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Studies on the biochemical properties of soybean (*Glycine max*) genotypes in response to charcoal rot incidence

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Abstract
Despite being one of the most economical source of protein and packed with several nutraceutical components, soybean does suffer from shortfalls that limit its utilization in food uses. Given the fluctuating production of soybean in the recent years due to several biotic and abiotic stresses, it has become important to breeding for quality traits in tandem with overcoming other biotic/ abiotic stress that affect crop productivity. Diseases on soybean is one of the major constrain in production in Maharashtra. Charcoal rot is a soil borne, rated as number one or two factors causing economic loss due to root and stem disease of soybean. In general, 30-50 % losses occur every year in soybean worldwide and recorded up to 77% in India considering the losses it has become important to study the biochemical properties of the crop for breeding for quality traits in tandem with overcoming other biotic/ abiotic stress that affect crop productivity. In the present study protein, proline, carbohydrate and oil content was evaluated. The highest protein content was recorded in the genotype AMS MB 5-19 (43.22 g/100g) and the lowest level was recorded in TAMS-38 (31.31 g/100g) genotype. Highest content of proline was found in AMS MB 5-19 (24.42 g/100g) and lowest amount of proline was found in AMS 475 (21.42 g/100g). Highest carbohydrate was found in AMS MB 5-19 (19.22 g/100g) and lowest amount of carbohydrate was found in AMS 475 (6.31g/100g). Highest oil content was found in AMS MB 5-19 (21.01%) and lowest amount of oil % was found in BRAGG (18.84%).

Keywords: Soybean, pulse crop, biotic stress resistance, charcoal rot, resistance breeding

Introduction
Soybean [*Glycine max*. (L.) Merrill] is the cheapest source of vegetable oil and protein. It contains about 40% protein, well balanced in essential amino acids, 20% oil rich with poly unsaturated fatty acid specially omega 6 and Omega 3 fatty acids, 6-7% total mineral, 5-6% crude fiber and 17-19% carbohydrates (Chauhan *et al*., 1988) [3]. Soybean crop can be attacked by more than 100 pathogens (Sinclair and Shurtleff, 1975) [5]. The major economically important diseases of the soybean are rust, wilts, leaf spots, rots, web blight, powdery mildew, bacterial and viral diseases. Among the fungal diseases, charcoal rot caused by fungus *Macrophomina phaseolina* is a common, widespread, destructive and economically important disease in soybean, causing obstruction of xylem vessels and wilting leading to the yield loss, quality deterioration of seed and oil (Abawi and Pastor-Corrales, 1990) [1]. Macrophomina phaseolina is an important fungus that causes charcoal rot having broad host range like soybean, common bean, mung bean, sorghum, maize, cotton, peanut, sesame, cowpea, chickpea and cluster bean producing the symptoms of dry root rot, dry weather wilt, ashy stem blight and seedling blight (Su *et al*., 2001) [6]. Macrophomina phaseolina is capable of infecting soybean at any crop growth stage, but usually, it infects at post flowering stage. The disease cycle for M. phaseolina begins with germination of microsclerotia when temperatures are between 28 °C and 32 °C. In India, the charcoal rot, which is used to be a minor disease of soybean until 2004, became serious due to altered weather conditions particularly on the account of longer drought spells during crop growth period. There are reports of few epiphytotics to occur in areas where temperature ranges from 35–40 °C during the crop season, disease can cause up to 80 percent yield losses. Therefore, developing soybean cultivars with higher yield, resistance to major pests and diseases and improved quality has become the major objective of soybean breeding program across the country. Keeping in view, the severity and losses caused by disease, present investigation was undertaken with the objective to study the biochemical status of core soybean genotypes in response to charcoal rot.

Biochemical analysis was done so as to understand the metabolic behavior of the crop under different conditions. Genotypes showing good potential may be useful in breeding programme through hybridization and selection.

