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Studies on Effect of Different Concentration of Gibberellic Acid (GA_3) on Seed Germination and Protein Profile in Mung Bean (*Vigna radiata*) under Saline Condition (NaCl)

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Abstract

A laboratory experiment was carried out during summer season of 2013-14 at Department of Biochemistry and Molecular Biology, MGM College of Agricultural Biotechnology, Aurangabad to study effect of different concentrations of gibberellic acid (GA_3) on seed germination and protein profile in mung bean (*Vigna radiata*) under saline condition (NaCl). The experiment was laid out in completely randomized design (CRD) with five treatments of GA_3+NaCl (D/ W), 300mM NaCl+0 mg/l GA_3, 300mM NaCl+50 mg/l GA_3, 300mM NaCl+100 mg/l GA_3, 300mM NaCl+150 mg/l GA_3) with four replications. After 7 days the seed germination percentage was 10 per cent, 78.2 per cent, 84.6 per cent, 81.7 per cent and 93 per cent with radicle length (7.48, 0.30, 0.31, 0.40 and 0.43 cm) and plumle length (2.38, 0, 0, 0, and 0 cm), respectively. The amount of protein content also changed with increase in GA_3 concentration under saline condition. The overall germination, length of radicle and the lenghth of plumule was affected due to the increase in GA_3 concentration under saline condition. The treatment T4 (300 mM NaCl+ 150 mg/l GA_3) was found better after treatment T0 (control D/W) for per cent germination and radical length. When SDS-PAGE was carried out it was observed that the change in protein expressed under GA_3 and salt stress, it showed the variation in banding pattern of proteins on SDS-PAGE, in some protein samples which was treated with different concentrations of GA_3 and constant NaCl.

Keywords : Germination, Length of plumule and radicle, Protein profile, Mung bean

Mung bean is a pulse crop, belongs to the family *Fabaceae*, genus *Vigna* and species *radiata*. It is currently grown on about 10 million ha in world. Mung bean is native to the Indo-Burma region with India, Burma, Thailand and Indonesia producing almost 90 per cent of total world's production. The present scenario indicates its plantation in countries like India, Pakistan, Bangladesh, Srilanka, Thailand, Laos, Malaysia, South China and Indonesia (Mensah *et al.,* 2009).

Mung bean is hardy deep rooted crop. The deep tap root system enhances its capacity to withstand drought conditions. Mung bean is a *kharif* crop and grows in months of June to October. The optimum temperature required for growth of mung bean is about 28-30^{0}C. It does not require large amounts of water (600-1000 mm). It is sensitive to water logging. Mung bean can grow on a wide range of soils but prefers well drained loams or sandy loams with pH ranging from 5-8. Maturity period of mung bean ranges from 60-65 days after sowing (Mohammed *et al.,* 2007).

India is largest producer with more than 50 per cent of world production but consumes almost its entire production. In India 34.4 lakh ha (18.07 per cent) area is cultivated under mung bean. Total production was 14 lakh tons in 2012. The leading mung bean

producing states are Rajasthan (Area-30.81 per cent and Production-34.67 per cent), Maharashtra (Area-19.51 per cent and Production-30.92 per cent), Andhra Pradesh (Area-12.79 per cent and Production-18.08 per cent), Karnataka (Area-15.35 per cent and Production-9 per cent), Orissa (Area-7.41 per cent and Production 15.17 per cent), Tamil Nadu (Area-4.97 per cent and Production-4.50 per cent), Uttar Pradesh (Area-2.09 per cent and Production-3.33 per cent). [Indian Institute of Pulses Research, Kanpur, 2011-12].

Mung bean is economically and nutritionally an important legume and is a major source of protein. Mung bean (100g raw) contains 60g carbohydrate, 24g protein, 1.3g fat, 16.4g fibre and other elements such as Zn, Ca, K, Mg and Iron in trace amounts (Hossain *et al.,* 2008).

Agricultural productivity and eco-environment severely affected by salinity (Bhivar *et al.,* 1984). It is estimated that about 20 per cent of irrigated land, which yields one third of the world's food, is affected by salinity. Salinity affected area in world is about 400 million ha and in India is about 8565.2 ha. Salinity affected states in India is Uttar Pradesh (1295 ha), Gujrat (1214.4 ha), Rajasthan (1122 ha), Andhra Pradesh (813.3 ha), West Bengal (800 -ha), Maharashtra (800 ha), Punjab (519 ha), Haryana (455 ha), Karnataka (404 ha), Orissa (400 ha), Bihar (400 ha), Tamil Nadu (340), Madhya Pradesh (242 ha) and Kerala (26 ha) (CSSRI-2010). Moreover a significant proportion of recently cultivated agricultural land has become saline because of elevated levels of irrigated water (Munns, 2005). Therefore need of developing salt tolerant cultivars is unavoidable.

