

## Morphological, Physiological and Biochemical Responses During Germination of the Cowpea (*Vigna unguiculata* Cv. Pitiuba) Seeds under Salt Stress

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**Abstract:** The study had the aimed at evaluating the morphological, physiological and biochemical responses, besides investigating the possible tolerance mechanism utilized by the *Vigna unguiculata* cv. Pitiuba seeds during germination under conditions of the severe and moderate salt stress (75 and 150 mM NaCl, respectively). The experimental design was randomized entirely, with 3 salt stress levels (0, 75 and 150 mM NaCl), in which was evaluated the germination percentage, mean germination time, seed fresh and dry matter, seed water content, root length, total soluble carbohydrates and total soluble proteins. The germination percentage was significantly affected by the salt stress, in which was showed 99, 98 and 95.8% of germination in the treatments under concentrations of 0, 75 and 150 mM NaCl, respectively. The root length was 100.1, 58.4 and 28.1 mm seedling<sup>-1</sup> in the treatments under salt stress of 0, 75 and 150 mM NaCl, respectively. The total soluble carbohydrates under salt stress of 0, 75 and 150 mM NaCl presented 517.6, 556.1 and 6.6 mg g<sup>-1</sup> DM, respectively. The results suggested that the cultivar Pitiuba tolerates moderate salt stress, because the carbohydrates are used to carry out the osmotic adjustment of the genotype in salt stress condition.

**Key words:** *Vigna unguiculata* • Saline stress • NaCl • Seed • Germination

### INTRODUCTION

The species *Vigna unguiculata* (L.) Walp. has as main characteristics be rustic, it presents high protein tenor in grain, besides of this, the crop is used in the Brazil in agricultural areas under environmental stress [1, 2], in which this areas presented small index of rainfall, as well as it high temperature and soils with low fertility and high salinity [3, 4].

The cultivar Pitiuba was developed by the Empresa Brasileira de Pesquisa Agropecuária (Embrapa-Meio Norte) with the objective of make possible the implantation of this species in areas that normally not might be explored by the others crops, besides it be an economic alternative for farmers installed in this regions. Studies carried out by Costa *et al.* [5] with two genotypes of *Vigna unguiculata* corroborated the high adaptability of the cultivar Pitiuba under unfavorable environmental conditions.

The stress is defined as all external factor that provokes loss in the yield or in the developments of a

species [6], in which the saline stress is important, because occurs with frequency in regions with small index rainfall and combined with high temperature and small air humidity, as well as the evaporation rate is higher, when compared with the infiltration rate of the water in the soil and this way, it provokes the salt accumulation, mainly NaCl, in the soil superficial layers [7].

The germination is a biologic process that demands as pre-requirement the seed viability, which it needs of structures and physiologic pathway ready to active the metabolism, besides external conditions specifics, in which the salt concentrations in the substrate can change the metabolic activity during the imbibition process of the seed [8].

The study had the aimed at evaluating the morphological, physiological and biochemical responses, besides investigating the possible tolerance mechanism utilized by the *Vigna unguiculata* cv. Pitiuba seeds during germination under conditions of the severe and moderate salt stress (75 and 150 mM NaCl, respectively).

## MATERIALS AND METHODS

**Plant material and seed treatments:** The *Vigna unguiculata* (L.) Walp. seeds cultivar Pitiuba were got in the Empresa Brasileira de Pesquisa Agropecuária (Embrapa Meio-Norte), harvested in the 2007 season. The experiment was conducted during the month of december of 2007, in the Laboratório de Fisiologia Vegetal Vegetal Avançada (LFVA) of the Universidade Federal Rural da Amazônia (UFRA), city of Belém, state Pará, Brasil.

The preparation of the seeds begun with harvest and removal of the impurities, after the seeds were imbibed in solution of 2-(metoxi-carbomol) benzimidazol ( $C_9H_7N_3O_2$ ) at 5 ppm by 5 min, subsequently it were placed on filter paper for drying by the period of 24 h and conditioned in plastic flasks under temperature of 10°C until the execution of the experiment [9].