Material and Methods

Plant material: A set of 14 diverse soybean genotypes were used for screening. The collected genotypes included promising varieties, indigenous, mutants, few pre released collections, advanced breeding lines as well as obsolete varieties. It varied in maturity, seed color, flower colour, seed size, and reaction to charcoal rot disease as well as other yield attributing traits. Specific features of the genotypes are presented in (Table 1).

Table 1: Soybean genotypes included in the study

S.N	Genotypes	Parents	Remarks
1	AMS MB 5-19	Mutant of Bragg	Developed by Mutation breeding and characteristically fixed at M8 generation.
2	AMS MB 5-18	Mutant of Bragg	Developed by Mutation breeding and characteristically fixed at M8 generation.
3	AMS – 1001	Mutants	Pre released variety
4	AMS – 77	Mutant of JS 93-05	Developed by Mutation breeding and characteristically fixed at M5 generation.
5	AMS – 353	Mutants	Pre released variety
6	AMS – 358	Mutant of JS 93-05	Developed by Mutation breeding and characteristically fixed at M5 generation.
7	BRAGG	Parental genotype	Parental genotypes
8	AMS – 243	Mutant of Bragg	Developed by Mutation breeding and characteristically fixed at M8 generation.
9	JS - 93-05	Parental genotype	Parental genotypes
10	AMS 99-33	Mutants	Pre released variety
11	AMS 38-24	TAMS 38 x RKS 24	Recombinant breeding, entry fixed at F2 generation.
12	AMS -475	Mutant of JS 93-05	Developed by Mutation breeding and characteristically fixed at M5 generation.
13	JS – 335 (R)	(Check-Resistant)	High yielding variety, most popular
14	TAMS -38 (S)	(Check-Susceptible)	Highly susceptible variety

R=Check Resistant
S=Check Susceptible

Fungus isolates: Isolates of Macrophomina phaseolina were collected from the charcoal rot infected soybean plants as well as from soil found in the experimental fields of RRC, Amravati. The isolates were cultured, purified and re-infected the soybean plants to confirm its identity and virulence. Pure culture of *M. phaseolina* was maintained in controlled condition and used for inoculating the target genotypes. Isolation of *Macrophomina phaseolina*, infected soybean seedling and plants with symptoms of charcoal rot was collected from fields of different regions of Maharashtra and was brought and cultured in the laboratory of Department of Plant Pathology, Dr. PDKV, Akola.

Biochemical characters

Biochemical analysis was done so as to understand the metabolic behavior of the crop under different conditions; it involved Protein content, Proline content, Carbohydrate content and Oil content (%)

1. Protein content - Maehre *et al*. (2016) [4]

Protein assay protocol- Salt / Alkaline extraction method of protein estimation, Weighed 0.5 gm of seed powder. Mixed with 30 ml of 0.1 M sodium hydroxide (NaOH) in 3.5 % sodium chloride (NaCl) using a homogenizer. The homogenates were incubated at 60 ºC for 90 mins. Centrifuged at 4000 rpm for 30 min at 4 ºC. The supernatants were frozen and kept at -20 ºC until analyses.

Protein profiling: Sodium dodecyl sulfate polyacrylamide gel electrophoresis, (SDS PAGE) profiling was done on a vertical slab gel electrophoresis system according to Laemmli (1970); using a 5% stacking gel and 12% separating gel.

30 % Acryamide stock: It was prepared by dissolving 29.2 g of acrylamide and 0.8 g bisacrylamide in water to make the final volume 100 ml. Running gel buffer: (1.5 M Tris-CI, pH 8.8) It was prepared by dissolving 18.2 g of Tris base in 80 ml of water. The pH of solution was adjusted to 8.8 with 1 N HCI and water was added to make the final volume 100 ml.

Stacking gel buffer: (0.5 M Tris-CI, pH 6.8) It was prepared by dissolving 6.06 g of Tris base in 80 ml water. The pH of solution was adjusted to 6.8 with 1 N HCI and water was added to make the final volume 100 ml.