Some researchers reported that salt stress (NaCl) caused a decrease in germination, shoot and root length in mung bean. Mensah *et al.* (2003) found that seedling emergence, fresh and dry mass of both shoots and yield decreased with increased salinity. Wahid *et al.* (2004) found that the reduced levels of N, P, K & Ca due to salinity. Akbari *et al.* (2008) found that the gibbrellic acid decreases the adverse effects of salinity on green gram germination.

GA_3 is known to be importantly concerned in the regulation of plant responses to the external environment. GA_3 has also been shown to alleviate the effects of salt stress on water use efficiency (Iqbal *et al.,* 2001).

Material and Methods

Treatment Details :

Statistical Design	Completely Randomised Design (CRD).
Number of treatments	05
Number of replication	04

Table 1

Treatment detail

Treatment	Concentrations of NaCl+GA_3(mg/l)
T_0	Control (D/W)
T_1	300 mM NaCl + 0 mg/l GA_3
T_2	300 mM NaCl + 50 mg/l GA_3
T_3	300 mM NaCl + 100 mg/l GA_3
T_4	300 mM NaCl + 150 mg/l GA_3

Biometric Observations

Seeds were observed daily with respect to calculate the length of plumule, radicle and percentage of germination.

Observation	Frequency	Day after Germination
Length of plumule (cm)	Daily	Up to 7 days
Length of radicle (cm)	Daily	Up to 7 days
Percentage of germination	Daily	Up to 7 days
Protein Profile of germinated seeds		After 7days

Methods

Germination of Seeds under Salt Stress

- Mung Bean (MOOG BM 2002-01) seeds were collected from Aurangabad district and used as seed sample
- The mung bean seeds were surface sterilized with 0.01M HgCl2 solution for three minutes

- Then seeds were washed thoroughly with distilled water for 10 min.
- The seeds were transferred (10 seeds per plate) on sterile petri dishes containing respective solution of treatment applied on filter papers
- The seeds were allowed for germination up to seven days
- Data for seed germination was recorded daily up to seven days and germination percentage was calculated. The data based on the above mentioned biometric parameters was recorded

Germination percentage : Total number of seeds germinated up to seven days was used to obtain germination per cent and was calculated as below :

$$\text{Germination percentage} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds}} \times 100$$

Extraction and Quantification of Protein from Germinated Seeds

Extraction

- Extraction of proteins from germinated seeds was carried out by using phosphate buffer (NaH_2PO_4 - 0.0718, $NaHPO_4$ - 1.2006 gm)
- 500 mg of the sample was weighed and ground well with a pestle and mortar in 5-10 ml of buffer
- The sample was subjected to centrifugation at 10,000 rpm for 20 min and the supernatant was used as a protein sample for quantification and gel electrophoresis

Quantification of Protein Concentration by Folin-Lowry's Method

Principle

The blue colour developed by the reduction of the phosphomolybdic-phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured at 540 nm in the Lowry's method.

Reagents

Reagent A – 2 per cent sodium carbonate in 0.1N sodium hydroxide.

Reagent B – 0.5 per cent copper sulphate ($CuSo_4.5H_2o$) in 1 per cent potassium sodium tartrate.

Reagent C / Alkaline copper solution – Mix 50 ml of A and 1 ml of B prior to use.

Reagent D /Folin- ciocalteu's reagent – A mixture consisting of 100 gm sodium tungstate ($Na_2MoO_4.2H_2o$), 700 ml water, 50 ml of 85 per cent phosphoric acid, 100 ml of hydrochloric acid in a 1.5 L flask. Add 150 gm lithium sulphate, 50 ml water and a few drops of bromine water were refluxed gently for 10 hrs. Then the mixture is boiled for 15 min without condenser to remove excess bromine, cooled and diluted to 1 L then filtered and used.

Protein stock Solution - 50 mg of BSA was weighed accurately and Dissolved in distilled water and made the final volume up to 50 ml in standard flask.

Working Standard Solution - Dilute 10 ml of the stock solution to 50 ml with distilled water in a standard flask, one of this solution contains 200 µg proteins.

