satt618 produced specific allelic bands making them candidate markers for linkage with seed storability and testa colour (Hosamani *et al.,*2013).

Significant progress has been made in soybean genomics to target important genes, which has provided a deeper insight into the soybean genome structure and organization. Many reports about the construction of soybean genetic linkage maps using various markers have been published. Based on the construction of soybean genetic maps, quantitative trait loci (QTL) for a number of agronomic traits in soybean have been mapped. Singh *et al.*,(2008) reported that the four SSR markers (Satt434, Satt538, Satt281 and Satt598) are associated with seed coat permeability and electrolyte leaching in soybean. Another set of four SSR markers (Satt538, Satt285, Satt600 and Satt434) has been reported to be significantly associated with seed longevity in an F2:3 soybean population in a cross involving good and poor storer genotypes.

3. MATERIAL AND METHODS

The materials used and methods adapted during the present investigation entitled “Biochemical analysis concerning seed longevity in soybean [*Glycine max* (L.) Merill]” are described here under.

Material:

The seeds of soybean germplasm and the seeds of crosses effected were obtained from the soybean breeder, ARS, Kasbe- Digras, Sangli, Maharashtra.

The details of genotypes obtained for the present investigation entitled “Biochemical analysis concerning seed longevity in soybean [*Glycine max* (L.) Merill]”, is as under. Table1.

Sr.No.	**Genotypes**	**Pedigree**	**Salient features**
1	Birsa Soya1	_	High seed longevity, Black seed coat
2	EC 241780	_	Yellow seed coat, Rust resistance
3	Kalitur	_	High seed longevity, Black seed coat
4	Phule Agrani (KDS 344)	JS 335 X EC 241780	Yellow seed coat, Recent released variety

5	MAUS 71	JS 71-05 X JS 87-38	Yellow seed coat, Low seed longevity
6	Phule Kalyani (DS 228)	JS 335 X Ankur	Local Check. Low seed longevity
7	JS 335	JS 78-77 X JS 71-05	National check
8	KDS 1025	Birsa Soya 1 X EC241780	Brown seed coat
9	KDS 1031	Birsa Soya 1 X EC241780	Brown seed coat
10	KDS 1029	Birsa Soya 1 X EC241780	Yellow seed coat
11	KDS 1038	Birsa Soya 1 X EC241780	Brown seed coat
12	KDS 1034	Birsa Soya 1 X EC241780	Black seed coat
13	KDS 1036	Birsa Soya 1 X EC241780	Yellow seed coat
14	KDS 1027	Birsa Soya 1 X EC241780	Black seed coat
15	KDS 1024	Birsa Soya 1 X EC241780	Yellow seed coat

16	KDS 1035	Birsa Soya 1 X EC241780	Brown seed coat
17	KDS 1032	Birsa Soya 1 X EC241780	Yellow seed coat
18	KDS 726	JS 93 05 X EC 241780	Bold seeded genotype, Yellow seed coat

Methods

3.1 Physical characteristics

3.1.1 Proportion of seed coat

The soybean varieties were categorized into bold, medium and small seeded types on the basis of 100-seed weight by testing 10 replicated random samples from each variety.

The seed coat from seeds were removed carefully with the help of forceps after applying gentle stroke to seeds which were placed in between muslin cloth. The seed coat and cotyledons were collected and weighed separately. The total weight of seed coat and cotyledon was considered as the seed weight. The proportion of seed coat was calculated as follows,

$$\frac{\text{Weight of seed coat} \times 100}{\text{Weight of seed coat and cotyledon}}$$

3.1.2 Seed Volume

The volume of seed was measured by following volume expansion principle. A 100 ml volumetric flask was filled with toluene up to the level of 50 ml and 5-g seeds were added in to it. The increase in volume was measured and density of seed was calculated as weight of whole seed per unit volume. The seed volume was expressed in g ml^{-1}.

3.1.3 Mechanical strength

Mechanical strength of the seed coat was measured as the first break point on the graph of seed cracking using the texture analyzer. The seed was placed on the stationary metallic plate of the instrument. The plane perpendicular to the plane of the hilum was parallel to the surface of the plate. The force was applied by moving the piston. The increase of force is plotted along the Y-axis and duration of force applied (till the seed is cracked) is plotted along the X-axis. The mechanical strength expressed in Newton.

3.1.4 Seed Permeability

The seeds used for permeability studies were initially checked with dissecting microscope and only those with no damage were classed as 'intact' and were used for water uptake measurement. For each genotype, three to five seeds were tested .Single seeds were weighed, immersed in tap water for a specific time, removed from the water, blotted with cellulose tissue, weighed again, and returned to the water. Seeds were weighed at

5-min. intervals for the 30 min, and at 30 min intervals for 1 h, then weight was taken after another 1 h. A penultimate weight was taken at 2 hrs 30 min. The rate of water uptake was calculated by expressing it as weight increase (g) per gram seed (initial) weight.

3.1.5 Electrical conductivity

Five grams of seeds from each treatment in three replications were weighed and soaked in 25 ml distilled water in a beaker and kept at 25 ±1^{0}C temperature. After 24 hour of soaking the solution was decanted and the volume was made up to 25 ml by adding distilled water. The electrical conductivity of the leachate was measured dSm^{-1} (Presley, 1958).

3.2 Determination of oil content

The oil was extracted as per the procedure given in standard methods of biochemical analysis (Thimmaiah, 2004).

3.2.1 Reagent

1. Petroleum ether or ethyl ether or Hexane

3.2.2 Procedure

1. Ten gram of dried seed samples of each treatment with three replications were weighed and packed in Whatman No. 40 filter paper and kept in thimble assembled to condenser and extraction flask.
2. The dry pre weighed solvent flask was connected beneath the apparatus and added the required volume of solvent

(petroleum ether, b.p. 40-60 ^{0}C) and thumble was connected to condenser.

3. Heating rate was adjusted to give a condensation rate of 2-3 drop and extracted for 6 hr.
4. Thimble and flasks were removed from the condenser apparatus.
5. For the removal of excess ether from the solvent, flask was kept on a hot water bath and then dried the flask at 105^{0}C for 30 min.
6. Flasks were cooled in desiccators and after cooling of flasks weight was taken.

Calculation:

$$\text{Oil content in sample (\% dry wt. basis)} = \frac{b - a \times 100}{\text{Wt. of sample (g)}}$$

Where, a = Pre-weighed solvent flask

b = After weighed solvent flask

3.3 Determination of Tocopherol (vit.E) content(Emmerie-Engel reaction)

3.3.1 Reagents

1. Absolute alcohol

2. Xylene
3. 2, 2-dipyridyl (1.2 g/L in n-propanol)
4. Ferric chloride solution (1.2 g/L in ethanol)
5. Standard solution (D L-α-tocopherol, 10 mg/L in absolute alcohol)
6. Sulphuric acid. (0.1 N)

3.3.2 Extraction of tocopherol

The plant sample (2.5 gm) was homogenised in 50 ml of 0.1 N sulphuric acid and were allowed to stand overnight. The contents of flask were shaken vigourously and filtered through Whatsman no. 1 paper. The suitable aliquot of the filtrate were used for the estimation of tocopherol.

3.3.3 Procedure

Into three stoppered centrifuge tubes 1.5ml of standard and 1.5 ml of water were pipeted out separately. To all the tubes, 1.5ml of ethanol and 1.5 ml of xylene were added, mixed well and centrifuged. Xylene (1.0 ml) layer was transformed into another stoppered tube. To each tube, 1.0 ml of dipyridyl reagent was added and mixed well. The mixture was pipeted out into a cuvette and the extinction was read at 460 nm. Ferric chloride solution (0.33 ml) was added to all the tubes and mixed well. The red colour development was read exactly after 15 minutes at 520 nm in spectrophotometer.

3.4 Estimation of Lignin

The seed coat lignin content was determined as per titration method given by (Hussain *et al.,* 2002).

3.4.1 *Reagents*

The following chemical reagents were used

1. Potassium permanganate solution (0.05N): 7.9g potassium permanganate dissolved in distilled water and the volume was made to 1000 ml.
2. Sulphuric acid solution (4N): 108.6 ml of conc. sulphuric acid dissolved in distilled water and make the volume 1000 ml.
3. Potassium iodide solution (1N): 166 g potassium iodide dissolve in distilled water and make the volume 1000 ml
4. Sodium thiosulphate solution (0.1N): 24.81 g sodium thiosulphate dissolved in distilled water and make the volume 1000 ml.
5. Starch indicator solution (0.2%): 0.2 g starch indicator dissolve in 100 ml distilled water.

3.4.2 Procedure

1. Soybean seed coat sample of 0.1 gm were dispersed in 6 ml distilled water and ground to fine paste by using mortar and pestle.

2. The disintegrated sample was transferred to 250 ml conical flask and distilled water was added to make the total volume to 120 ml.
3. One ml of potassium permanganate solution and 15 ml of sulphuric acid solution were mixed together and added immediately to the disintegrated fibre sample.
4. Thus, the total volume was made to 150 ml with distilled water. The reaction was allowed to proceed at 25°C temperature for exactly 10 minutes in temperature bath maintained with 25^0C temperature.
5. Then 3 ml of potassium iodide solution was added and the free iodine was titrated with standard sodium thiosulphate solution using starch as an indicator.

A blank titration was made using the same volume of water and reagents.

The Kappa Number was then calculated from the following equation:

$$\text{Kappa Number(k)} = \frac{p \times f}{W}$$

Where,

p = Milliliter of 0.1N potassium permanganate consumed by the experimental soybean seed coat sample.

w = Weight in gram of moisture free seed coat sample.

f = Factor for correction to 50% permanganate consumption, which has been shown in the Table 2.

Using Table 2 as a guide

%Consumed	0	1	2	3	4	5	6	7	8	9
10	0.911	0.913	0.915	0.918	0.920	0.923	0.925	0.927	0.929	0.931
20	0.934	0.936	0.938	0.941	0.943	0.945	0.947	0.949	0.952	0.954
30	0.958	0.960	0.962	0.964	0.966	0.968	0.970	0.973	0.975	0.977
40	0.979	0.981	0.983	0.985	0.987	0.989	0.992	0.994	0.996	0.998
50	1.000	1.002	1.004	1.006	1.009	1.011	1.013	1.015	1.017	1.019
60	1.022	1.024	1.026	1.028	1.030	1.033	1.035	1.037	1.040	1.042
70	1.044									

3.5 Calcium Content

3.5.1 Laboratory analysis

Most methods involve destruction of the tissues organic component, thereby converting the elements to a soluble form for analysis. The calcium content was determined as per the method of Huang and Shuttle (1985).

3.5.2 Wet digestion procedure: HNO_3, 30% H_2O_2

1. Weighted 0.5 gm of 20 mesh plant tissue into a beaker or digestion tube. Added 5 ml conc.HNO_3, and digestion tube was allowed to stand digestion overnight.
2. Set temperature at 125^0C and digest for one hour.
3. Removed the beaker or tube and let it cool.
4. Repeat cooling and 30% H_2O_2 additions until digest is clear.
5. Add more HNO_3 as needed to keep digest from going dry.
6. When the digest is colourless, reduce the temperature to 80 ^{0}C. Remove watch glass from beaker on the funnel from digestion tube. Let the digest to go almost to dryness. Residue should be white or colourless.
7. Cool. Add 1:10 HCL to bring to the final desired volume.
8. A clear solution is ready for elemental assay.
9. The final volume made 100 ml with distilled water. for determination of calcium standards of 5/10/15 ppm were made and were used for determination of calcium by using atomic absorption photometer (Zoroski and Burau, 1977).

3.6 Determination of antioxidative enzymes

3.6.1 Ascorbate peroxidase

Ascorbate peroxidase activity was assayed as per the method described by Nakano and Asada (1981).

3.6.1.1 Reagents

1. Phosphate buffer(100mM, p^H =7.0) : It was prepared by mixing 39ml of 0.2M NaH_2PO_4 and 61 ml of 0.2M Na_2HPO_4 and the final volume made to 100ml with distilled water.

2. Ascorbic acid (3mM): It was prepared by dissolving 0.013g ascorbic acid in distilled water and final volume made to 25ml with distilled water.

3. Hydrogen peroxide (3mM): 20ml H_2O_2 was dissolved in distilled water and final volume made to 50 ml.

4. Ethylenediaminetetraacetic acid(3mM) : It was prepared by dissolving 0.219g of EDTA in 25ml of distilled water

3.6.1.2 Enzyme Extraction

Enzyme extract for Ascorbate peroxidase was prepared by grinding 0.5g of cotyledon sample with 2ml of 100 mM Potassium phosphate buffer (p^H=7.5). The homogenate was centrifuged at 15000rpm for 15 min at 4°C and the supernatant was used as the enzyme source.

3.6.1.3 Procedure

The reaction mixture contained: 2.3ml Phosphate buffer, 0.2ml ascorbic acid, 0.2ml EDTA, 50µl enzyme extract, 50µl H_2O_2 and 0.3ml distilled water.

The reaction was started with addition of 0.2 ml of hydrogen peroxide. Decrease in absorbance after 30 sec. was measured at 290 nm in UV- visible spectrophotometer.

3.6.1.4 Protein estimation

The protein contained in the crude enzyme extract was estimated according to the method of Lowry *et al.,* (1951).

3.6.2 Superoxide dismutase

Superoxide dismutase activity was measured immediately in fresh extract as per the method described by Dhindsa *et* al., (1981).