**Experiment design and procedures:** The experimental design was randomized entirely, with 3 salt stress level (0, 75 and 150 mM NaCl), in which the salt stress was simulated with the concentrations of 5 and 150 mM NaCl, as well as was add up in the NaCl solutions (Sigma Chemicals) and distilled water autoclaved at 120 °C, 1 atm by 20 min. In the salt stress at 0 mM NaCl (control) was add up only distilled water.

The seeds were placed in plastic container transparent, type gerbox, with the dimensions (length×width×height; 20×20×4 cm), in which were previously lined with sterile sheets of filter paper, it were dampened with the solutions (NaCl and water) until to reach 2.5 times the dry paper weight. The containers containing the seeds were hermetically closed, after were placed put into germination chamber, type BOD, model TE-401 (Tecnal Equipamentos), with controlled temperature at 25°C and dark.

**Morphological and physiological measurements:** The germination, mean germination time (MGT) in the seeds were measured according Edmond and Drapala [10], describe by Silva and Nakagawa [11] and adapted in the Laboratório de Fisiologia Vegetal Avançada, in which was calculated as:

$$MGT = \sum SNGI \times IT / TNSG, \text{ where:}$$

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MGT = Mean germination time

SNGI = Seed number germinated in the interval

IT = Interval time

SNG = Total number of seed germinated

The seeds also were measured the fresh weight of germinated seed, dry weight of germinated seed, in which the seeds were placed in oven at 65° C by 72 h and after measured the dry weight, root length and seed water content, according Romero-Aranda *et al.* [12] and adapted in the Laboratório de Fisiologia Vegetal Avançada, in which was calculated as:

$$SWC = FWS - DWS$$

Where:

SWC = FWS – DWS, where

SWC = Seed water content

SFW = Seed fresh weight

SDW = Seed dry weight

The seeds were considered germinated when presented root length equal or higher at 1.0 cm and the readings were carried out in the 3rd, 5th and 7th day to evaluate mean germination time and only in the 7th day in the others parameters, in which the treatments were constituted by 6 repetitions and each experimental unit by 100 seeds.

**Biochemical measurements:** The germinated seeds were placed in oven at 65°C by the period of 72 h, in which it were triturated and the powder was collected to carry out the extraction. The total soluble carbohydrates extraction were make with 50 mg of powder and 5 mL of distilled water at 100°C by 30 min. In the total soluble proteins were utilized 100 mg of powder and 5 mL of Tris-HCl buffer at 25 Mm/pH 7.6, being shakes by 2 h at 25°C. After the extractions, the samples were centrifuged at 2000 rpm by 10 min and were collected the supernatants. The quantifications were carried out in spectrophotometer (Quimis, model Q798DP), in which the total soluble carbohydrates were quantified in agreement with Dubois *et al.* [13] at 490 nm and total soluble proteins according the method of Bradford [14] at 595 nm. The seeds were collected in the 7th day after the experimental implementation, with 6 repetitions and each experimental unit was compost by 20 seeds.

**Data analysis:** The variance analysis was applied in the results and when occurred significant difference the values were submitted by Tukey test at 5% significance, as well as were calculated standard error in all the points and treatments evaluated. The statistical analyses were performed with software SAS [15] and based on the statistical theories suggested by Gomes [16].

## RESULTS

**Germination and mean germination time:** The variance analysis reveals a significant reduction of the germination percentage of *Vigna unguiculata* seeds submitted to saline stress (Fig. 1 A), as well as the germination rates showed were 99, 98 and 95.8% in the treatments under concentrations at 0, 75 and 150 mM NaCl, respectively. This way, the progressive drop in the germination percentage is directly affected by the increase of the salt stress.

The mean germination time was significantly changed, according the ANOVA, in which the seeds submitted to saline stress at 0, 75 and 150 mM NaCl have the mean germination time of 3.96, 4.11 and

4.17 days, respectively (Fig. 1 B). The salt stress simulated in this experiment provoked the increase of the time necessary to germination *Vigna unguiculata* seeds.