Procedure

- 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard solution were pipetted out into a series of test tubes
- 0.11 ml and 0.2 ml of the sample extracts was pipetted out in two other test tubes
- The volume in all test tubes was made to 1 ml. A tube with 1 ml of water served as the blank.
- 5 ml of reagent C was added to each tube including the blank. It was mixed well and allowed to stand for 10 min.
- 0.5 ml of reagent D was added in each test tube and mixed well. All test tubes were incubated at room temperature in dark for 30 min for the development of blue color.
- Reading was taken at 660 nm by using spectro-photometer
- A standard graph was drawn and the amount of protein in the sample was calculated

Polyacrylamide Gel Electrophoresis of the Extracted Proteins: The separation of protein was carried out by using SDS PAGE.(Geok *et al*., 2012).

Principle

SDS is an anionic detergent which binds strongly to, and denatures proteins. The number of SDS molecules bound to a polypeptide chain is approximately half the number of amino acid residues in that chain. The protein- SDS complex carries net negative charges hence move towards the anode and the separation is based on the size of the protein.

Reagents

A] 3X SDS- PAGE Sample Buffer: - 0.2 M Tris- HCL (pH 6.8), 4.5 % SDS, 12 % ß-mercaptoethanol, 30% Glycerol, 0.06% Bromophenol Blue.

B] 10x Laemmli Buffer (500 ml):- Glycine 72.15 g, Tris Base 15.25 gm, SDS 5 g.

C] Fixing solution (500 ml):- Methanol 150 ml, Acetic Acid 50 ml, H_2O 300 ml.

D] Staining solution (500 ml):- Methanol 250 ml, Acetic Acid 50 ml, Coomassie Brilliant Blue R250 1.25 g, H_2O 200 ml.

E] Destain solution (500 ml):- Methanol 40 ml, acetic acid 10 ml, H_2O 50 ml.

F] 70% Ethanol

Procedure

1. The glass plates and spacers were cleaned and dried thoroughly, then they were assembled properly and the assembly was held together with bulldog clip. The assembly was clamped in an upright position. White petroleum jelly or 2 per cent agar (melted in boiling water bath) was then applied around the edges of the spacers to hold them in place and the chamber between the glass plates was sealed.

2. A sufficient volume of separating gel mixture [30mL for a chamber of about (18×9×0.1cm)] was prepared by mixing the following (Table No.2).

TABLE 2

Composition of separating gel

Chemicals	For 15% gel	For 10% gel
Stock acrylamide solution	20 mL	13.3 mL
Tris-HCl(pH8.8)	8 mL	8 mL
Water	11.4 mL	18.1 mL
Ammonium persulphate solution *	0.2 mL	0.2 mL
SDS10%	0.4 mL	0.4 mL
TEMED	20 µL	20 µL

(*Degas on a water pump for 3-5 min and then add: Ammonium persulphate, SDS, TEMED)

3. Above chemicals were mixed gently and carefully, the gel solution was poured in the chamber between the glass plates. Distilled water was layered on the top of the gel and left to set for 30-60 min.

4. The stacking gel (4%) was prepared by mixing the following solutions (total volume 10 mL) (Table No.3)

TABLE 3

Composition of stacking gel

Chemicals	Amount
Stock acrylamide solution	1.35mL
Tris-HCl(pH6.8)	1 mL
Water	7.5 mL
Ammonium persulphate solution *	50 µL
SDS 10%	0.1 mL
TEMED	10 µL

(*Degas on a water pump for 3-5 min and then add: Ammonium persulphate, SDS, TEMED.) The water from the top of the gel was removed and washed with a little stacking gel solution. The stacking gel mixture was poured and the comb was placed in the stacking gel and the gel was allowed to set (30-60 min).

5. After the stacking gel has polymerized, the comb was removed without distorting the shapes of the well. The gel was installed carefully after removing the clips, agar etc. In the electrophoresis apparatus .The apparatus was filled with electrode buffer and trapped the air bubbles at the bottom

of the gel were removed. The cathode was connected at the top and the DC-power was turned on briefly to check the electric current. The electrode buffer and the plates were kept closed using a suitable facility so that heat generated during the run is dissipated and does not affect the gel and resolution.

6. The protein concentration in each sample was adjusted using the 5-strength sample buffer and watering in such a way that the same amount of protein is present per unit volume. Again the volume concentration should be such as to give a sufficient amount of protein (50-200 µg) in a volume (20-50 µL) not greater than the size of the sample well.

7. The sample solutions were cooled and the required volume was taken in a microsyringe and carefully injected into a well. The position of wells on the glass plate was marked with a marker pen and (presence of bromophenol blue in the sample buffer facilitate easy loading of the samples). Similarly, few wells were loaded with standard marker proteins in the sample buffer.