3.6.2.1 Reagents

1. Nitro blue tetrazolium (2.25mM): It was prepared by dissolving 0.018g of nitro blue tetrazolium in distilled water.
2. Riboflavin (60µM): It was prepared by dissolving 0.002g of riboflavin in distilled water and final volume made to 100ml.
3. L-methionine (200mM): It was prepared by dissolving 0.447g of L methionine in distilled water and then final volume made to 15ml.
4. Ethylenediaminetetraacetic acid (3mM): It was prepared by dissolving 0.219g of EDTA in 25ml of distilled water.
5. Phosphate buffer(0.1M, p^H =7.5) : It was prepared by mixing 16ml of 0.2m NaH_2PO_4 and 84ml of 0.2M Na_2HPO_4 and final volume made to 100ml with distilled water.

3.6.2.2 Enzyme Extraction

Enzyme extract for Superoxide dismutase was prepared by grinding 0.5g of cotyledon samples with 2ml of 100 mM Potassium phosphate buffer (p^H=7.5). The homogenate was centrifuged at 15000rpm for 15 min at 4°C and the supernatant was used as the enzyme source.

3.6.2.3 Procedure

The reaction mixture contained : 1.5ml Phosphate buffer, 0.2ml methionine, 0.1ml EDTA, 0.1ml Sodium carbonate, 0.1ml enzyme extract, 0.1ml NBT, 0.9ml distilled water, and 0.1ml riboflavin.

The reaction was started by adding 0.1ml of riboflavin and placing the tubes under two 15w fluorescent lamps for 15min. A complete reaction mixture without enzyme, which gives the maximal colour, served as control. Switching of the lights and putting the tubes into dark stopped the reaction. The non-irradiated complete reaction mixture served as blank. The enzyme activity was expressed in Units mg^{-1} of soluble protein min^{-1}.

3.6.2.4 Protein estimation

The protein contained in the crude enzyme extract was estimated according to the method of Lowry *et al.,* (1951)

3.6.3 Catalase

The enzyme analysis of catalase was performed as per the method described by Luck *et. al.*,(1947).

3.6.3.1 Extraction of enzyme

Fresh cotyledon sample (1 gm) was homogenized in 10 ml of 0.1M Potassium phosphate buffer (pH 7.0) in prechilled morter and pestle. The extract was centrifuged at 15000 rpm for 20 min at 4^0c and the supernatant used as enzyme source.

3.6.3.2 Procedure

The assay mixture in a total volume of 3 ml contained 0.5 ml of 0.2 M Potassium phosphate buffer (pH 7.0), 0.3 ml of 50mM hydrogen peroxide and 0.1 ml enzyme extract and final volume was made three ml by adding distilled water the reaction was started by adding the enzyme and change in optical density was measured at 240nm.

3.6.4 Glutathione reductase

The glutathione reductase activity was measured by the method of Sadasivam and Manickam (1996).

3.6.4.1 Extraction of enzyme

Fresh cotyledon sample (1 gm) was homogenized in 10 ml of 0.1M Potassium phosphate buffer (pH 7.0) in prechilled morter and pestle. The extract was centrifuged at 15000 rpm for 20 min at 4^0c and the supernatant used as enzyme source.

3.6.4.2 Reagents

1. Oxidised glutathione (20mM)

Dissolve 0.305g Oxidized glutathione in 25ml distilled water

2. 5, 5-dithiobis-2-nitrobenzoic acid (30 mM)

Dissolve 0.294g 5, 5-dithiobis-2-nitrobenzoic acid in 25ml distilled water.

3. Nicotinamide adenine dinucleotide phosphate (2mM)

Dissolve 0.O83g Nicotinamide adenine dinucleotide phosphate in 50ml distilled water

4. Potassium Phosphate buffer (0.2 M, pH 7.5) It was prepared by mixing 150 ml of 0.2 M KOH and 250 ml of 0.2 M KH_2PO_4 containing 100 ml of distilled water and final volume was made to 500 ml after adjusting pH to 7.5.

3.6.4.3 Procedure

The assay mixture in a total volume of 3 ml contained 1 ml of 0.2 M Potassium phosphate buffer (pH 7.5), 0.350 ml of 5, 5-dithiobis-2-nitrobenzoic acid (30 mM), 0.350ml of oxidised glutathione (20mM), 0.100ml Nicotinamide adenine dinucleotide phosphate (2mM) and 0.100 ml enzyme extract final volume was made 1.100 ml by adding distilled water the reaction was started by adding the enzyme and change in optical density was measured at 412 nm.

3.7 Lipid peroxidation

Lipid peroxidation is oxidative degradation of lipid fatty acid by reactive oxygen species. The level of lipid

peroxidation is measured in terms of thiobarbituric acid reactive substance (TBARS) content (Heath and Packer, 1968).

3.7.1 Reagent

1. Trichloroacetic acid (0.1 %): Trichloroacetic acid solution was prepared by dissolving 0.1 g TCA in water and then the volume was made to 100 ml.
2. Thiobarbituric acid reagent: Five gram TBA was dissolved in small amount of 20 % TCA and then the volume was made to 100 ml by 20 % TCA.

3.7.2 Procedure

1. **Sample extraction**: Cotyledon sample (0.2 g) was homogenized in 4 ml of 0.1 % TCA. The homogenate was centrifuged at 15000 g for 15 min and the supernatant was used for the estimation of TBARS content.
2. **Assay:** Four ml of 0.5 % TBA in 20% TCA was added in 10 ml aliquot of the supernatant. The mixture was heated at 95^0 C for 30 min in the lab electric oven and then cooled in an ice bath. After cooling the aliquot was centrifuged at 10,000 X g for 10 min. The absorbance of the clear supernatant was recorded at 532 nm. The values of non specific absorption were recorded at 600 nm and were substracted from the values recorded at 532 nm. The TBARS content was calculated by using extinction coefficient E = 155 m M^{-1} cm^{-1}.

3.8 Isolation of genomic DNA from Soybean

The isolation of genomic DNA from fresh young seedlings of different soybean cultivars and their crosses were

carried out by a modified cetyl trimethylammonium bromide (CTAB) method described by Keim *et al.* (1988).

Reagents:

1. CTAB buffer 1 liter :100ml of 1 M Tris (pH.8.0), 280ml of 5M NaCl, 40ml of 0.5 M EDTA, 20g CTAB, 40g PVP and 5ml β-Mercaptoethanol.
2. Chloroform : isoamyl alcohol (24:1)
3. Ethanol : 70 % (v/v)
4. TE (10/1) buffer : 10 mM Tris-HCl, 1 mM EDTA (pH 8.0)
5. Ammonium acetate : 7.5 M

Procedure

1. About 0.5 g of fresh young leaves of different soybean cultivars and their crosses genotypes were taken and cut into small pieces (about $10mm^2$) with blade and powdered in liquid nitrogen (N_2) with prechilled mortar and pestle.
2. The powder was homogenized in prechilled 1.5 ml CTAB buffer with mortar and pestle and transferred in 2 ml tubes.
3. The tubes were incubated for 60 min at 65^0C in a thermostatic water bath.
4. The contents in the tubes were mixed after every 15 min by inversion during incubation.
5. After incubation the tubes were allowed to cool at room temperature.

6. An equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently by inversion. The tubes were then centrifuged at 10,000 x g for 10 min at 4^0C in a high speed refrigerated centrifuge (Kubota 6500, Japan).
7. Then aqueous phase was carefully recovered and transferred to a fresh tube.
8. An equal volume of isopropanol and 50µl of 7.5 M ammonium acetate was added into the contents of fresh tube. The tube was gently vortexed and incubated at -20^0C for overnight for precipitation of DNA.
9. The tubes were centrifuged at 12,000 x g for 10 min at 4^0C.
10. Collected the pellet carefully and washed with 70 % ethanol. The DNA pellet was air dried till the last traces of ethanol was evaporated.
11. The pellet was resuspended in suitable volume of TE (10/1) buffer.

3.8.1 Purification of genomic DNA

Reagents

1. Sodium acetate (pH.4.8): 3 M
2. Ribonuclease A (10 mg/ml)
3. Proteinase K (20mg/ml)
4. Isopropanol
5. Ethanol : 70 % (v/v)
6. Choroform : Isoamyl alcohol (24:1)

Procedure:

1. For purification, 500 µl of DNA sample was taken in a fresh eppendorf tube.
2. To this 10µl of RNAase (10 mg / ml) was added and incubated at 37^0C for 1 hr with occasional gentle shaking.
3. After incubation 20µl of Proteinase K and 100µl of 3 M sodium acetate (pH. 4.8) was added and again incubated for 60^0C for 1 hr.
4. After incubation equal volume of Choroform : Isoamyl alcohol was added.
5. The tubes were then centrifuged at 12,000 rpm for 10 min at 4^0C
6. Upper layer was removed and transferred into another fresh eppendorf tube.
7. To the supernatant equal volume of isopropanol was added.
8. The tubes were chilled at -20^0 C for 1 hr/overnight.
9. Then the tubes were centrifuged at 12,000 rpm for 10 min at 4^0C.
10. Supernatant was decanted carefully and DNA pellets were washed with 70% ice cold ethanol and air dried.
11. Purified DNA was then dissolved in suitable aliquot of TE buffer.

3.8.2 DNA amplification by SSR primers

The sequences of oligonucleotide of nine SSR primers were synthesized and screened for polymorphism in seven cultivated good and poor storer varieties *viz.,* Birsa soya 1, Kalitur, DS 228, KDS 1034, KDS 1035, KDS 1027 and EC 241780. Primer sequences, annealing temperature are described,

Table . Sequences of the SSR primers

Sr. No	Primer Name	Primer sequences	T_a (°C)
1.	Satt 371	F : TGCAAACTAACTGGATTCACTCA	57.6
		R : GAGATCCCGAAATTTTAGTGTAACA	
2.	Satt 453	F : GCGGAAAAAAAACAATAAACAACA	57
		R: TAGTGGGGAAGGGAAGTTACC	
3.	Satt 618	F : GCGGTGATATTACCCCAAAAAAATGAA	61.9
		R:GCGCTAGTTTCTAGTGGAAAGATGGAT	
4.	Satt 400	F : TTGGTCATCAAACTGTTA	56
		R : CATAAGGGGTCCCACTCTA	
5.	Satt 143	F : GTGCCACAAATTTAAAATTACTCA	52
		R : TCCCTCCCTTTTGATTTACAC	
6.	Satt162	F : GGGAAGAAGTATATGCTACATCAA	57
		R : GGGTTAATTTTTATCTTCTAATAGTTT	
7.	Satt 286	F : GCGGCGTTAATTTATGCCGGAAA	60
		R : GCGTTTGGTCTAGAATAGTTCTCA	
8.	Satt 523	F : GCGATTTCTTCCTTGAAGAATTTTCTG	56
		R: GCGCTTTTTCGGCTGTTATTTTTAACT	
9.	TR	F : GGAAGAAAGTATTGGTCTGT	56
		R : AGGAGAGAGTGGAGAGATTA	

PCR amplification was performed with simple sequence repeat primers obtained from Operon Technologies, Inc., Alameda USA. Amplification was performed in a 0.2 ml PCR tubes having 25 µl reaction volume as described by Hosamani *et al.,* (2013) with some modification. PCR reaction was carried out in a total of 25 µl volume containing 50ηg templet DNA, 1.0 µl of each forward and reverse primer. The detailed composition mixture for PCR was given below:

Sr.No.	Constituents	Stock concentration	Volume of PCR reaction mixture per tube (25µl)
1.	Taq buffer A (Tris with 15 mM $MgCl_2$)	10X	2.5 µl
2.	Taq DNA polymerase	3U/ µl	0.5 µl
3.	dNTPs	10mM	1.0 µl
4.	Primer	10uM	F- 1 .0 µl R- 1.0 µl
5.	Template DNA	25ηg/ µl	2 µl
6.	Sterile distilled water	--	17.00 µl
7.	Total volume	--	25 µl

Procedure

The 25 µl reaction mixture was gently vortexed and spinned down. The DNA amplification was carried out on a thermal cycler (Eppendorf, Mastercycle gradient, Germany) with the following conditions: initial denaturation at 94^0C for 5 minutes followed by 35 amplification cycles. Each amplification cycle was initially at 94^0C for 1 min followed by annealing temperature as per the TM values of primers for 30 sec and primer extension at 72^0C for 30 sec. A final extension at 72^0C for 7 minutes was given at the end of the cycles and the samples were held at 4^0C till retrieval. The PCR conditions particularly the annealing temperature of each primer was standardized. The amplified product was stored at 4^0C. The amplified products were separated by 2.5% agarose gel electrophoresis in 1X TBE buffer. The size of the amplified fragments was estimated using

100 bp DNA ladder. Amplified bands were visualized after staining with 0.5µg/ ml ethidium bromide. Gel photographs were taken under UV light in GelDoc system (Image Quant Las 4000 mini).

3.9. Statistical analysis

The data on biochemical constituent was analyzed by using completely randomized design (Panse and Sukatme, 1985).

4. RESULTS AND DISCUSSION

In the present investigation entitled "Biochemical analysis concerning seed longevity in soybean [*Glycine max* (L.) Merill]" seven genotypes of soybean comprising of two (Birsa soya 1, Kalitur) with higher seed longevity; two genotypes (MAUS 71, DS 228) with low seed longevity; one rust resistant genotype (EC 241780); one recently released variety Phule Agrani (KDS 344) and one variety JS 335 a national check were used. Eleven crosses were effected using Birsa soya 1 with EC 241780 (10 crosses) and one cross effected between JS 9305 and EC 241780. The F_3 progenies of the crosses exhibiting variable seed coat colour *viz.,* yellow (5), brown (4) and black (2) were also analyzed for different biochemical parameters which are related to better seed longevity. The genomic DNA of soybean genotypes with higher seed longevity (Birsa soya 1, Kalitur) and low seed longevity (DS 228) and crosses exhibiting better morpho-physiological parameters, strength and biochemical composition were amplified using 9 SSR markers which have been reported to be linked with the seed longevity or seed coat colour. The results thus obtained are presented and discussed in the light of the available literature in the chapter under appropriate sub-heads.