**Fresh matter and dry matter:** The fresh weight of germinated seed was significantly modified, in which the treatments under influences of salt stress at 0, 75 and 150 mM NaCl presented the weights of 0.41, 0.50 and 0.41 g. germinated seed<sup>-1</sup>, respectively. The test applied reveals that the treatment under moderate salt stress (75 mM NaCl) is higher and statistically different of the others (Fig. 2 A), in which this treatment proves that the salt might be accumulated under conditions of salt stress during the germination process.

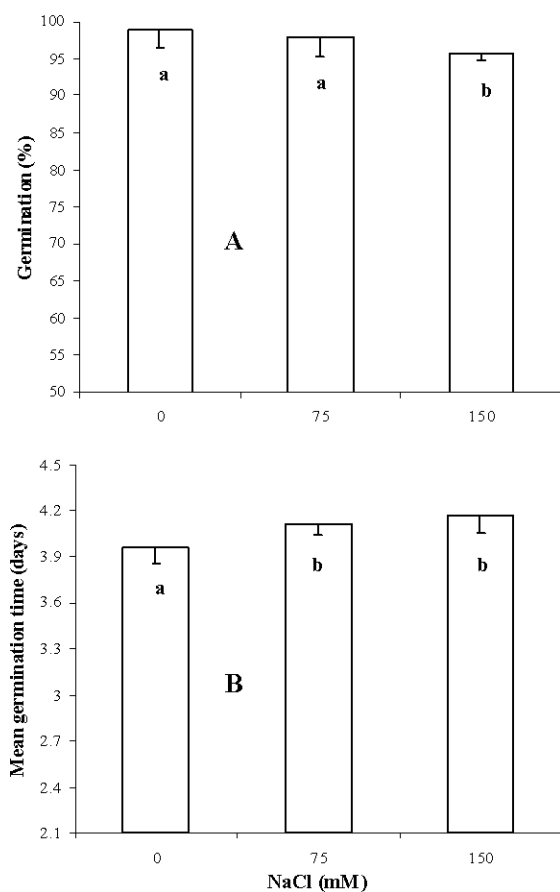


Fig. 1: Germination (A) mean germination time (B) in *Vigna unguiculata* (L.) Walp seeds cv. Pitiuba under salt stress at 0, 75 and 150 mM NaCl. Averages followed by the same letter do not differ among themselves by the Tukey test at 5% of probability. The bars represent the mean standard error

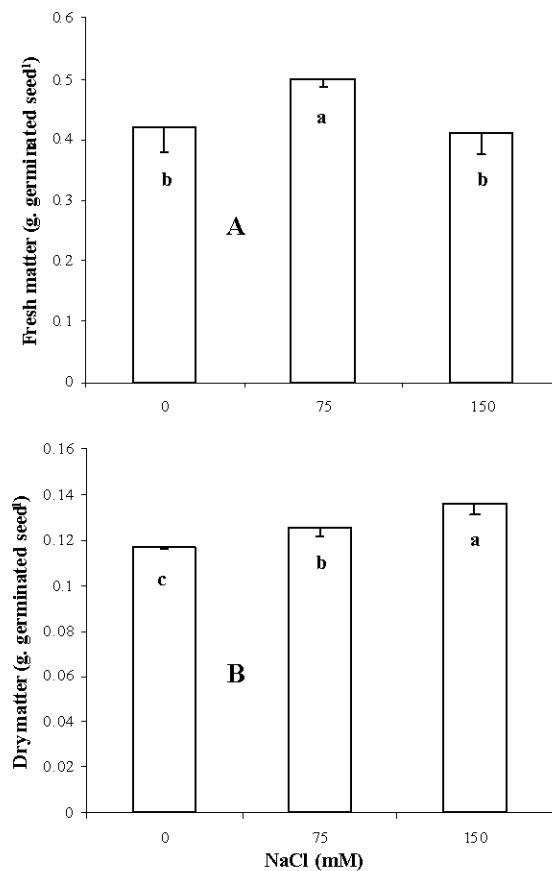


Fig. 2: Fresh matter (A) and dry matter (B) in *Vigna unguiculata* (L.) Walp seeds cv. Pitiuba under salt stress at 0, 75 and 150 mM NaCl. Averages followed by the same letter do not differ among themselves by the Tukey test at 5% of probability. The bars represent the mean standard error