Sodium dodecyl sulphate (SDS) solution (10%, w/v): It was prepared by dissolving 10 g of SDS in water to a final volume 100ml Ammonium persulfate (APS) solution (10%; w/v): It was prepared fresh by dissolving 100 mg APS in 1 ml water.

TEMED (N, N, N', N'-tetramethylethylenediamine): It was used undiluted. Electrode Buffer (5X): It was prepared by dissolving 15 g Tris base, 72 g glycine and 5 g of SDS in 1 L water. The pH of solution was set at 8.3 with 1 N HCl.

Staining solution: Prepared by dissolving 1 g Coomassie Brilliant Blue R 250 in 500 ml methanol and 200 ml glacial acetic acid and finally distilled water was added to make the final volume 1000 ml. The solution was stored at room temperature.

1	Coomassie Brilliant Blue R 250	1 g
2	Methanol	500 ml
3	Glacial acetic acid	200 ml
4	D/W	Added to make 1L

Destaining solution: Destining solution is same as staining solution without Coomassie Brilliant Blue R 250. It was stored at room temperature.

1	Methanol	500 ml
2	Glacial acetic acid	200 ml
3	D/W	Added to make 1L

Sample buffer (pH 6.8):

1	0.5 M Tris pH 6.8	1 g
2	50% glycerol	500 ml
3	1% Bromophenol Blue	200 ml
4	10 % SDS	1
5	D/W	Added to make 5 ml vol

Composition of the Resolving gel (12 %) mixture (ml):

1	ddH2O	6.6
2	1.5 M Tris (pH 8.8)	5
3	30% acrylamide/Bis	8
4	10 % SDS	0.2
5	10% APS	0.2
6	TEMED	0.2
	Total Volume	20 ml

Composition of the stacking gel (5 %) mixture (ml):

1	ddH2O	6.6
2	1.5 M Tris (pH 8.8)	3
3	30% acrylamide/Bis	2.04
4	10 % SDS	0.2
5	10% APS	0.2
6	TEMED	0.2
	Total Volume	12 ml

Gel casting: The resolving gel mixture was immediately poured into glass sandwich upto the mark taking the precaution that air bubbles do not enter into it. The stacking gel mixture was made after polymerization of the resolving gel and poured over the resolving gel in such a manner as to allow escape entry of any air bubble. The comb was inserted into it for well formation. It was kept undisturbed till polymerization.

Sample preparation: 50 µg of protein was added to equal amount (1: 1, v/v) of the sample buffer and placed in a boiling water bath for 3 min at 95 °C. The samples were run on SDS-PAGE gels at 25º C for Stacking gel to the power supply at 90 volt and resolving gel at 70 volts per well Run the gel. It will take 6 hours. The unstained protein molecular weight marker (Fermentas #SM0431) was used for precise sizing of proteins.

Coomassie staining: Coomassie staining is able to detect protein bands containing about 0.2 µg or more protein. For low abundant protein, silver staining is a better choice since it is about 100-fold more sensitive than Coomassie staining Incubate the gel in staining solution with shaking for 30 min or longer (can leave it overnight). Remove the dye solution (it can be reused for many times) and rinse the gel with water 1-2 times to remove the dye. Immerse the stained gel into the destaing solution. Transfer the gel to water (can keep it in water for several days). Destaining is done by immersing the stained gel into the destaing solution.

Analysis of gel: Protein bands of different intensities and positions were obtained after destaining. Photography was done and bands were counted by keeping the gel on a light box.

2. Proline content - Bates *et al.* (1973) [2]

Procedure: The frozen plant material was homogenized in 3% aqueous sulphosalicylic acid (0.01g/ 0.5 ml) and the residue was removed by centrifugation at 12 000 rpm for 10 min. 1 ml of the homogenized tissue reacts with 1 ml acid-ninhdrin and 1 ml of glacial acetic acid in a test tube for 1 hour at 100°C and the reaction was terminated in an ice bath. The reaction mixture is extracted with 2 ml toluene, mixed vigorously and left at room temperature for 30 min until separation of the two phases. The chromophore-containing toluene (1 ml, upper phase) is warmed to room temperature and its optical density is measured at 520 nm using toluene for a blank. The proline concentration is determined from a standard curve using D-Proline.