4.1 Physical characteristics of seed

4.2 Oil content

4.3 Vitamin E content (Tocopherol)

4.4 Lignin content

4.5 Calcium content

4.6 Antioxidative enzymes

4.7 Lipid peroxidation

4.8 Molecular identification and validation of candidate markers for seed longevity in soybean

4.1 Physical characteristics of seed

4.1.1 Proportion of seed coat

The proportion of seed coat is of primary importance in providing protection against mechanical damage during harvesting and processing, which cause significant loss in viability of seed lot. The seed coat proportion of seven genotypes and eleven F_3 progenies of the crosses ranged between 8.85 to 10.85% with the lowest seed coat proportion of 8.85% in DS 228 and the highest seed coat proportion of 10.85% in KDS 1032. The seed coat proportion in Birsa soya 1 and Kalitur, the two black seed coat soybean genotypes was 10.11 and 10.49% respectively. The F_3 progenies of some crosses involving Birsa soya 1 and EC 241780 as parents also exhibited higher seed coat proportion *viz.,* 10.81% in KDS 1038, 10.85% in KDS 1032, 10.29% in KDS, and 10.32% in KDS 1034, which was better than either of parents Birsa soya 1 (10.11%) and EC 241780 (9.70%) (Table 1).

The black- seeded varieties Kalitur and Birsa soya1 had been reported to have higher seed coat to seed proportion of 11.25 and 10.21% respectively (Kuchlan *et al.,* 2010). The genotype JS 335 a national check reported a seed coat proportion of 9.05% which is slightly higher than reported earlier *i.e.* 8.31%. In the present investigation soybean variety MAUS 71 a yellow seeded variety with low seed longevity however recorded higher seed coat proportion of 10.72%. The soybean genotypes with black-seed coat colour and thicker seed coat have been reported to be resistant to field weathering (Dassou and Kueneman, 1984).

4.1.2 Seed volume

The volume of soybean was measured by volume expansion principle and expressed as weight per unit volume. The seed volume varied between 0.80 to 0.98 g ml^{-1}. The black seeded variety is Birsa soya 1 and Kalitur recorded seed volume of 0.96 and 0.92 g ml^{-1} and genotype with low seed longevity MAUS 71 recorded seed volume of 0.82 g ml^{-1} (Table 1). Lower seed density has been associated with lower seed quality (Hoy and Gamble, 1985). A variation in soybean seed density from 1.13 to 1.91 has been reported with higher seed density in Birsa soya 1 (1.186 g/ml) and JS 335 1.191g/ml (Kuchlan *et al.,* 2010).

4.1.3 Mechanical strength

The mechanical strength of 18 soybean seeds was evaluated as the first break point on the graph of seed cracking using texture analyzer. The mechanical strength of seed varied from 72 to 132 Newton. The black seeded and high seed longevity recorded higher mechanical strength. The soybean genotype with low seed longevity *viz.,* DS 228 and MAUS 71 recorded low values 72 and 87 (N) of mechanical strength. The mechanical strength of newly released variety KDS 344 was 121(N). The F_3 progenies of the cross Birsa soya1 and EC 241780 recorded mechanical strength range of 76 to 132(N) with highest mechanical strength of 132 (N) in KDS 1034 and lowest mechanical strength of 76(N) in KDS 1036 (Table 1). Cotyledons were seen as a dark inner circular mass and the seed coat as outer black circular line in x-radiographs. The light area between these two zones was the space between the seed coat and cotyledon, which showed considerable variation in different varieties. This gap was found to be positively correlated with seed coat strength. The mechanical strength of seed were highest in KDS 1034 and KDS 1025 *i.e.*, 132(N) and 128(N) respectively (Table 1).

The mechanical strength of soybean seed coat varying in the range of 9.319 to 13.639 (kg) has been reported with highest mechanical strength of seed coat of 13.639 (kg) in Birsa soya 1 (Kuchlan *et al.,* 2010). The lesser gap between the seed coat and cotyledons is correlated with better mechanical strength, (Wolf *et al.,* 1981).

Table 1. Physical characteristics of soybean seed

Sr.No.	Genotype	Seed coat proportion(%)	Seed volume (gml^{-1})	Mechanical strength (N)
1	Birsa soya1	10.11	0.96	104
2	EC 241780	9.70	0.98	107
3	Kalitur	10.49	0.92	102
4	KDS 344	9.93	0.80	121
5	MAUS 71	10.72	0.82	87
6	DS 228	8.85	0.92	72
7	JS 335	9.05	0.90	120
8	KDS 726	9.42	0.96	94
9	KDS 1032	10.85	0.82	94
10	KDS 1029	9.40	0.98	94
11	KDS 1035	10.26	0.94	122
12	KDS 1034	10.32	0.96	132
13	KDS 1025	9.95	0.86	128
14	KDS 1031	10.16	0.88	110
15	KDS 1038	10.81	0.84	123
16	KDS 1024	10.29	0.82	94
17	KDS 1036	9.45	0.86	76
18	KDS 1027	10.19	0.90	110
	Range	8.85-10.85	0.80-0.98	72-132
	GM	9.99	0.89	105.44
	SE	0.0057	0.0360	0.5773
	CD 5%	0.0165	0.1032	1.6559

4.1.4 Electrical conductivity of seed leachate

The electrical conductivity of the seed leachate varied from 0.30 to 0.63 dSm^{-1}. The electrical conductivity of seed leachate of Birsa soya 1 was 0.30 dSm^{-1} while that of Kalitur was 0.45 dSm^{-1}. The highest electrical conductivity was recorded in EC 241780. The seed leachate conductivity of DS 228, a soybean variety of low seed longevity was 0.58 dSm^{-1}. The F_3 progenies of the cross (Birsa soya 1 x EC 241780) recorded electrical conductivity in the range of 0.30 to 0.55 dSm^{-1} with lowest in KDS 1034 and highest in KDS 1024 (Table 2).

During the present investigation, a soybean variety MAUS 71 with low seed longevity recorded minimum electrical conductivity of 0.30 dSm^{-1}. Loeffler *et al.,* (1988) reported that good storer soybean genotypes with lower electrolyte leackage were characterised by smaller seed size, black testa colour, and better germination, as compared to poor storer genotypes, which recorded higher electrolyte leakage, larger seed size, and yellow testa colour.

4.1.5 Seed permeability

The seed permeability of seven genotypes and eleven F_3 progenies of crosses ranged between 30 to 97% with the lowest seed permeability of 30% in KDS 1038 and highest seed permeability of 97% in KDS 1034. The seed permeability of black seed coat genotypes Birsa soya 1 and Kalitur was 57 and 81% respectively. The F_3 progenies of some crosses involving Birsa

soya 1 and EC 241780 as parents also exhibited lower seed permeability *viz.,* 30% in KDS 1038, 42% in KDS 1035, 47% in KDS 1024 which was less than either of parents Birsa soya 1 (57%) and EC 241780 (93.2%) (Table 2).

Rolston, (1978) and Tran and Cavanagh, (1984) reported that it is biological beneficial for long term survival and can be important for wild plants soybean (*Glycine max)* and many other legumes have hard seeds. Tyler (1997) observed that hard seededness in soybeans can protect against seed decay and improve agronomic qualities under certain conditions but hard seededness is undesirable for the food processing industry.

4.2 Oil content

The oil content was extracted as per the method of Thimmaiah (2004). The oil content of the soybean seed varied from 17.3 to 20.9%. The oil content of Birsa soya 1 was 20% while that of Kalitur was 19.72%. The highest oil content of seed was recorded 20% in KDS 1034, 19.66% in KDS 1038 and 19.31% in KDS 1032. The F_3 progenies of the cross (Birsa soya 1 × EC 241780) recorded oil content in the range of 18.00 to 20.90% with highest in KDS 1024 and lowest in KDS 1036 (Table 3).

Table 2. Physical characteristics of seed

Sr.No.	Genotype	Electrical Conductivity (dSm^{-1})	Seed Permeability (%)
1	Birsa soya1	0.30	57
2	EC 241780	0.63	93.2
3	Kalitur	0.45	81
4	KDS 344	0.58	90
5	MAUS 71	0.30	49
6	DS 228	0.59	67
7	JS 335	0.43	61
8	KDS 726	0.52	32
9	KDS 1032	0.42	64
10	KDS 1029	0.39	50
11	KDS 1035	0.33	42
12	KDS 1034	0.30	97
13	KDS 1025	0.33	57.2
14	KDS 1031	0.33	75.1
15	KDS 1038	0.34	30
16	KDS 1024	0.55	47
17	KDS 1036	0.53	58
18	KDS 1027	0.51	52
	Range	0.30-0.63	30-97
	GM	0.43	61.47
	SE	0.0033	0.2837
	CD 5%	0.0095	0.8137

During the present investigation, a soybean variety MAUS 71 with low seed longevity recorded minimum oil content of 17.3%.

Raut *et al.*, (1998) concluded that highest mean oil content was recorded in yellow-coloured seeds (19.12%), followed by green (18.28%) and black seeds (18.21%). Statistically significant differences were found among genotypes for protein and oil contents. Protein content ranged from 331 to 448 g kg^{-1} whereas oil content constituted more than 198 g kg^{-1} in all cultivars. Protein and oil contents varied significantly between locations; cultivars grown at Manfredi showed the highest values. There was no negative correlation between protein and oil contents as indicated previously (Leffel and Rhodes, 1993; Liu *et al.,* 1995).

4.3 Vitamin E content

The tocopherol content in oil of soybean seed varied from 18.66 to 41.03 µg ml^{-1} oil, with the lowest vitamin E content of 18.66 µg ml^{-1} in KDS 1024 and the highest vitamin E content of 41.03 µg ml^{-1} in KDS 1034. The tocopherol content in Birsa soya 1 and Kalitur, these two black seed coat soybean genotypes was 35.74 µg ml^{-1} and 34.74 µg ml^{-1} respectively. The F_3 progenies of some crosses involving Birsa soya 1 and EC 241780 as parents also exhibited higher vitamin E content *viz.,* 37.03 µg ml^{-1} in KDS 1027, 31.85 µg ml^{-1} in both KDS 1035 and KDS 1031, 27.25 µg ml^{-1} in KDS 1025 (Table 3).

Table 3. Oil and vitamin E content in soybean seed.

Sr.No.	Genotype	Oil Content (%)	Vitamin. E (μg ml^{-1} of oil)
1	Birsa soya 1	20.00	35.74
2	EC 241780	19.56	20.96
3	Kalitur	19.72	34.74
4	KDS 344	18.80	22.66
5	MAUS 71	17.30	19.37
6	DS 228	18.15	27.25
7	JS 335	18.79	29.55
8	KDS 726	18.73	20.96
9	KDS 1032	19.31	20.37
10	KDS 1029	18.40	20.96
11	KDS 1035	19.00	31.85
12	KDS 1034	20.00	41.03
13	KDS 1025	19.18	27.25
14	KDS 1031	19.00	31.85
15	KDS 1038	19.66	20.96
16	KDS 1024	20.90	18.66
17	KDS 1036	18.00	20.37
18	KDS 1027	19.00	37.03
	Range	17.3-20.9	18.66-41.03
	GM	20.4827	26.5311
	SE	0.6052	0.5773
	CD 5%	1.7359	1.6559

Seneratna *et al.,* (1988) Kalpana and Madhava Rao, (1994) suggested that soybean seeds have a lower tocopherol content following ageing, suggesting that tocopherol is consumed and protects the seed against free radical damage.

4.4. Lignin content

The seed coat lignin content was determined as per the titration method. The lignin content ranged between 4.6 to 16 %.The black seeded variety Birsa soya 1 and Kalitur recorded lignin content of 15.01% and 12.30%, and the genotype with low seed longevity MAUS 71 and DS 228 recorded lignin content 5.90% and 6.60% respectively (Table 4). The F_3 progenies of the cross (Birsa soya 1 × EC 241780) recorded lignin content in the range of 5.12 to 16 % with lowest in KDS 1036 and highest in KDS 1034.

During the present investigation, a soybean genotype KDS 1034 with black seed coat recorded maximum lignin percentage compared to other genotypes. Breeding soybean for high seed quality is an important approach for developing cultivars for tropical regions, and the lignin content in the seed coat was found to be one of the screening parameters for this trait (Krzyzanowski, 1998).

Table 4. Lignin content in soybean seed coat

Sr.No.	Genotype	Lignin content (%)
1	Birsa soya 1	15.01
2	EC 241780	08.70
3	Kalitur	12.30
4	KDS 344	07.00
5	MAUS 71	05.90
6	DS 228	06.60
7	JS 335	04.60
8	KDS 726	08.60
9	KDS 1032	12.80
10	KDS 1029	05.70
11	KDS 1035	15.80
12	KDS 1034	16.00
13	KDS 1025	15.03
14	KDS 1031	13.30
15	KDS 1038	13.20
16	KDS 1024	08.30
17	KDS 1036	05.12
18	KDS 1027	08.00
	Range	4.6-16
	GM	10.38
	SE	0.5773
	CD 5%	1.6559

It has been reported that the soybean seed coat is very thin and low in lignin content, and provides little protection to the fragile radicle that lies in a vulnerable position directly beneath the seed coat (Gupta *et al.,* 1973, Agrawal and Menon 1974, França Neto and Henning 1984). Lignin is known to impart mechanical strength to the seed coat (Alvarez et al. 1997). Black-seeded varieties 'Birsa Soya 1' (1.0131) and 'Kalitur' (0.9689) had significantly higher amount of lignin than other varieties. Among yellow-seeded varieties, 'PS 1029' (0.8054), 'NRC 7' (0.7725), and 'JS 335' (0.7691) had significantly higher amount of lignin in seed coat, whereas variety 'PK 472' (0.7288), which is highly susceptible to mechanical injury, had significantly lower amount of lignin content among all varieties (Kuchlan *et al.,*2010).