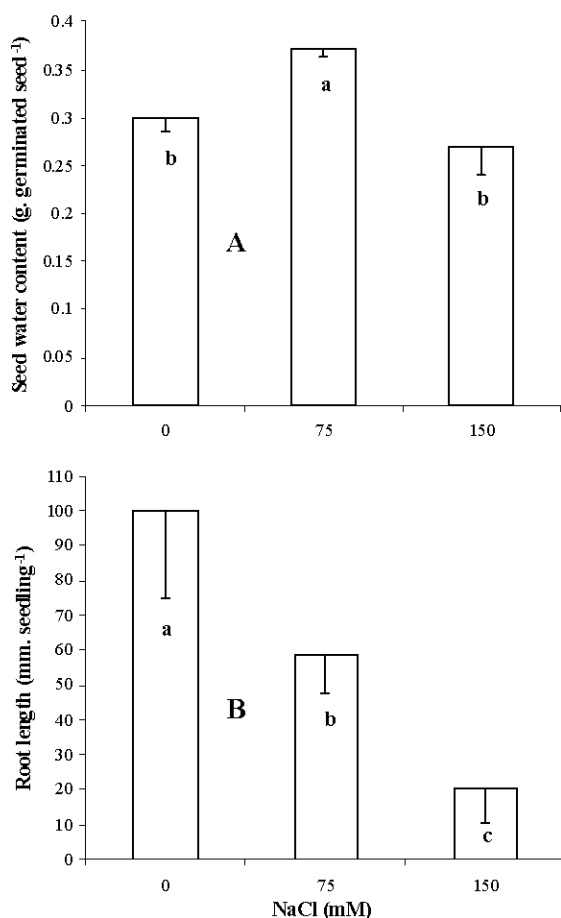


Fig. 3: Seed water content (A) and root length (B) in *Vigna unguiculata* (L.) Walp seeds cv. Pitiuba under salt stress at 0, 75 and 150 mM NaCl. Averages followed by the same letter do not differ among themselves by the Tukey test at 5% of probability. The bars represent the mean standard error.

The dry weight of germinated seed was significantly increased, in which was got the weights of 0.11, 0.12 and 0.13 g. germinated seed<sup>-1</sup> in the treatments submitted to saline stress at 0, 75 and 150 mM NaCl, respectively. The Fig. 2 B reveals that dry matter increased with the increase of the salt stress level.

**Water content and root length:** The seed water content was significantly modified by the action of salt stress induced to *Vigna unguiculata* seeds (Fig. 3 A), in which the treatments under concentrations at 0, 75 and 150 mM NaCl had the water content of 0.30, 0.37 and 0.27g. germinated seed<sup>-1</sup>, respectively. These results