8. The current was turned on to 10-15mA for initial 10-15 min until the samples travel through the stacking gel. Then the run was continued at 30mA until the bromophenol blue reaches the bottom of the gel (about 3h).

9. After the run is complete, the gel was removed carefully from between the plates and immersed in staining solution for overnight with uniform shaking. The proteins absorb the Coomassie brilliant blue.

10. The gel was transferred to a suitable container with at least 200-300 mL destaining solution and shaken gently and continuously. Dye that was not bound to proteins was thus removed. The destainer was changed frequently, particularly during initial periods, until the background of the gel was colourless. The proteins fractionated into band were seen coloured blue. As the proteins of minute quantities are stained faintly, destaining process was stopped at appropriate stage to visualize as many bands as possible.

11. Migration was measured using the migration distance of protein marker and extracted protein samples. The relative mobility of each protein was calculated by using formula.

$$\text{Mobility} = \frac{\text{Distance moved by protein}}{\text{Distance moved by tracking dye}}$$

Results and Discussion

This study was designed to evaluate the changes in protein content of mung bean seeds germinated at various concentrations of GA_3 under saline condition. The seeds of variety MOOG BM 2002-01 were kept for germination at different concentrations of GA_3 under saline condition. The result is depicted in Table 1.

Data presented in Table 4 indicated that the germination of mung bean seeds of variety MOOG BM 2002-01 was influenced significantly by the varying concentrations of GA_3 under saline condition. The treatment T_0 recorded highest length of radicle and e radicle length was found increased from treatment T_1 to T_4. The treatment T_1 showed highest percentage of germination and germination percentage was found increased from T_1 to T_4. This might be due to the effect of gibbrellic acid which decreases the

Table 4

Effect of different concentrations of GA3 under saline conditions on Mung Bean seeds germination

Treatment	Length of radicle	Percentage of germination
T_0 (D/W)	7.48	100
T_1 (300mM NaCl+0 mg/l GA_3)	0.30	78.21
T_2 (300mM NaCl+50 mg/l GA_3)	0.31	84.64
T_3 (300mM NaCl+100 mg/l GA_3)	0.39	86.78
T_4 (300mM NaCl+150 mg/l GA_3)	0.43	93.56
Mean	1.782	88.63
S.E±	0.063	3.31
C.D	0.189	9.98

adverse effect of salinity on green gram germination (Akbari *et al.,* 2008).

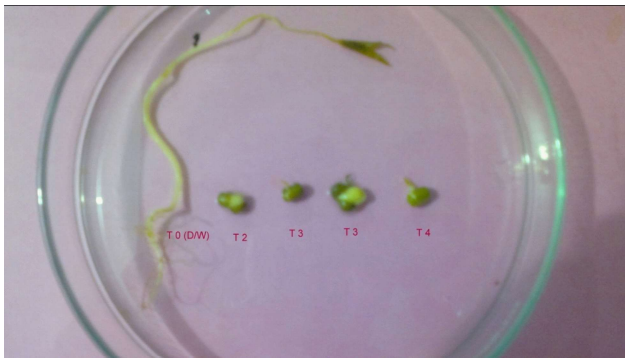

Plate 1 : Germination of seeds after different treatments

From the data represented in Table 5 it is found that amount of protein in germinating seeds under GA_3 and saline condition increased with increase in GA_3 concentration upto 100 mg/l and then decreased at 150 mg/l GA_3. Seeds germinated under distilled water showed lowest amount of proteins (0.53) as compared

TABLE 5

Protein percentage of *vigna radiata* as influenced by different concentrations of GA_3 under saline condition

Treatment	Amount of Protein content (mg/ml)
Control (D/W)	0.53
T_1 (300mM NaCl+0 mg/l GA_3)	1.01
T_2 (300mM NaCl+50 mg/l GA_3)	1.21
T_3 (300mM NaCl+100 mg/l GA_3)	1.43
T_4 (300mM NaCl+150 mg/l GA_3)	0.78
Mean	0.992
S.E±	0.104
C.D	0.313

to other treatments. This was due to the expression of more proteins to resist saline condition. Seeds germinated under treatment T_4 showed lower protein amount (0.78 mg/ml) than the treatments T_1, T_2 and T_3 and higher protein amount than control. From above data the increased GA_3 concentration under saline condition increases the amount of protein in germinated seeds upto a certain level and then decreases. When seeds are under saline condition, some proteins are expressed to resist salt due to these protein

Further, to know which of the protein were inhibited under salt stress, SDS PAGE analysis was carried out. SDS PAGE showed major proteins and their molecular mass contributing to protein profile of seeds. Table 6 depicts number of proteins obtained and their molecular mass.