4.5 Calcium content

The calcium content was determined as per the method of wet digestion procedure, by using atomic absorption photometer. The calcium content of seed coat varied from 2079 to 2688 ppm. The calcium content of the seed coat of Birsa soya 1 was 2639 ppm while that of Kalitur was 2305 ppm. The highest calcium content of seed coat 2688 ppm was recorded in KDS 1038 (Table 5). The calcium content of DS 228, a soybean variety having low seed longevity was 2128 ppm. The F_3 progenies of the cross (Birsa soya 1 × EC 241780) recorded calcium content in the range of 2097 to 2688 ppm with lowest in KDS 1027 and highest in KDS 1038.

Kuchlan *et al.,* (2010) reported that yellow seeded good storer variety, namely 'JS 80-21', had the highest amount of calcium in the seed coat. Significantly higher amount of calcium in seed coat was found in intermediate storer variety 'Pusa 16' (7.929 mg/g seed coat), black-seeded 'Birsa Soya 1' (7.032 mg/g. Next to these varieties were yellow-seeded good storer variety 'JS 335' (6.347 mg/g), intermediate storer variety 'NRC 2' (6.273 mg/g), and black-seeded good storer variety 'Kalitur' (5.914 mg/g). Yellow-seeded poor storer variety 'PK 472' which is most susceptible to mechanical stress recorded lowest Ca content (4.528 mg/g) followed by another poor storer variety 'PS 1029' (4.896 mg/g). Though, 'NRC 55' is an intermediate storer variety, it had significantly low (4.939mg/g) amount of calcium in seed coat of the two black-seeded varieties, bold-seeded 'Birsa Soya 1' (7.032 mg/g) had significantly higher amount of calcium content in seed coat than the small-seeded 'Kalitur' (5.914 mg/g). Chen *et al.,* (1993), (2001) reported that high calcium content appears to be the main cause of stone seeds. In texture studies, calcium content in soybean seed was found positively correlated with seed hardness, but this correlation was not always consistent because of environmental effects such as soil type and temperature variations.

Table 5. Calcium content in soybean seed coat

Sr.No.	Genotype	Calcium content (ppm)
1	Birsa soya1	2639
2	EC 241780	2337
3	Kalitur	2305
4	KDS 344	2303
5	MAUS 71	2340
6	DS 228	2128
7	JS 335	2106
8	KDS 726	2228
9	KDS 1032	2252
10	KDS 1029	2079
11	KDS 1035	2630
12	KDS 1034	2628
13	KDS 1025	2460
14	KDS 1031	2448
15	KDS 1038	2688
16	KDS 1024	2114
17	KDS 1036	2330
18	KDS 1027	2097
	Range	2079-2688
	GM	2323.05
	SE	23.4591
	CD 5%	67.2846

4.6 Antioxidative enzymes

4.6.1 Ascorbate peroxidase

The ascorbate peroxidase (E.C.1.11.1.1) plays the most essential role in scavenging ROS and protecting cells in higher plants, algae, and other organisms.

The ascorbate peroxidase activity of seven genotypes and eleven F_3 progenies of the crosses ranged between 0.166 to 0.786 µ moles of ascorbate oxidized mg^{-1} of soluble protein min^{-1}, with the lowest ascorbate peroxidase activity of 0.166 µ moles of ascorbate oxidized mg^{-1} of soluble protein min^{-1} in KDS 1034 and highest ascorbate peroxidase activity was 0.786 µ moles of ascorbate oxidized mg^{-1} of soluble protein min^{-1} in KDS 344. The ascorbate peroxidase content in black seed coat genotypes Birsa soya 1 and Kalitur was 0.498 and 0.359 µ moles of ascorbate oxidized mg^{-1} of soluble protein min^{-1} respectively (Table 6). The F_3 progenies of some crosses involving Birsa soya 1 and EC 241780 as parents also exhibited higher APX activity *viz.*, 0.764 in KDS 1038, 0.666 in KDS 1032, 0.608 in KDS 1029, which was better than either of the parents Birsa soya 1(0.498 µ moles of ascorbate oxidized mg^{-1} of soluble protein min^{-1}) and EC 241780 (0.215 µ moles of ascorbate oxidized mg^{-1} of soluble protein min^{-1}).

Asada (1992, 1999) and Shingeoka *et al.,* 2002) reported that ascorbate peroxidase (APX) is an enzymatic antioxidant present in practically all sub cellular compartments. The main hydrogen peroxide detoxification system in plant

Table 6. Ascorbate peroxidase activity in cotyledon of soybean

Sr.No.	Genotype	Ascorbate peroxidase (µmoles of ascorbate oxidised mg $^{-1}$ of protein min^{-1})
1	Birsa soya1	0.498
2	EC 241780	0.215
3	Kalitur	0.359
4	KDS 344	0.786
5	MAUS 71	0.554
6	DS 228	0.397
7	JS 335	0.313
8	KDS 726	0.525
9	KDS 1032	0.666
10	KDS 1029	0.608
11	KDS 1035	0.283
12	KDS 1034	0.166
13	KDS 1025	0.385
14	KDS 1031	0.300
15	KDS 1038	0.764
16	KDS 1024	0.220
17	KDS 1036	0.322
18	KDS 1027	0.519
	Range	0.166-0.786
	GM	0.436
	SE	0.0011
	CD 5%	0.0032

chloroplast is ascorbate glutathione cycle, in which APX is a key enzyme. APX utilizes AsA as specific electron donor to reduce H_2O_2 to water. The APX and ascorbate glutathione cycle also plays a role in ROS scavenging in cytosol, mitochondria and peroxisomes. The APX stimulation has also been verified in several plants subjected to Cd, Zn, Cu, Pb and Fe treatment. Increased activities of SOD and APX and other antioxidative enzymes under heavy metals treatments may be considered as circumstantial evidence for tolerance mechanism evolved by the plant species (Patra and Panda, 1998; Prasad *et al.,* 1999). Cicek and Cakirlar (2008) reported that salt treatment decreased remarkable ascorbate peroxidase activity in soybean at the 25^0C.

4.6.2 Superoxide dismutase

One of the major classes of antioxidant enzymes, which protect the cellular and sub cellular components against harmful reactive oxygen species (ROS), is superoxide dismutase (SOD). SODs play pivotal role in scavenging highly reactive free oxygen radicals and protecting cells from toxic effects.

The superoxide dismutase activity of soybean cotyledon varied from 8.68 to 21.52 Units mg^{-1} of protein. The SOD activity of Birsa soya 1 was 20.08 Units mg^{-1} of protein while that of Kalitur was 16.05 Units mg^{-1} of protein. The highest superoxide dismutase activity was recorded in KDS 1025 *i.e.* 21.52 Units mg^{-1} of protein, lowest activity was recorded in KDS 1035 *i.e.* 8.68 Units mg^{-1} of protein (Table 7). The F_3 progenies of the cross (Birsa soya 1 × EC 241780) recorded superoxide dismutase

activity in the range of 8.68 to 21.52 Units mg^{-1} of protein. The SOD activity recorded 19.24 Units mg^{-1} of protein in KDS 1027, 19.23 Units mg^{-1} of protein in KDS 1032 genotypes.

Our results regarding the Hosamani *et al.,* (2013) concluded that superoxide dismutase (SOD) is first in the series of antioxidant system, which mollifies the superoxide radicals (O•), produced during electron transport process, into H_2O_2 and oxygen. It plays an important role in the process of free radical reaction and in seed ageing. Among 33 genotypes, freshly harvested seeds of poor-storer genotypes recorded higher SOD activity (8.629 U/min/g seed) as compared to good-storer genotypes (7.579 U/min/g seed). With the progress of the storage period, SOD activity significantly increased in good-storer genotypes (8.716 U/min/g seed) and decreased drastically in poor-storer genotypes (5.387 U/min/g seed). Monk *et al.,* (1998) reported that superoxide dismutase (SOD) represents a key element of the enzymatic system that protects the plant cell against deleterious peroxidation reactions. SOD activity enhances under a variety of stressful conditions including Cu, Al, Mn, Fe and Zn toxicities (Prasad, 1997) for maintenance of overall defense system of plants subjected to oxidative damage (Slooten *et al.,* 1995).

Table 7. Superoxide dismutase activity in cotyledon of soybean

Sr.No.	Genotype	Superoxide dismutase (Units mg $^{-1}$ of soluble protein min^{-1})
1	Birsa soya1	20.08
2	EC 241780	10.81
3	Kalitur	16.05
4	KDS 344	17.39
5	MAUS 71	16.90
6	DS 228	13.05
7	JS 335	12.17
8	KDS 726	13.43
9	KDS 1032	19.23
10	KDS 1029	17.69
11	KDS 1035	08.68
12	KDS 1034	12.72
13	KDS 1025	21.52
14	KDS 1031	11.70
15	KDS 1038	17.92
16	KDS 1024	10.96
17	KDS 1036	16.25
18	KDS 1027	19.24
	Range	8.68-21.52
	GM	15.76
	SE	0.5773
	CD 5%	1.6559

4.6.3 Catalase

Catalase (E.C. 1.11.1.6) is tetrameric heme containing enzyme with the potential to directly dismutate H_2O_2 into H_2O and O_2 and is indispensable for ROS detoxification during stress conditions.

The catalase activity in cotyledons of soybean genotypes varied from 1.590 to 5.753 µ moles of H_2O_2 decomposed mg^{-1} of protein min^{-1}. The catalase activity in Birsa soya 1 and Kalitur, the two black seed coat soybean genotypes was 4.053 and 5.387 µ moles of H_2O_2 decomposed mg^{-1} of protein min^{-1} respectively. The F_3 progenies of some crosses involving Birsa soya 1 and EC 241780 as parents also exhibited higher catalase activity *viz.*, 5.753 µ moles of H_2O_2 decomposed mg^{-1} of protein min^{-1} in KDS 1038, 5.022 µ moles of H_2O_2 decomposed mg^{-1} of protein min^{-1} in KDS 1025, 4.484 µ moles of H_2O_2 decomposed mg^{-1} of protein min^{-1} in KDS 1027, and 4.426 in KDS 1029 (Table 8).

Catalase (CAT) enzyme is essential for the removal of the potentially toxic H_2O_2 produced under various stress conditions and hence, for the avoidance of oxidative-stress related damage, by breaking hydrogen peroxide molecules to oxygen and water. It is located mainly in glyoxysomes. CAT is an important component of a plant's defense system. Similar to GR, the freshly harvested seeds of poor-storer genotypes recorded higher CAT activity (873.5 µmol/min/g seed) as compared to good-storer genotypes (180.4 µmol/min/g seed) advancement of

Table 8. Catalase activity in cotyledon of soybean

Sr.No.	Genotype	Catalase (μ moles of H_2O_2 decomposed mg $^{-1}$ protein min^{-1})
1	Birsa soya1	4.053
2	EC 241780	3.477
3	Kalitur	5.387
4	KDS 344	4.920
5	MAUS 71	4.020
6	DS 228	3.394
7	JS 335	2.607
8	KDS 726	3.943
9	KDS 1032	3.617
10	KDS 1029	4.426
11	KDS 1035	3.641
12	KDS 1034	4.119
13	KDS 1025	5.022
14	KDS 1031	2.897
15	KDS 1038	5.753
16	KDS 1024	1.590
17	KDS 1036	3.503
18	KDS 1027	4.484
	Range	1.590-5.753
	GM	3.936
	SE	0.6203
	CD 5%	1.7792

storage period, CAT activity significantly increased in good-storer genotypes (289.8 µmol/min/g seed) and decreased drastically in poor-storer genotypes (228.5 µmol/min/g seed) (Hosamani *et al.,* 2013). This result correalats with experiments in *Phaseolus aureus* (Shaw, 1995); *Pisum sativum* (Dalurzo *et al.,* 1997) and *Amaranthus levidus* (Battacharjee, 1998) following the application of Cd to growth medium.

4.6.4 Glutathione reductase

Glutathione reductase (E.C.1.6.4.2) is flavoprotein oxidoreductase found in both prokaryotes and eukaryotes. It is a potential enzyme of the ASH-GSH cycle and plays an essential role in defence against ROS by sustaining the reduced status of GSH.