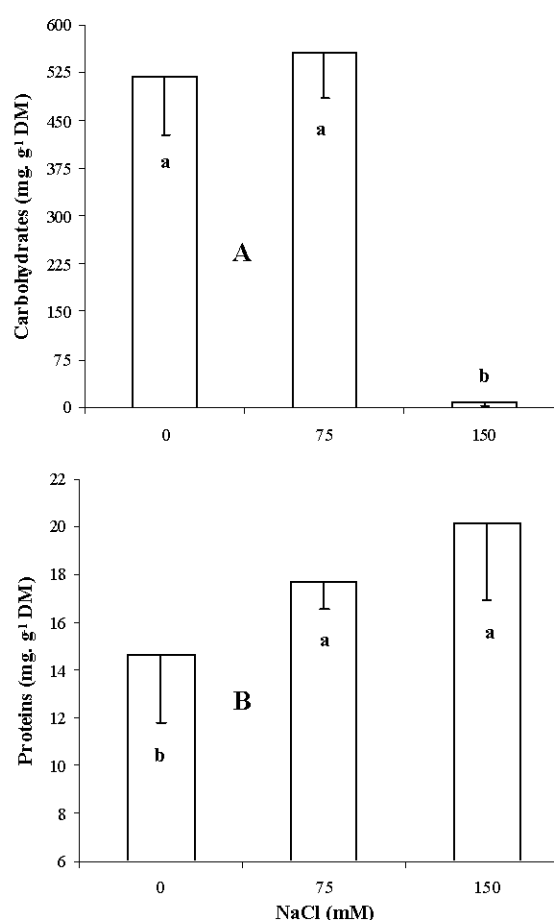


Fig. 4: Total soluble carbohydrates (A) and total soluble proteins (B) in *Vigna unguiculata* (L.) Walp seeds cv. Pitiuba under salt stress at 0, 75 and 150 mM NaCl. Averages followed by the same letter do not differ among themselves by the Tukey test at 5% of probability. The bars represent the mean standard error

reveal that the treatment that used the salt solution at 75 mM NaCl had higher seed water content, as well as it was statistically different of the others treatments.

The root length was influenced by the saline stress submitted during germination of this species (Fig. 3 B), in which the root lengths were 100.1, 58.4 and 28.1 mm. seedling<sup>-1</sup> in the treatments under salt stress at 0, 75 and 150 mM NaCl, respectively. These results reveal that the root length is strongly reduced with increase of the amount of salt in the substrate.

**Total soluble carbohydrates and total soluble proteins:** The total soluble carbohydrate levels of the

*Vigna unguiculata* seeds were significantly modified, according the variance analysis, in which the treatments under saline stress at 0, 75 and 150 mM NaCl present 517.6, 556.1 and 6.6 mg g<sup>-1</sup> DM, respectively (Fig. 4 A). These results reveal increase of the total soluble carbohydrate levels under moderate salt stress (75 mM NaCl) and strong drop in the levels of this parameter under severe salt stress (150 mM NaCl), when compared with control treatment (0 mM NaCl).

The total soluble protein level presents in the seeds was influenced by the salt concentration in the substrate, in which the treatments with 0, 75 and 150 mM NaCl were showed the values of total soluble proteins of 14.6, 17.6 and 20.0 mg g<sup>-1</sup> DM, respectively (Fig. 4 B). This way, the proteins levels were increased with the increase of the salt stress artificially induced by the NaCl in the substrate. Fig. 4.

## DISCUSSION

The progressive drop showed in the germination of *Vigna unguiculata* seeds cultivar Pitiuba was provoked by the salt stress, in which this stress probably damaged the seed hydration and depending of the salt concentration present in the substrate might occurs an effect toxic in the embryo and endosperm membrane responsible by the water assimilation and translocation [17]. Moreover, the solutions (NaCl + water) used in this experiment to simulate artificially the saline stress (moderate and severe) carried out different water potentials in the substrate and consequently different water conductancies, in which it interferes in the capacity of water absorption by the seed [18]. Similar results were found by Dantas *et al.* [19] working with *Phaseolus vulgaris* seeds under saline stress.

The increase in the mean germination time of seed was promoted by the progressive reduction of the water conductivity in the substrate, in which the activity of the hydrolytic enzyme is depends of the supplement and assimilation velocity of water. Study carried out by Uriyo [20] found high correlation among the  $\alpha$ -amylase activity and the germination process of *Vigna unguiculata* seeds. The  $\alpha$ -amylase and  $\beta$ -amylase are the mains enzymes responsible by the carry out the starch hydrolysis, as well as these enzymes liberate the ATP that will be used in the root protrusion and consequently the seed germination [21]. Results on the increase in the mean germination time were found by Lobato *et al.* [22] investigating water stress in *Sorghum bicolor* seeds.