3. Total carbohydrates assay protocol (Anthrone method)

Carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound with anthrone forms a green colored product with an absorption maximum at 630nm.

Procedure: Weighed 100 mg of the sample into a boiling tube and hydrolyze by keeping it in boiling water bath for 3 hours with 5mL of 2.5 N-HCl and cool to room temperature. Make up the volume to 100mL and centrifuge. Collect the supernatant and take 0.5 and 1mL aliquots for analysis. Prepare the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1mL of the working standard. '0' serves as blank. Make up the volume to 1mL in all the tubes including the sample tubes by adding distilled water. Then add 4mL of anthrone reagent and cool rapidly and read the green to dark green color at 630nm. Draw a standard graph by plotting concentration of the standard on the *X*-axis versus absorbance on the *Y*-axis. From the graph calculate the amount of carbohydrate present in the sample tube.

4. Oil content

Oil content was determined by using NMR (Nuclear Magnetic Resonance) machine at Instrumental cell, Oilseed Research Unit, Dr. P.D.K.V, Akola.

Results and Discussions

Biochemical analysis was done so as to understand the metabolic behavior of the crop under different conditions. Genotypes showing good nutrient potential may be useful in breeding programme through hybridization and selection. All results of the estimated protein, proline, carbohydrate and oil content are given in (Table 2).

Table 2: Total protein, proline, carbohydrate, and oil content in the soybean genotypes

S.N	Genotype	Protein (g/100g)	Proline (g/100g)	Carbohydrate (g/100g)	Oil content (%)
1	AMS MB 5-19	43.22	24.42	19.22	21.01
2	AMS MB 5-18	42.86	24.02	17.86	19.78
3	AMS - 1001	41.57	23.82	16.57	18.93
4	AMS - 77	41.21	23.22	16.21	19.24
5	AMS - 353	40.98	23.02	15.05	19.57
6	AMS - 358	40.05	22.82	13.12	19.47
7	BRAGG	38.12	22.62	12.18	18.84
8	AMS - 243	37.18	22.22	11.89	19.44
9	JS - 93-05	36.89	22.02	10.58	20.25
11	AMS 99-33	35.58	21.82	9.65	19.61
11	AMS 38-24	34.65	21.42	8.88	19.21
12	AMS -475	33.88	21.42	6.31	19.84
13	JS – 335 (R)	32.25	23.22	15.98	19.88
14	TAMS -38 (S)	31.31	21.42	7.25	19.69

▪	GM	40.30	22.68	13.36	19.30
▪	Range	43.22-31.31	21.42-24.42	6.31-19.22	18.84-21.01
▪	SE(m)	0.57	0.33	0.61	0.33
▪	CD @1%	2.30	1.33	2.44	1.33
▪	F Test	S	S	S	S

R=Check Resistant
S=Check Susceptible

A. Protein content (g/100g)

Proteins are the most complex and abundant of the macro molecules. Within cells, many proteins function as enzymes in the catalysis of metabolic reactions, while others serve as transport molecules, storage proteins, electron carriers, and structural components of the cell. especially important in seeds, as they make up as much as 40 % of the seed's weight and serve to store amino acids for the developing embryo. The highest protein content was recorded in the genotype AMS MB 5-19 (43.22 g/100g) and the lowest level was recorded in TAMS-38 (31.31 g/100g) genotype, respectively.

SDS-PAGE profiling

SDS-PAGE electrophoresis was performed on all the twelve soybean genotypes, along with two additional genotypes *viz.* NRC 101 and field pea variety to check the presence or absence of specific protein band of KTI and to determine molecular weight of protease inhibitor.