The Glutathione reductase activity in cotyledons of soybean genotypes and eleven F_3 progenies of crosses ranged between 2.89 to 9.40 nmoles of TNB formed min^{-1} mg^{-1} of protein, with the lowest glutathione reductase activity of 2.89 n moles of TNB formed min^{-1} mg^{-1} of protein in EC 241780 and the highest glutathione reductase activity of 9.40 nmoles of TNB formed min^{-1} mg^{-1} of protein in KDS 1032. The GR activity in Birsa soya1 and Kalitur black seed coat soybean genotypes was 6.23 and 6.83 nmoles of TNB formed min^{-1} mg^{-1} of protein respectively. The F_3 progenies of some crosses involving Birsa soya 1 and EC 241780 as parents also exhibited higher GR activity *viz.,* 9.40 in KDS 1032, 9.05 in KDS 1038, 8.30 in KDS 1036, which was better than either of the parents Birsa soya 1

(6.23 nmoles of TNB formed min^{-1} mg^{-1} of protein) and EC 241780 (2.89 nmoles of TNB formed min^{-1} mg^{-1} of protein) (Table 9). Enzyme glutathione reductase (GR) takes part in the control of endogenous hydrogen peroxide through an oxidoreduction cycle involving glutathione and ascorbate. In freshly harvested seeds of 33 genotypes, the poor storers recorded significantly higher GR activity (4.424 µmol/min/g seed) as compared to good-storer genotypes (1.681 µmol/ min/g seed). As the storage period progressed, GR activity increased significantly in good-storer genotypes (2.134 µmol/min/g seed) on one hand and decreased significantly in poor-storer genotypes (1.807 µmol/min/g seed) (Hosamani *et al.*,2013). An enhanced activity of glutathione reductase in response to increasing concentrations of $HgCl_2$, a result which can often be exacerbated by the addition of GSSG and ameliorated by NADPH has been reported (Halliwell and Foyer, 1978; Serrano *et al.,* 1984). Activation of the ascorbate glutathione cycle has been found to be essential in stressed plants to combat oxidative stress.

4.7 Lipid peroxidation

The peroxidation of lipid is considered as the most damaging process known to occur in every living organisms. The membrane damage is taken as a single parameter to determine the level of lipid destruction under various stresses. During LPO, products are formed that include small hydrocarbon fragment such as ketones, MDA(malondialdehyde) etc. and compound

which react with thiobarbituric acid TBA to form coloured products called Thiobarbituric acid reactive substances

Sr.No.	Genotype	Glutathione reductase (n moles TNB formed $min^{-1}mg^{-1}$ protein)
1	Birsa soya1	6.23
2	EC 241780	2.89
3	Kalitur	6.83
4	KDS 344	5.90
5	MAUS 71	5.83
6	DS 228	6.06
7	JS 335	3.51
8	KDS 726	3.32
9	KDS 1032	9.40
10	KDS 1029	6.82
11	KDS 1035	3.47
12	KDS 1034	4.30
13	KDS 1025	5.00
14	KDS 1031	3.16
15	KDS 1038	9.05
16	KDS 1024	4.32
17	KDS 1036	8.30
18	KDS 1027	6.88
	Range	2.89-9.40
	GM	5.65
	SE	0.04
	CD 5%	0.11

(TBARS).

The level of lipid peroxidation is measured in terms of thiobarbituric acid reactive substance (TBARS) content. The lipid peroxidation content of cotyledon of soybean varied from 20.96 to 24.51 nmoles of MDA gm^{-1} fresh weight. The lipid peroxidation content of Birsa soya 1 was 20.96 nmoles of MDA gm^{-1} fresh weight while that of Kalitur was 22.00 nmoles of MDA gm^{-1} fresh weight. The highest lipid peroxidation 24.51 nmoles of MDA gm^{-1} fresh weight was recorded in KDS1038 (Table 10). The lipid peroxidation in DS 228, a soybean variety having low seed longevity was 24.51 nmoles of MDA gm^{-1} fresh weight. The F_3 progenies of the cross (Birsa soya 1 and EC 241780) recorded lipid peroxidation activity in the range of 22.70 to 23.99 nmoles of MDA gm^{-1} fresh weight with the lowest in KDS 1035 and highest in KDS 1038.

Priestly, (1986), Wilson and McDonald, (1986); Hendry, (1993) suggested that lipid peroxidation and associated free radical oxidative stresses are widely considered to be major contributors to seed deterioration. They affect the structure and function of membranes, including the inactivation of membrane bound proteins and the alteration of membrane permeability. Accumulation of volatile aldehydes during seed ageing, which are released upon hydration, is an indicator of lipid peroxidation [Zhang *et al.*,(1993)]. The level of volatile aldehydes released by the poor-storer genotypes was higher than good-storer genotypes, though it increased in all the genotypes during

Table 10. Lipid peroxidation in cotyledon of soybean

Sr.No.	Genotype	Lipid peroxidation (nmoles malondialdehyde produced g^{-1} fw)
1	Birsa soya1	20.96
2	EC 241780	24.25
3	Kalitur	22.00
4	KDS 344	23.47
5	MAUS 71	24.25
6	DS 228	24.51
7	JS 335	22.67
8	KDS 726	24.51
9	KDS 1032	23.20
10	KDS 1029	23.23
11	KDS 1035	22.70
12	KDS 1034	23.73
13	KDS 1025	23.40
14	KDS 1031	23.21
15	KDS 1038	23.99
16	KDS 1024	23.22
17	KDS 1036	23.73
18	KDS 1027	23.80
	Range	20.96-24.51
	GM	24.56
	SE	0.03
	CD 5%	0.09

ageing. Rudrapal and Nakamura, (1988) reported that exposure slowed deterioration under accelerated and natural ageing of freshly harvested eggplant and radish seeds to halogen vapour fatty acids in membranes making them less prone to peroxidative attack. Lipid peroxidation has been suggested as the cause of loss of sunflower and soybean seed viability (Bailley, *et al.,* 1996; Sung, 1996).

4.8 Identification and validation of candidate markers for seed longevity in soybean

Many reports about the construction of soybean genetic linkage maps using different markers have been published (Cregan *et al.,* 1999). Based on the construction of soybean genetic maps quantitative trait loci (QTL) for a number of agronomic traits in soybean have been mapped (Hyten *et al.,* 2004). A few SSR markers could clearly discriminate between the good and the poor storer *viz.,* Satt371, Satt453, and Satt618 produced specific allelic bands with respect to storability and testa colour.

Total 9 SSR primer either related to seed coat colour and/or storability of soybean seeds *viz.,* Satt371, Satt453, Satt618, Satt400, Satt143, Satt162, Satt286, Satt523, TR were screen to observe the differences in the allelic size of the fragment in parents (Birsa soya 1, Kalitur, DS 228, EC 241780 and F_3 of the crosses Birsa soya 1 × EC 241780). Out of the 9 SSR primer tested Satt371, Satt162, Satt453 and Satt523 shown very distinct allelic size fragment in good (Birsa soya) and poor

storer (DS 228). The amplified allelic fragment also observed in the F_3 progenies of the crosses which exhibited either black, brown seed coat colour and they were also promising based on their biochemical composition and other seed character.

Satt371- This SSR marker has been reported to produce two distinct allelic size fragments 180 and 160bp. The allelic size fragment 180 bp was reported in good storer soybean genotypes with black testa colour and an allelic size fragment of 160 bp was reported to be present in all the 19 yellow testa colour poor storer soybean genotypes.

The genomic DNA extracted from good storer and black seed coat colour genotypes (Birsa soya 1, Kalitur) and yellow seed coat colour with poor seed longevity (EC 241780) along with the three F_3 progenies of the cross (Birsa soya 1 × EC 241780) *viz.,* KDS 1027, KDS 1034, and KDS 1035 were amplified using Satt371 SSR primer. It was observed that an allelic size fragment of around 220 bp was distinctly observed in both birsa soya 1, Kalitur and the same fragment was also present in KDS 1034and KDS 1027.

Satt453- This SSR primer has been reported to produce specific allelic bands with respect to storability and testa colour. Hosamani *et al.,* (2013) as reported a distinct band corresponding to 200 bp was observed in black testa colour soybean genotypes *i.e.* Birsa soya 1 and Kalitur and allelic size fragment of 220 bp was observed in yellow seed coat colour poor storer genotype. The 220 bp size allelic fragment was also seen in

EC 241780 however in the F_3 progenies *i.e.* KDS 1034 and KDS 1027 bands corresponding to both the allelic size were observed.

Satt523 – This SSR primer has been related to black test colour and it was observed that a band corresponding to an allelic size 200 bp was observed in black seed coat colour genotype *i.e.* Birsa soya 1 and band corresponding to allelic size of 180 bp in DS 228 which was yellow testa colour.

Satt162- The SSR primer has been related to black testa colour and it was observed that a band corresponding to an allelic size 180 bp was observed in black seed coat colour genotype *i.e.* Birsa soya 1 and a band corresponding to allelic size of 200bp in DS 228 however Kalitur the other black seed coat colour genotype did exhibit a band correspond to 200 bp. The F_3 progenies of the crosses particularly KDS 1034 and KDS 1027 exhibited a corresponding to 180 bp both are black testa colour. In the brown seed coat colour F_3 progenies *i.e.* KDS 1035 both the fragments *i.e.* 200 and 180 bp were observed. Similarly the same pattern was also observed in EC 241780.

5. SUMMARY AND CONCLUSIONS

The eighteen soybean genotypes comprising seven genotypes and eleven crosses in which black, yellow and some are brown testa color genotypes were analyzed for different biochemical parameters. The genomic DNAs of soybean genotypes with higher seed longevity (Birsa soya 1, Kalitur) and low seed longevity (DS 228) and crosses exhibiting better morphophysiological parameters, strength and biochemical composition were amplified using 9 SSR markers which have been reported to be linked with the seed longevity or seed coat colour.

The results, thus obtained during the investigations are summarized in this section.

5.1 Physical characteristics

5.1.1 Proportion of seed coat

The seed coat proportion of seven genotypes and eleven F_3 progenies of the crosses ranged between 8.85 to 10.85 %. In the F_3 progenies of some crosses involving Birsa soya 1 and EC 241780 as parents, the seed coat proportion ranged between 9.40 to 10.85%. The higher seed coat proportion was recorded in KDS 1032 and lowest in DS 228.

Based on this Birsa soya 1 genotype which is having black testa colour appears to be good storer and DS 228 which is having yellow testa colour appears to be poor storer. The parents

Birsa soya 1 and EC 241780 recorded the seed coat proportion of 10.11 and 9.70% respectively. The F_3 progenies of some crosses involving these two parents when analyzed recorded higher seed coat proportion than Birsa soya 1 and EC 241780 in KDS 1032 (10.85%) and KDS 1038 (10.81%).

5.1.2 Seed volume

The seed volume of seven genotypes and eleven F_3 progenies of the crosses ranged between 0.80 to 0.98 g ml^{-1}. The seed volume of F_3 progenies of some crosses involving Birsa soya 1 and EC 241780 as parents varied between 0.82 to 0.98 g ml^{-1}. The seed volume was higher in EC 241780 and less in KDS 344.

5.1.3 Mechanical strength

The mechanical strength of eighteen soybean genotypes in which seven genotypes and eleven F_3 progenies of the crosses ranged between 72 to 132 Newton. The mechanical strength of eleven F_3 progenies of some crosses involving Birsa soya 1 and EC 241780 as parents varied between 76 to 132 Newton. The F_3 progenies of the crosses recorded higher mechanical strength than the parents in KDS 1034(132 N) and KDS 1025 (128 N). The higher mechanical strength recorded in KDS 1034 and less in DS 228.

Based on this KDS 1034 F_3 progeny genotype which is having black testa colour appears to be good storer and DS 228 which is having yellow testa colour appears to be poor storer.

5.1.4 Electrical conductivity

The electrical conductivity of seven genotypes and eleven F_3 progenies of the crosses ranged between 0.30 to 0.63 dSm^{-1}. The electrical conductivity in Birsa soy 1 and Kalitur, a two black seed coat genotypes was 0.30 and 0.45 dSm^{-1} respectively. The higher electrical conductivity was recorded in EC 241780 and less in Birsa soya1. Based on this Birsa soya 1 which is having black testa colour, high seed longevity appears to be better storer and EC 241780 poor storer with low seed longevity.

5.1.5 Seed permeability

The seed permeability of seven genotypes and eleven F_3 progenies of the crosses ranged between 30 to 97. The seed permeability of Birsa soya 1 and Kalitur, the two black seed coat soybean genotypes was 57 and 81% respectively. The higher seed permeability was recorded in KDS 1038 and less in KDS 1034.

5.2 Oil content

The oil content of the eighteen soybean genotypes in which seven genotypes and eleven F_3 progenies of the crosses ranged between 17.3 to 20.9 %. The oil content of the F_3 progenies of some crosses involving Birsa soya 1 and EC 241780 as parents varied between 18.00 to 20.90 %. The higher oil content was recorded in KDS 1024 and less in MAUS 71. Based on this MAUS 71 which having low seed longevity appears to be poor storer.

5.3 Vitamin E content

The tocopherol content in the seed of seven genotypes and eleven F_3 progenies of the crosses ranged between 18.66 to 41.03 µg ml^{-1} of oil. The vitamin E content of Birsa soya 1 and Kalitur, the two black seed coat soybean genotypes was 35.74 and 34.74 µg ml^{-1} of oil. The higher vitamin E content was recorded in KDS 1034 and less in KDS 1024. Based on this KDS 1034 genotype which is having black testa colour appears to be better storer.

5.4 Lignin content

The lignin content in the seed coat of seven genotypes and eleven F_3 progenies of the crosses ranged between 4.6 to 16 %. The lignin content of the F_3 progenies of some crosses involving Birsa soya 1 and EC 241780 as parents varied between 5.12 to 16 %. The higher lignin content was recorded in the KDS 1034 which is having black testa colour appears to be better storer. The lignin content in seed coat of Birsa soya 1 and Kalitur, a black seed coat genotypes was 15.01 and 12.30 %. The parents Birsa soya 1 and EC 241780 recorded the lignin content of 15.01 and 8.70% respectively. The F_3 progenies of some crosses involving these two parents when analyzed recorded higher lignin content than Birsa soya 1 and EC 241780 in KDS 1034 (16%) and KDS 1035 (15.80%).