The increase and maintenance of the seed fresh matter is due the tolerance in conditions under salt stress, in which the treatment under moderate stress (75 mM NaCl) was showed an increase in this parameter and under severe stress (150 mM NaCl) was showed maintenance of the fresh matter, when compared with control treatment (0 mM NaCl). These results reveal the tolerance of *Vigna unguiculata* cultivar Pitiuba to saline stress and demonstrate that the utilization of solutions until 75 mM NaCl can significantly increase the seed fresh matter. Studies conducted by Sivritepe *et al.* [23] indicate that the application of saline solutions in small concentrations might prevent the membrane damage during germination. Similar results on increase of fresh matter were found by Dantas *et al.* [24] evaluating the saline stress effects in three cultivars of *Vigna unguiculata*.

The progressive increase in the seed dry matter is due the increase in the salt concentration in the substrate, in which the NaCl is accumulated in the seed cotyledon walls and consequently it increase the seed dry matter [25]. During the seed imbibition process occurs the absorption of solution that contain NaCl and water, in which the salt acts directly in the biochemical processes linkages the seed germination and depending of the intensity of the saline stress occurs until deactivation/denaturation of the enzymes [26].

Saline stress at 75 and 150 mM NaCl promote increase and decrease, respectively, in the seed water content, when compared with the control treatment (0 mM NaCl). Indicating that in the salt stress at 75 mM NaCl occurs absorption of solution (NaCl and water), but this absorption not provokes intoxication by the ion accumulation (NaCl) in the seed cotyledon walls. Moreover, it proves the tolerance of the cultivar Pitiuba in moderate salt stress [27].

The minor growth of rootlets showed with the increase of the severity of the salt stress occurred due the smaller rate of cell division and probable ethylene production, in which this hormone presents as consequence the smaller growth of the seedling shoot. Besides of this, it provokes the reduction of the length and increase the diameter of rootless [28]. The results showed in this experiment on the reduction of rootless growth are similar with the found by Rodrigues *et al.* [29] investigating the seed germination of *Oryza sativa* and Dantas *et al.* [19] with *Phaseolus vulgaris*.

The increase in the total soluble carbohydrates levels of the treatment under moderate stress (75 mM NaCl), compared with control treatment, it was provoked by the two factors, in which as main factor might be considered the starch hydrolysis promoted by the activity of the  $\alpha$ -amylase enzyme and consequently production of several soluble carbohydrates [20]. Simultaneously as secondary factor occurred the osmotic adjustment promoted by the *Vigna unguiculata* seed cultivar Pitiuba, because with the increase of this organic solutes the water flux remains of substrate in direction to seed and consequently the water conductivity remains constant and adequate with the objective of keeps the water supplement. However the strong drop in the total soluble carbohydrates levels under action of severe salt stress (150 mM NaCl), compared with control treatment, it corresponds the not starch degradation and the not possibility of to carry out the osmotic adjustment, because under this condition probably there be intoxication of the cell walls and of the several seedling structures as embryo and hypocotyl radicle axis, besides deactivation of the enzymes [8].

The reduction in the total soluble proteins levels showed in the control (0 NaCl), when compared with the treatment under severe salt stress (150 mM NaCl), it demonstrates that under normal conditions occurs drop in this parameter during the germination. Beside of this, the grain of this species is rich in protein, in which is necessary to use the pathway of the protein degradation with the objective of forms enzymes, amino acids, nucleic acids (DNA and RNA) and others proteins [30] during the germination process. In the treatment under action of 75 mM NaCl occurs moderate reduction, compared with the treatment under severe stress (150 Mm NaCl), because as the seedling structures suffer reductions, an example is the root length, the demand by nitrogen compounds (enzymes, amino acids, nucleic acids and proteins) also is small, it promotes consequently smaller breakdown of total soluble proteins [31]. Similar results on the drop in the protein levels in normal conditions (control) were found by Corte *et al.* [32] studying the mobilization of reserves in *Caesalpinia peltophoroides* seeds.

The results suggest that the cultivar Pitiuba supports the moderate salt stress, because in this cultivar was showed through of biochemical parameters the capacity of this genotype carry out the osmotic adjustment and consequently decreasing the salt stress effects on morphological and physiological characteristics during the seed germination process.

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