SDS-PAGE separated total soybean protein into several bands (Fig.1).

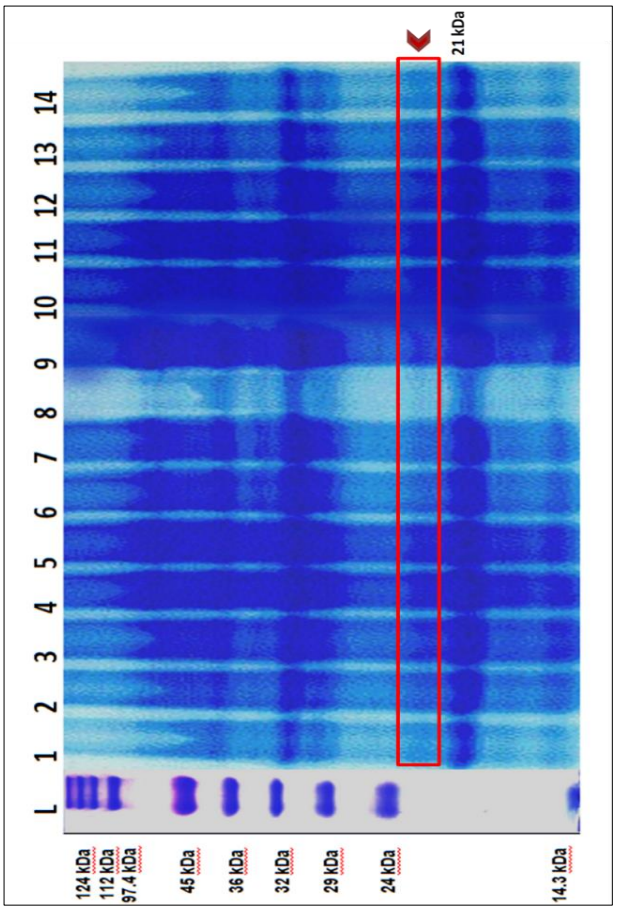

Fig 1: Protein profile of seed powder obtained by SDS-PAGE

B. Proline content (g/100g)

Proline, an amino acid, plays a highly beneficial role in plants exposed to various stress conditions. Stressful environment results in an overproduction of proline in plants which in turn imparts stress tolerance by maintaining cell turgor or osmotic balance, thus preventing oxidative burst in plants. Literature states that proline content in the plant under stress condition has direct relation with its resisting capacity to the stress. Highest content of proline was found in AMS MB 5-19 (24.42 g/100g) and lowest amount of proline was found in AMS 475 (21.42 g/100g).

C. Sugar content (Carbohydrate) (g/100g)

Carbohydrates are the important components of storage and structural materials in the plants. The basic units of carbohydrates are the monosaccharides which cannot be split by hydrolysis into more simpler sugars. The carbohydrate content can be measured by hydrolyzing the polysaccharides into simples sugars by acid hydrolysis and estimating the resultant monosaccharides. Higher carbohydrate indicated better plant health status. Highest carbohydrate was found in AMS MB 5-19 (19.22 g/100g) and lowest amount of carbohydrate was found in AMS 475 (6.31g/100g).

D. Oil content (%)

Oil content was determined by using NMR (Nuclear Magnetic Resonance) machine at Instrumental cell, Oilseed Research Unit, Dr. P.D.K.V, Akola. Highest oil content was found in AMS MB 5-19 (21.01%) and lowest amount of oil % was found in BRAGG (18.84%)

Conclusions

The result indicated that geometric mean of protein, proline, carbohydrate and oil content was 40.30g/100g, 22.68 g/100g, 13.36 g/100g, and 19.30 % respectively for all the selected genotypes. Range observed was 43.22-31.31g/100g for protein, 21.42-24.42g/100g for proline, 6.31-19.22 g/100g for carbohydrate and 18.84-21.01 % for oil content. F test for all the evaluated parameters was found to be significant.

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