5.5 Calcium content

The calcium content of the seed coat of soybean seven genotypes and eleven F_3 progenies of the crosses ranged between 2079 to 2688 ppm. The calcium content of Birsa soya 1 and Kalitur, a two black seed coat colour soybean genotype was recorded 2639 and 2305 ppm respectively. Birsa soya 1 and Kalitur good storer genotypes. The higher calcium content was recorded in KDS 1038 and less in KDS 1029. Based on this KDS 1038 genotype is better storer which is black testa colour and better seed longevity. The parents Birsa soya 1 and EC 241780 recorded the calcium content of 2639 and 2305 ppm respectively. The F_3 progenies of some crosses involving these two parents when analyzed recorded higher calcium content than Birsa soya 1 and EC 241780 in KDS 1038 (2688 ppm).

5.6 Antioxidative enzymes

5.6.1 Ascorbate peroxidase

The ascorbate peroxidase activity in the cotyledon of soybean of seven genotypes and eleven F_3 progenies of the crosses ranged between 0.166 to 0.786 µ mole ascorbate oxidized mg^{-1} of soluble protein min^{-1}. The ascorbate peroxidase activity of the F_3 progenies of some crosses involving Birsa soya 1 and EC 241780 as parents varied between 0.166 to 0.764 µ mole ascorbate oxidized mg^{-1} of soluble protein min^{-1}. The higher ascorbate peroxidase activity was recorded in KDS 344 (Phule Agrani) which is recently released genotype of soybean. The

higher ascorbate peroxidase activity was recorded in black testa colour genotypes as compared to yellow testa colour genotypes.

5.6.2 Superoxide dismutase

The superoxide dismutase activity in the cotyledon of soybean of seven genotypes and eleven F_3 progenies of the crosses ranged between 8.68 to 21.52 Units mg^{-1} of protein. The higher superoxide dismutase activity was recorded in KDS 1025 and less in KDS 1035. The superoxide dismutase activity Birsa soya 1 and Kalitur, a two black seed coat genotypes *i.e.* 20.08 and 16.05 Units mg^{-1} of protein respectively. Based on this KDS 1025 which is having brown seed coat colour appears to be better storer.

5.6.3 Catalase

The catalase activity in the cotyledon of soybean of seven genotypes and eleven F_3 progenies of the crosses ranged between 1.590 to 5.753 µ moles of H_2O_2 decomposed mg^{-1} of protein min^{-1}. The catalase activity in the F_3 progenies of some crosses involving Birsa soya 1 and EC 241780 as parents varied between 1.590 to 5.753 µ moles of H_2O_2 decomposed mg^{-1} of protein min^{-1}. The catalase activity Birsa soya 1 and Kalitur, a two black seed coat genotypes was 4.053 and 5.387 µ moles of H_2O_2 decomposed mg^{-1} of protein min^{-1}. The higher catalase activity was recorded in the KDS 1038 and less in KDS 1024. Based on this the cross KDS 1038 which is having black testa colour appears to be better storer.

5.6.4 Glutathione reductase

The glutathione reductase activity in the cotyledon of soybean of seven genotypes and eleven F_3 progenies of the crosses ranged between 2.89 to 9.40 nmoles of TNB formed min^{-1} mg^{-1} of protein. The glutathione reductase activity of the F_3 progenies of some crosses involving Birsa soya 1 and EC 241780 as parents varied between 3.16 to 9.40 nmoles of TNB formed min^{-1} mg^{-1} of protein. The higher glutathione reductase activity was recorded in the KDS 1032 and less in EC 241780. The glutathione reductase activity Birsa soya 1 and Kalitur, a two black seed coat genotypes *i.e.* 6.23 and 6.83 nmoles of TNB formed min^{-1} mg^{-1} of protein respectively. Based on this KDS 1032 cross which is having black testa colour appears to be better storer and EC 241780 which is having yellow testa colour appears to be poor storer.

5.7 Lipid peroxidation

The lipid peroxidation rate in the cotyledon of soybean seven genotypes and eleven F_3 progenies of the crosses ranged between 20.96 to 24.51 nmole malondialdehyde produced g^{-1} of fresh weight. The lipid peroxidation rate of the F_3 progenies of some crosses involving Birsa soya 1 and EC 241780 as parents varied between 22.70 to 24.51 nmole malondialdehyde produced g^{-1} of fresh weight. The significantly more lipid peroxidation rate was recorded in yellow testa colour genotype *i.e.* DS 228 which is poor storer, while lowest lipid peroxidation rate was recorded in

black testa colour genotypes and also in F_3 progenies exhibiting black or brown seed coat colour.

5.8 Identification and validation of candidate markers for seed longevity in soybean

The Satt371 SSR primer produced an allelic size fragment of around 200 bp which was distinctly observed in both Birsa soya 1 and Kalitur and the same fragment was also present in KDS 1027, KDS 1034. The F_3 progenies of the crosses particularly KDS 1034 and KDS 1027 exhibited a 180 bp allelic size with Satt162 primer which are both black testa colour crosses. In the brown seed coat colour F_3 progenies KDS 1035 both the fragments *i.e.* 200 and 180 bp were observed. Similarly the same pattern was also observed in EC 241780.

Conclusion

Amongst eighteen genotypes and crosses of soybean, the cross KDS 1034 exhibited higher mechanical strength, vitamin. E content, lignin content and calcium content. The KDS 1034 reported an allelic size band of 180 bp, which also reported in black seed coat colour genotype *i.e.* Birsa soya 1 with Satt162 primer and also in Satt523 primer. The cross KDS 1038 exhibited higher activity of catalase, ranked second in respect of seed coat proportion, APX and glutathione reductase. The KDS 1038 contain higher calcium content and showed relatively less lipid peroxidation rate. Based on this the crosses KDS 1034 and KDS 1038 having black and brown testa colour respectively may be utilized as good storer with better seed longevity genotypes.

6. Literature Cited

Agrawal, PK., Dadlani M (eds) (1992). Techniques in Seed Science and Technology. South Asian Publisher. 114–120.

Agrawal, P.K. and Menon, K. (1974). Lignin content and seed coat thickness in relation to seed coat cracking. Seed Research. 2 : 64-66.

Allen, RD., Webb RP., and Schake SA (1997). Use of transgenic plants to Study antioxidant defenses. Free Radical, Biol. Med. 23: 472-479.

Alscher, RG., Dooahoe JL., Cramer Cl (1997) Reactive oxygen species and antioxidants: relationships in green cells. Plant Physiol. 100: 224-233.

Alvarez, P.J.C., Krzyzanowski, F.C., Mandrino, J.M.G.,Franca-Neto, J.B. 1997. Relationship between soybean seed coat lignin content and resistance to mechanical damage. Seed Science and Technology, Zurich. 25 : 209-214.

Anonymous, (1996). International Rules for Seed Testing. *Seed Sci. Technol.* 29(Supl.):1-335.

Asada, K. 1992. Ascorbate peroxidase - A hydrogen peroxide scavenging enzyme in plants. Physiol. Plantarum. 85 : 235-241.

Asada, K (1999). The water-water cycle in chloroplasts: Scavenging of activeoxygen and dissipation of excess

photons. Annu. Rev. Plant Physiol. Plant Mol Biol. 50: 601.

Arechavaleta-Medina F, Snyder HE.(1981). Water imbibitions by normal and hard soybeans. Journal of American Oil Chemical Society: 976-979.

Aver'yanov, A.A., and Lapikova V.P. (1994). Active oxygen and fungitoxicity of plant phenols. In: *Natural Phenols in Plantn Resistance. Acta Horticulturae.*, Wageningen, I nt. Soc. for Horticultural Sci. pp. 336-339.

Bailly, C., Benamar, A., Corbineau, F. and Come, D. (1996). Changes in malondialdehyde content and in superoxide dismutase, catalase and glutathione reductase activities in sunflower seeds as related deterioration during accelerated ageing. Physiol. Planta. 97:104–110.

Bailly, C., Bogatek-Leszczynska R., Come D., and Corbineau F. (2002). Changes in activities of antiox-idant enzymes and lipoxygenase during growth of sunflower seedlings from seeds of different vigour. Seed Sci Res .12:47–55.

Bailly, C., El-Maarouf-Bouteau H.,Corbineau F.(2008). From intracellular Signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. C RBiol . 331:806–814.

Bailly, C., Kranner I. (2011). Analyses of reactive oxygen species and antioxidants in relation to seed longevity and germination. Methods Mol Biol. 773:343–367.

Baskin, J.M., Baskin C.C., and Li X. (2000). Taxonomy, anatomy and evolution of physical dormancy. Plant Species Biology. 15: 139–152.

Battacharjee, S. (1998). Membrane lipid peroxidation, free radical scavengers and ethylene evolution in Amaranthus as affected by lead and cadmium. Biol.Plant. 40: 131-135.

Bellani, LM., Salvini L., Dell'Aquila A., and Scialabba A. (2012). Reactive oxygen species release, vita-min E, fatty acid and phytosterol contents of artificially aged radish (*Raphanus sativus* L.) seeds during germination. Acta Physiol Plant .34:1789–1799.

Benites, C. I., Reis S. M. P. M., and Oliveira A. O. (2005). Avaliação de métodos deneutralização do destilado da desodorização do óleo de soja (DDOS). In: II Simpósio Internacional Tendências eInovaçõesemTecnologia de Óleos e Gorduras. Florianópolis/SC. SociedadeBrasileira de Óleos eGorduras. p.25-28.

Bewley, JD, Black M. (1994). Seeds: physiology of development and germination. New York: Plenum Press. 147–197.

Beyer, W.F. Jr. and Fridovich, I. (1937). Assaying for superoxide dismutase activity; some large consequences of minor changes in conditions Anal Biochem. 161: 559-566.

Borher, J. R. Z., Gonçalves L. A. G., and Felício P. E. (2002). α-andγ-tocopherol levels in Nelore steer blood plasma

after a single oral treatment of soybean oil deodorizer distillate (SODD).Meat Science, Oxford . 61, p. 301-306.

Bowler, C., Van Montagu M., and Inze D. (1992). Superoxide dismutase and stress tolerance. Annu Rev Plant Physiol Plant Mol. Biol. 43: 83–116.

Caballero Avarez , P J . (1994). Relacao entre o conteu-do de lignin no tegumento da semente de soja e Suarelacaoaodano mecanico. Londrina : UEL/EMBRAPA/IAPAR,.43p. Tese de Mestrado.

Campbell, M.M. and Sederoff, R. (1996). Variation in lignin content and composition : Mechanisms of control and implications for the genetic improvement of plants. Plant Physiol. 110 : 3-13.

Carbonell, S.A.M.and Krzyzanowsky F. C.(1995).The pendulum test for screening soybean genotypes for seeds resistant mechanical damage. Seed Sci Tech. 23: 331-339.

Carlson,J.B.(1973).Morphology.In*Soybeans:Improvement,production, and uses*, B. E. Caldwell, ed., 16. Madison, WI: American Society of Agronomy.

Chachalis D, Smith ML. (2000). Imbibition behavior of soybean [*Glycine max* (L.) Merr.]. accessions with different testa characteristics. Seed Sci. Technol. 28: 321–331.

Chachalis, D., Smith ML. (2001). Seed coat regulation of water uptake during imbibition in soybean [*Glycine max* (L.) Merr.]. Seed Sci. Technol. 29: 401–412.

Chaoui, A., Mazhoudi S., Ghorbal M.H., and El Ferjani E (1997). Cadmium and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities inbean (*Phaseolus vulgaris* L.). Plant Sci. 127:139-147.

Chen, P., Buss G.R., and Diehl K.C. (1993). Physical and chemical characteristics associated with hardness of small-seeded soybean for natto. ASACSSA- SSSA International Annual Meetings.

Chen, P., Huhn M.R., Buss G.R., Gunduz I., and Wilson J.H. (2001). Interrelationship among seed quality traits of specialty soybeans for natto and tofu. ASACSSA-SSSA International Annual Meetings.

Cicek, N and Cakirlar, H. (2008). Changes in some antioxidant enzyme activities in six soybean cultivars in response to long term salinity of two different temperature. Gen. Appl. Physiol. 34:267 280.

Cregan, P. B., Jarvik T., Bush A. L., Shoemaker R.C., Lark K. G.,Kahler A. L., Kaya N., Vantoai T. T.,Lohnes D. G., Chung J. and Specht J. E. (1999). An integrated genetic linkage map of the soybean genome. Crop Sci. 39: 1464-1490.

Dalurzo, H.C., Sandalio L.M., Gomez M., and Rio del L.A. (1997). Cadmium in filteration of detached pea leaves: Effect

on its activated oxygen metabolism. Phyton Ann Rei Bot. 37: 59-64.

Dassou, S., and E. A. Kueneman. (1984). Screening methodology for resistance to field weathering in soybean seed. *Crop Science.* 24:774–778.

De, GreytW., and Kellens, M. (2000). Refining practice. In:Edible Oil Processing. Hamm W., Hamilton R. J. ed. Danvers: Blackwell. 281p.

De Paula, M; M Perez-Otaola, M Darder, M Torres, G Frutos, C J Martinez- Honduvilla. (1996). Function of ascorbate-glutathine cycle in aged sunflower seeds. Physiol Plant 96: 543-550.

Dhakal M. R. and Pandey A.K.(2001).Storage potential of niger (Guizotia abyssinica Cass.) seeds under ambient conditions. Seed Sci. Tech.,29(1), pp.205-213.

Dhindsa, R.S., Plumb-Dhindsa, P. and Thorpe, T.A. (1981). Leaf senescence : Correlated with increased levels of membrane permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase. Journal of Experimental Botany. 32 : 93-101.