TABLE 6

The R_f values of protein and their molecular weight obtained by SDS PAGE analysis

NaCl concentrations	Number of proteins	R_f value	Molecular weight (k Da)
Marker proteins	Band 1	0.28	66
	Band 2	0.47	43
	Band 3	0.61	29
	Band 4	0.75	14
T_0 Control	Band 1	0.23	70
(D/W)	Band 2	0.38	54
	Band 3	0.43	48
	Band 4	0.50	40.5
	Band 5	0.56	34.4
	Band 6	0.69	20.5
	Band 7	0.73	16
T_1 (300mM NaCl	Band 1	0.23	70
+0 mg/l GA_3)	Band 2	0.4	51.5
	Band 3	0.44	47.5
	Band 4	0.52	38.5
	Band 5	0.6	30
	Band 6	0.69	20.5
	Band 7	0.73	16
T_2 (300mM NaCl	Band 1	0.23	70
+50 mg/l GA_3)	Band 2	0.4	51.5
	Band 3	0.44	47.5
	Band 4	0.52	38.5
	Band 5	0.58	32
	Band 6	0.69	20.5
	Band 7	0.73	16
T_3 (300mM NaCl	Band 1	0.33	58.5
+100 mg/l GA_3)	Band 2	0.44	47.5

NaCl concentrations	Number of proteins	R_f value	Molecular weight (k Da)
	Band 3	0.52	38.5
	Band 4	0.58	32
	Band 5	0.66	23
	Band 6	0.69	20.5
	Band 7	0.75	14
T_4 (300mM NaCl +150 mg/l GA_3)	Band 1	0.26	67
	Band 2	0.43	48
	Band 3	0.46	45
	Band 4	0.53	37.5
	Band 5	0.61	29
	Band 6	0.67	22.5
	Band 7	0.72	17
	Band 8	0.75	14

Each band represents a single protein. The protein profile of particular seed under different concentrations of GA_3 under saline condition is contributed by various protein as obtained in SDS PAGE analysis. The maximum numbers of eight bands were obtained in the seeds germinated under 300mM NaCl +150 mg/l GA_3. Also variation in banding pattern occurred in different protein concentration up to certain level on SDS-PAGE. In case of the treatments T_0, T_1, T_2 and T_3 concentration showed same bands (7) but the molecular weight of proteins of that bands showed variation (Plate 2)

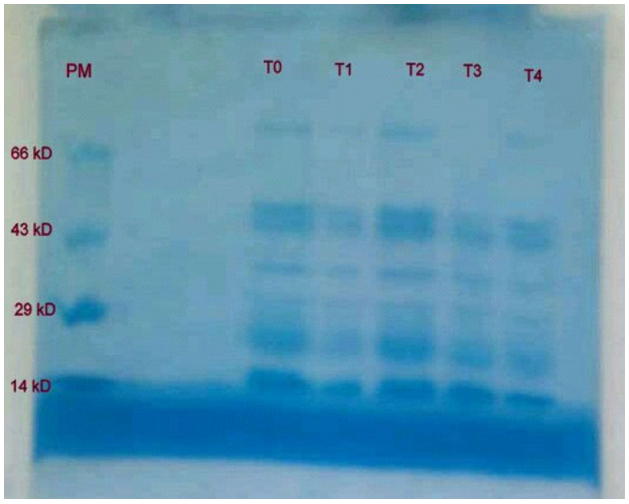

Plate 2 : SDS PAGE profile of the proteins

Molecular weight (k Da) of mung bean seed proteins as influenced by different GA3 concentrations under saline conditions (Table 4)

The above results showed that the proteins extracted from mung bean seeds showed maximum number of eight bands. The treatment T_4 was shown highest number of bands. However the bands showed proteins having slightly different molecular weight in some cases.

The mung bean seeds contain around 25 per cent to 30 per cent of protein and above results showed that under different concentrations of GA_3 under saline condition the protein content was affected due to the increase in GA_3 concentration. Seeds under salt stress were utilizing their storage and all other forms of protein to tolerate the salt stress and the germination was inhibited due to absence of all necessary proteins required for seed germination (Kumar *et al.,* 1983). However GA_3 stimulates m RNA to synthesize proteins necessary for seed germination. The change in banding pattern might be due to the effect of GA_3 concentrations under salt stress on seed germination and these expressed proteins functions as GA_3 stimulated proteins under saline condition (Iqbal *et al.,* 2001).

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