Fraczek T H., slipek Z and kurpaska S . (2005). Effect of seed coat thickness on seed hardness.Canadian Biosynthesis Engineering 47.

Franca neto, J.B.; Henning, A.A. (1984). Qualidades fisiologica e sanitaria de sements de soja. Londrina:Embrapa-cnpso. 39 p. (EMBRAPA-CNPSo. Circular Tecnica, 9).

Francisco Dübbern, H. De Souza and Marcos-Filho J. (2001).Seed Coat as a Modulator of Seed-Environment Relationships in Fabaceae. Rev. bras. Bot. São Paulo Dec. 24(4).

Fornazier R.F., Ferreira R.R., Vitoria A.P., Molina S.M.G., Lea P.J., and Azevedo R.A (2002). Effects of cadmium on antioxidant enzymes in sugar cane. Biol.Plantarum. 45: 91-97.

Gille, J. J. P. and Joenje, H. (1991). Biological significance of oxygen toxicity: An introduction. In :*Membrane Lipid Oxidation* (ed. C. Vigo-Pelfrey), pp. 1-32, CRC Press, Boca Raton.

Goel, A., Goel A.K., and Sheoran I.S. (2003). Changes in oxidative stress enzymes during artificial ageing in cotton (*Gossypium hirsutum* L.) seeds. J Plant Physiol.160:1093–100.

Gupta, P.C., Miller, D.A. ; Hittle, C.N. (1973). Note on the effect of threshing on seed damage, seed vigor and germination in two soybean varieties. Indian Journal of Agricultural Sci. n. 43, p.617-618.

Hampton, J .G and Tekrony D. M . (1995). Handbook of vigour test methods. 3rd ed., 1995. International seed testing association Zurich:117pp.

Halliwell, B., and Foyer CH (1978). Properties and physiological function of a glutathione reductase purified from

spinach leaves by affinity chromatography.Planta. 139: 9-17.

Heath, R.L. and Packer L. (1968). Photoperoxidation in isolated chloroplasts : kinetics and stoichiometry of fatty acid peroxidation. Arch BiochemBiophys .125:189–198.

Hendry, G.A.F. (1993). Oxygen, free radical processes and seed longevity. Seed Sci. Res., 3:141-153.

Hitomi, K. and Yoshiharu, S. (1998). Influence of phytate removal and structure modification on calcium binding properties of soybean globulins. Bio sci Biotechnol. Biochem.62 :341-346.

Hosamani, J., Dadlani. M, Santha. I. M., and Kumar M.B. (2013). Biochemical phenotyping of genotypes to establish the role of lipid peroxidation and antioxidant enzyme in seed longevity. Agric Res. 2(2):119-126.

Hosamani, J., Kumar M.B.A., Talukdar A., Lal S. K., and Dadlani M.(2013). Molecular characterization and identification of candidate markers for seed longevity in soybean [*Glycine max* (L.) Merill]. Indian J. Genet. 73(1):64-71.

Hoy, D. J., and E. E. Gamble. (1985). The effect of seed size and seed density on germination and vigour in soybeans (*Glycine max* (L.) Merrill.). *Canadian Journal of Plant Sci.* 65:1–8.

Hsu, J. L.; Sung J. M. (1997). Antioxidants role of glutathione associated with accelerated ageing and hydration of triploid watermelon seeds. Physiol Plant 100: 967-974.

Hu, D., Ma G., Wang Q., Yao J., Wang Y., Pritchard H.W, *et al.* (2012). Spatial and temporal nature of reactive oxygen species production and programmed cell death in elm (UlmuspumilaL.) seeds during controlled deterioration. Plant Cell Environ. 35:2045–2059.

Huang, C. L. and E.E. Shuttle.(1985). Digestion of plant tissue analysis by ICP emission spectroscopy. Commun. Soil. Sci. Plant Anal. 16: 943-958.

Hussain, M. A., Huq, M. E., Rahman S. M. and Zakaria A ,(2002). Estimation of Lignin in Jute by Titration Method *Pakistan Journal of Biological Sciences. 5: 521-522.*

Hyten, D. L., Pantalone V. R., Sams C. E., Saxton A.M., Landau-Ellis D., Stefaniak T. R. and Schmidt M.E. (2004). Seed quality QTL in a prominent soybean population. Theor. Appl. Genet. 109: 552-561.

Jin, D.H. and Yizhen, Z. (2000). Inhibitory effect of hydrolysates on calcium carbonate Crystallization. J. agric. Food Chem. 48:5450-5454.

Kaewnaree, P., Vichitphan S., Klanrit P., Siri B., and Vichitphan K. (2011). Effects of accelerated aging process on seed quality and biochemical changes in sweet pepper (*Capsicum annuum* Linn.) seeds. Biotech. 10:175–182.

Kalpana, R. and Madhava Rao, K.V.(1994). Absense of the role of lipid peroxidation during accelerated ageing of seeds of pigean pea (*Cajanas cajan* [L.] Millsp.)-Seed sci. Technol.22:253-260.

Keim, P., Olsen, T.C. and Shoe Maker, R.C. (1988). A rapid protocol for isolating soybean DNA. Soybean Genet Newslett. 15:150–152.

Khanna-Chopra, R.and Selote, D. S. (2006). Acclimation drought stress generates oxidative stress tolerance in drought resistant than susceptible wheat cultivars under field condition. Environ. Expt. Bot. 60 : 276-283.

Kibinza, S., Vinel D., Côme D., Bailly C., and Corbineau F.(2006). Sunflower seed deterioration as related to moisture content during aging, energy metabolism and active oxygen species scavenging. Physiol Plant.128:496–506.

Krzyzanowski, F. C. (1998). Relationship between seed technology research and federal plant breeding programs. Sci. agric.special issue Piracicaba.Print version ISSN01039016.l.scientia@esalq.usp.br.

Krzyzanowski, F.C., Fraca-Neto, J.B., Mandarino, J.M.G. and Kaster, M. (2001). Comparison between two gravimetric methods to determine the lignin content in soybean seed coat. Seed Sci. Technol, Zurich. 29 : 619-624.

Kuchlan, M.K. (2006). Identification of physical physiological and biochemical factors and molecular analysis for longevity of soybean seeds. PhD Thesis, Division of Seed Science and Technology, IARI, New Delhi.

Kuchlan, M.K., Dadlani M. and Samuel D.V.K. (2010). Seed coat properties and longevity of soybean seeds. Journal of New seeds. 11:239-249.

Larson, R. A. (1997). *Naturally Occurring Antioxidants.* Lewis Publ.,Boca Raton.

Leffel, B.C. and Rhodes, W.K. (1993). Agronomic performance and economic value of high-seed-protein soybean. J. Prod. Agrie. 6: 365-368.

Liu, K., Grthoefer, F. and Brown, E.A. (1995). Association of seed size with genotypic variation in the chemical constituents of soybeans. J. Am. Gil Chem.Soc. 72:189-192.

Loeffler, T.M., Tekrony D.M., and Egli D.B (1988). The bulk conductivity test as an indicator of soybean seed quality. J Seed Tech. 12:37–53.

Lu, X.X., Chen X.L., and Guo Y.H. (2005). Seed germinability of 23 crop species after a decade of storage in the National Gene bank of China. Agric. Sci China. 4:408–412.

Lowry, H. O., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with folin phenol reagent J. Biol Chem. pp 265-275.

Luck, H. and Silinger, A. (1947). Catalase in methods of enzymatic analysis, Z. Lebensmith University. pp. 108-341.

Lush, W.M., and Evans L.T. (1980). The seed coats of cowpea and other grain legumes: structure in relation to function. Field Crops Research. 3:267–286.

Ma, F., Cholewa E., Mohamed T., Peterson C.A., and Gijzen M. (2004). Cracks in the palisade cuticle of soybean seed coats correlate with their permeability to water. Annals of Botany. 94: 213–228.

MacRae, E.A., and Fergusam I.B (1985). Changes in catalase activity and H_2O_2 concentration in plants in response to low temp. Plant Physiol. 65: 51-56.

Matsumura, H., Nirasawa, S., Kiba, A. and Urasaki, N. (2003). Over expression of Bax inhibitor suppresses the fungal elicitor-induced cell death in rice (*Oryza sativa* L.) Cells.Plant Journal. 33: 425-434.

Mentreddy, S.R., Mohammed, A.I. and Joshee, N. (2002).Edamame : A nutritious vegetable crop. In J. Janick and A. Whipkey (Eds.), Trends in new crops and new uses Alexandria, V.A : ASHS Press. pp. 432–438.

Monk, L.S., Fogerstedt K.V., R.M.M (1998). Oxygen toxicity and SOD as an antioxidant in physiological stress. Plant Physiol. 76: 456-459.

Moussa, H.R (2005). Effect of cadmium on growth and oxidative metabolismof faba bean plants. ActaAgron. Hung 269-276.

Nakano, Y. and Asada, K. (1981). Hydrogen peroxide is scavenged by ascorbic acid specific peroxidase in spinach chloroplast. Plant Cell Physiol. 22 : 867.

Oracz, K., El-Maarouf-Bouteau H., Kranner I., Bogatek R., Corbineau F., and Bailly C. (2009). The mechanisms involved in seed dormancy alleviation by hydrogen cyanideunravel the role of reactive oxygen species as key factors of cellular signaling during germination. Plant Physiol. 150:494–505.

Panse, V .G. and Sukhatme, P. V. (1985). Statistical methods for agricultural workers. ICAR publication New Delhi.

Parish, D.J. and Leopold, A.C. (1978). On mechanism of aging in soybean seeds. Plant Physiol. 1978: 365-368.

Passardi, F., Bakalovic, N., Teixeira, F.K., Margis-Pinheiro, M., Penel, C. and Dunand, C. (2007). Prokaryotic origins of the non animal peroxidase super family and organelle-mediated transmission to eukaryotes. Genomics. 89 : 567-579.

Patra, J., and Panda B.B (1998). A comparison of biochemical responses to oxidative and metal stress in seedlings of barley *Hordeum vulgare* L. Environ Pollut. 101: 99-105.

Prasad, KVSK., Saradhi P.P., and Sharmila P. (1999). Concerted action of antioxidant enzymes and curtailed growth under zinc toxicity in *Brassica juncea*. Environ.Exp. Bot. 42: 1-10.

Prasad, T.K. (1997). Role of catalase in inducing chilling tolerance in pre- emergence maize seedlings. Plant Physiol. 114, 1369-1376.

Pereira, L. A. G., and C. H. Andrew. (1985). Comparison of non- wrinkled and wrinkled soybean seed coats by scanning electron microscopy. *Seed Sci Technol.* 13:853–859.

Prasad, M.N.V. (1997). Trace metals In: Prasad MNV (Ed) Plant Ecophysiol, wiley, New York, 207-249.

Presley, J. T. (1958). Relations of protoplast permeability of cotton seed viability and pre-deposition of disease. Pl. Disease Report.42: 582.

Priestley, D.A. (1986). Seed aging. Comstock Publishing Associates, Ithaca, NY.

Priestley, D. A., Cullinan, V. I. and Wolfe, J. (1985). Difference in seed longevity at species level. Pl. Cell Environ. 8: 557-562.

Pukacka, S., and Ratajczak E. (2006). Antioxidative response of ascorbate–glutathione pathway enzymes and metabolites to desiccation of recalcitrant *Acer saccharinum* seeds.J Plant Physiol.163:1259–1266.

Pukacka, S., and Ratajczak E. (2005). Production and scavenging of reactive oxygen species in Fagussylvatica seeds during storage at varied temperature and humidity. J Plant Physiol. 162:873–885.

Puntarulo, S., Galleano, M., Sanchez, R.A. and Boveris, A. (1991). Superoxide anion and hydrogen peroxide metabolism in soybean embryonic axes during germination. Biochem. Biophy.Acta.1074 : 277-283.

Rajjou, L., Lovigny Y., Groot SP., Belghazi M., Job C., and Job D. (2008). Proteome-wide characteri-zation of seed aging in Arabiadopsis. A comparison between artificial and natural aging protocols. Plant Physiol.148:620–641.

Raut, V. M., Taware S.P. and Halvankar G. B. (1998). Evaluation of soybean germplasm for seed oil content. Soybean Genetics Newslett. 25:71-72.

Rolston, M.P. (1978). Water impermeable seed dormancy. Botanical Review. 44: 365–396.

Rudrapal , D., and S. Nakamura. (1988). Use of halogens in controlling egg-plant and radish seed deterioration. Seed Sci. Technol. 16:115-121.

Sadasivam, M. and Manickam, A. (1996) . Biochemical methods , New age International Publishers. 185, 193.

Sandalio, L.M., Dalura H.C., Gomez M., Romero-Puertas M.C., and del Rio L.A (2001). Cadmium-induced changes in the growth and oxidative metabolism of pea plants. J Exp Bot. 52: 2115-2126.

Saio, K., Arai K., and Watanabe T. (1973). Fine structure of soybean seed coat and its changes on cooking. Cereal Sci. Today. 18:197–201.

Saio, K. (1976). Soybean resistant to water absorption. Cereal Foods World. 21:168–173.

Sen Gupta, A., Heinen, J., Holaday, A.S., Burte, J.J. and Allen, R.D. (1993). Increased resistance to oxidative stress in transgenic plants that over express chloroplastic Cu/Zn supeoxide dismutase. Proc. Natl. Acad Sci. 90 : 1629-1633.

Seneratna, T.G., J.F. Gusse., and B.D. Mckerise. (1988). Age-induced changes in cellular membranes of imbibed soybean axes. Physiol. Plant. 73:85-91.

Serrano, A., Rivas J., and Losada M. (1984). Purification and properties of glutathione reductase from the Cyanobacterium anabaena sp. Strain 7119. J. Bacteriol. 158: 317-324.

Shah, K., Kumar R.G., Verma S., and Dubey R.S (2001). Effect of cadmium on lipid peroxidation, superoxide anion generation and activities of antioxidant enzymes in growing rice seedling. Plant Sci. 161: 1135-1144.

Shaw, B.P (1995). Effects of Hg and Cd on the activities of antioxidative enzymes in the seedlings of *Phaseolus aureus*. Biol. Plant. 37: 587-596.

Shim, I.S., Momose Y., Yamamoto A., Kim D.W., and Usui K (2003). Inhibition ofcatalase activity by oxidative stress and its relationship to salicylic acid accumulation in plants. Plant Growth Regul. 39: 285-292.

Shigeoka, S., Ishikawa, T., Tamoi, M., Miyagawa, Y., Takeda, T., Yabuta, Y. and Yoshimura, K. (2002). Regulation and function of ascorbate peroxidase isoenzymes. J. Bot. 53 : 1305-1319.

Singh, R. K., Raipuria R. K., Bhatia V. S., Rani A., PushpendraT., Husain S. M., Satyavathi C. T., Chauhan G. S. and Mohapatra T.(2008). Identificationof SSR markers associated with seed coat permeability and electrolyte leaching in soybean.Physiol. Mol. Biol. Plants. 14: 173-177.

Slooten, L., Capiau K., Van Montago W., Sybesma M.C., and Inze D.(1995). Factors affecting the enhancement of oxidative stress tolerance in transgenic tobaccoover

expressing Mn-SOD in the chloroplast. Plant Physiol. 107: 737-750.

Somashekaraiah, SV., Padmaja K., and Prasad ARK. (1992). Phytotoxicity of cadmium ions on germinating seedlings of mung bean (*Phaseolus vulgaris*):Involvement of lipid peroxidation in chlorophyll degradation. Plant Physiol. 85:85-89.

Sosnowski, S., and Kuzniar P. (1999). Effect of Dynamic Loading on the quality of Soybean.Department of Agricultural Product. Int. Agrophysics. 13: 125-132.

Srivastava, A. K. (1976). Physiological studies on soybean seed viability during storage and its practical applicability. Seed Res. 4(1): 56-61.

Sung, J.M. (1996). Lipid peroxidation and peroxide-scavenging in soybean seeds during aging. Physiol Plant. 97:85–89.

Sveinsdóttir, H., Yan F., Zhu Y., Peiter-Volk T., and Schubert S. (2009). Seed ageing-induced inhibition of germination and post-germination root growth is related to lower activity of plasma membrane H+-ATPase in maize roots. J Plant Physiol.166:128–135.

Teixeira, F.K., Menezes-Benavente, L., Galvão, V.C., Margis, R. and Margis-Pinheiro, M. (2006). Rice ascorbate peroxidase gene family encodes functionally diverse isoforms localized in different subcellular compartments. Planta. 224 : 300-314.

Thimmaiah, S. R., (2004). Estimation of phenolics, Standard methods of biochemical analysis (eds), New Delhi, India: Kalyani Publisher.pp.287-302.

Tran, V.N., and Cavanagh A.K. (1984). Structural aspects of dormancy. In: Murray D.R, ed. Seed physiology, . Germination and reserve mobilization. Sydney: Academic Press. 1–44.

Tyler, J.M. (1997). Effect of impermeable seed coat on germination of seed for early maturing soybean. Seed Technology. 19: 45–50.

Vaglio, A., and Landriscina C (1999). Changes in liver enzyme activity in the teleost *Sparusaurata* in response to cadmium in toxication Ecotoxicol. Environ.Safety. 43: 111-116.

Ventelon-Debout, M., Delalande, F., Brizard, J.P., Diemer, H., Vandorsselaer, A. and Brugidou, C. (2004). Proteome analysis of cultivar specific deregulations of *Oryza sativa* indica and *Sativa japonica* cellular suspensions undergoing rice yellow mottle virus infection. Proteomics. 4 : 216-225.

Verma, V. D. and Ram H. H.(1986). Heritability estimates for seed quality traits in soybeans. Soybean Genetics News lett. 13: 67-70.

Vyas, R.P., Kumar R., Prakash V., and Katiyar R.P (1990). Germinability of soybean seeds after harvest in subsequent storage. Seed Res. 18:44–46.

Walters, C., Wheeler L.M., and Stanwood P.C. (2004). Longevity of cryogenically stored seeds. Cry-obiology. 48:229–244.

Wilson, D.O., and McDonald M.B (1986). A convenient volatile aldehydes assay for measuring seed vigour. Seed Sci. Technol. 14:269–271.

Wilson, R.F., Weissinger, H.H., Buck, J.A. and Faulkner, G.D. (1980). Involvement of phospholipids in polyunsaturated fatty acid synthesis in developing soybean cotyledons. Plant Physiol. 66: 545-549.

Wolf, W. J., F. L. Baker, and R. L. Bernard. (1981). Soybean seed coat structural features: Pits, deposits and cracks. *Scanning Electron Microscopy.* 3:531–534.

Yao, Z., Liu L., Gao F., Rampitsch C., Reinecke D.M., and Ozga JA, *et al.*, (2012). Developmental and seed aging mediated regulation of antioxidative genes and differential expres-sion of proteins during pre- and post-germinative phases in pea. Plant Physiol. 169:1477–88.

Zhang M, Lui Y, Tori I, Sasaki H, and Esashi Y (1993). Evolution of volatile compounds by seeds during storage periods. Seed Sci Tech 21:359–373.

Zoroski, R.J. and Burau R.G. (1997). Rapid nitric acid digestion method for multi elements tissue analysis, Soil Sci. Plant analysis . 2: pa 425-436.

7. VITA

Miss. PRIYANKA VISHWNATH PAWAR

A candidate for the degree

of

MASTER OF SCIENCE (AGRICULTURE)

in

BIOCHEMISTRY

2015

Title of Thesis	□	Biochemical analysis concerning seed longevity in soybean [*Glycine max* (L.) Merill]
Major field	□	Biochemistry
Biographical information:		
Personal	□	Born at Charegaon, Tal.Karad, Dist. Satara on 17th june, 1992.
		Daughter of Sau. Meena and Shri. Vishwnath Kundlik Pawar.
Educational	□	Passed S.S.C. from Bharat Vidya Mandir, Kodoli, Satara.
	□	Passed H.S.C. from Yashavantrao Chavan Institute of Science College, Satara.
	□	Passed B. Sc. (Agri) degree from College of Agriculture Kolhapur.
Address	□	A/P-Charegaon, Tal.Karad, Dist.

Satara.

Email:priyankavpawar1992@rediffmail.com

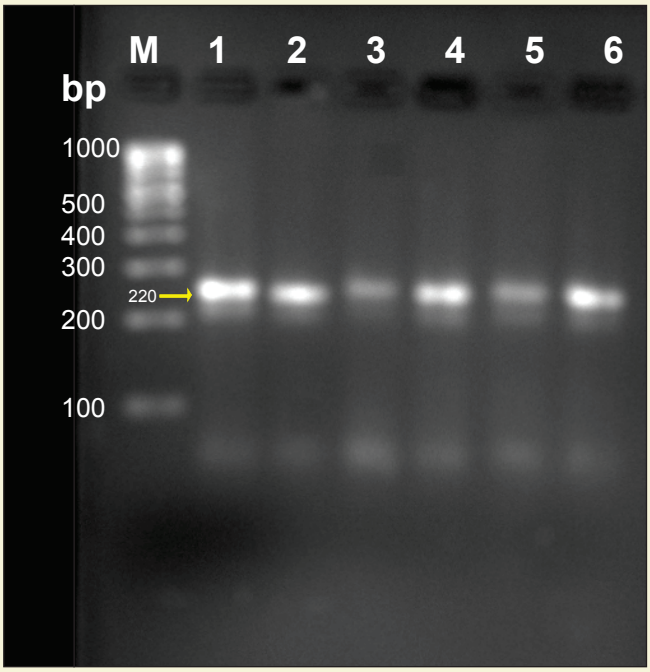

Fig. 1. - Amplification pattern of the soybean genotypes obtained using the SSR marker Satt 371.

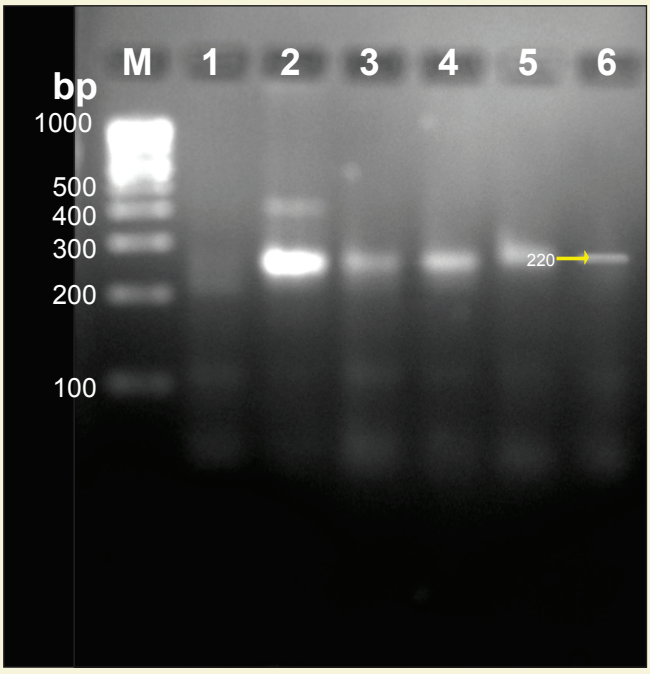

Fig. 2. - Amplification pattern of the soybean genotypes obtained using the SSR marker Satt 453.

Lane M: 100 bp ladder
Lane 1 : Birsa soya 1
Lane 2 : Kalitur
Lane 3 : KDS 1034
Lane 4 : KDS 1027
Lane 5 : KDS 1035
Lane 6 : EC 241780

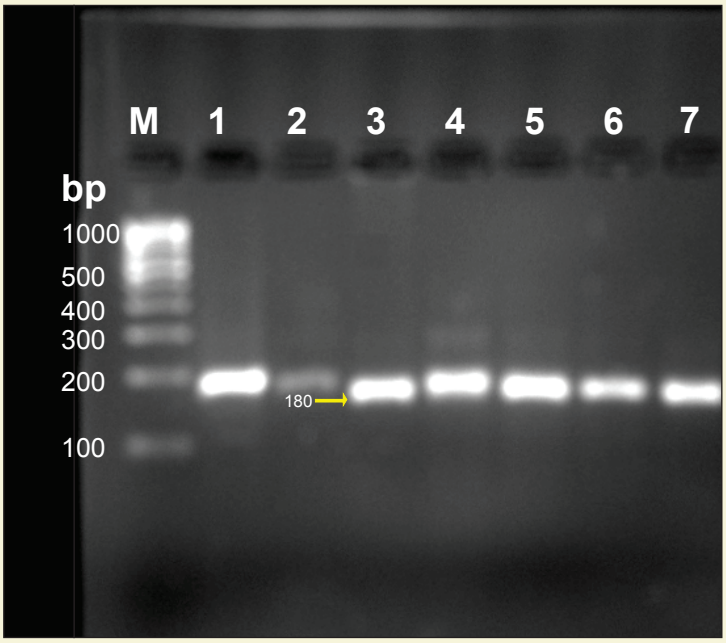

Fig. 3. - Amplification pattern of the soybean genotypes obtained using the SSR marker Satt 523.

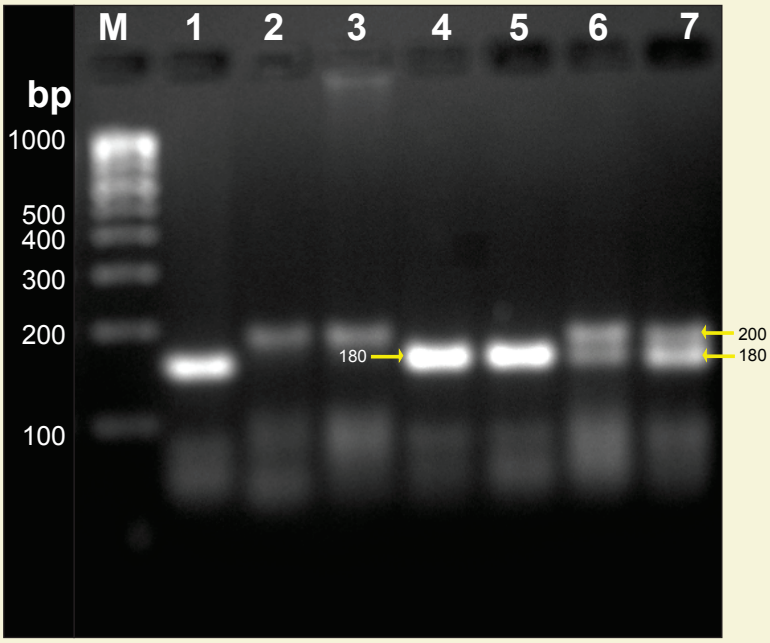

Fig. 4. - Amplification pattern of the soybean genotypes obtained using the SSR marker Satt 162.

Lane M: 100 bp ladder
Lane 1 : Birsa soya 1
Lane 2 : Kalitur
Lane 3 : DS 228
Lane 4 : KDS 1034
Lane 5 : KDS 1027
Lane 6 : KDS 1035
Lane 7 : EC 241780