Asian Journal of Plant Sciences

ISSN 1682-3974

science alert

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OPEN ACCESS Asian Journal of Plant Sciences

ISSN 1682-3974
DOI: 10.3923/ajps.2025.262.269

Research Article

Impact of Salt Stress on Chlorophyll, Carotenoids, Proline, Phenolics, Flavonoids and Antioxidant Activity in Yam Bean (*Pachyrhizus erosus* L. Urban)

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Abstract

Background and Objective: Salt stress is a major abiotic factor limiting plant growth and productivity, yet the physiological and biochemical responses of yam bean (*Pachyrhizus erosus* L. Urban) under salinity remain poorly understood. This study aimed to evaluate the effects of salt stress on chlorophyll, carotenoids, proline, phenolic, flavonoid and antioxidant activity in yam bean seedlings. **Materials and Methods:** Seedlings were germinated in sponges for 10 days and then transferred to Hoagland's nutrient solution supplemented with NaCl at concentrations of 0, 25, 50, 75 and 100 mM. Leaves and roots were harvested to measure chlorophyll, carotenoids, proline, phenolic, flavonoid contents and antioxidant activity. Data were analyzed using Microsoft Excel for basic statistics (mean, percentage, SD) and SPSS for One-way ANOVA and Duncan's Multiple Range Test (DMRT) at a 95% confidence level. **Results:** Salt stress reduced chlorophyll and carotenoid contents, with chlorophyll a, chlorophyll b and total chlorophyll decreasing by 23.72, 28.50 and 17.58%, respectively, at 75 mM NaCl compared to control. Proline accumulation increased with rising NaCl levels, reaching a maximum of 0.16 µg/g FW (20.71% increase) at 75 mM. Phenolic and flavonoid contents showed variable responses, with the highest levels in leaves at 50 mM NaCl (22.82 and 38.35 mg/g FW, respectively). Antioxidant activity was highest in leaves, peaking at 79.60% under 75 mM NaCl. Exposure to 100 mM NaCl for 45 days proved lethal to plants. **Conclusion:** Yam bean exhibits differential biochemical responses to salt stress, including reduced pigment levels, increased proline and enhanced antioxidant activity, highlighting its adaptive mechanisms. These findings contribute to understanding yam bean tolerance to salinity and may inform future strategies for cultivation under saline conditions.

Key words: Yam bean, salt stress, chlorophyll, proline, phenolic

Citation: Maneechai, S., R. Phannaen, O. Phuseerit and S. Chookhampaeng, 2025. Impact of salt stress on chlorophyll, carotenoids, proline, phenolics, flavonoids and antioxidant activity in yam bean (*Pachyrhizus erosus* L. Urban). Asian J. Plant Sci., 24: 262-269.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Yam bean (*Pachyrhizus erosus* (L.) Urban), family Leguminosae[1]. Yam bean is a legume with economic value, widely grown in tropical and subtropical regions, including the Northeastern Region of Thailand. The roots of this plant are often eaten as food. Moreover, the extract from the root of the yam has skin-lightening properties, which is why Indonesians use it in cosmetics[2]. In addition, many countries in the Americas and Asia consume yams as a vegetable or fruit root[3]. The nutritional value of 100 g of fresh yam bean tuber has been found to contain many bioactive compounds that are beneficial to health[4].

Salt-affected soils adversely affect agricultural areas in over 100 countries. Globally, approximately 831 million hectares (Mha) are affected by salt. Of the total world arable land, approximately 77 Mha are affected by salinity or salinized soils, with further increases expected in many parts of the world[5-7]. High concentrations of soluble salts reduce the concentration of chlorophyll and other photosynthetic pigments, such as carotenoids[8,9]. Salt stress also causes increased accumulation of abscisic acid, resulting in stomata closure and a reduced transpiration rate[10]. Under salt stress, there is an increased accumulation of abscisic acid that may affect the depolarization of stomatal guard cells and hydrogen peroxide production. Excessive production of hydrogen peroxide tends to result in the production of free radicals (ROS), which induce oxidative stress in plants[11]. Salt stress induces the production of ROS within plant cells, which may lead to cell dysfunction and death. Plants therefore embark on an adaptive response as a defense mechanism against stress damage by inducing the synthesis of secondary metabolites that act as antioxidants, particularly polyphenolic compounds[12]. Another way plants adapt to salt stress involves the accumulation of osmotic regulating solutions containing sugars and proline[9].

The objective of this research was to determine the phytochemical content of yam bean under salt stress, including the chlorophyll, carotenoids, proline, phenolics, flavonoids and antioxidants. The results of this study reveal the effects of salt stress on the phytochemicals, which may provide guidelines for the development of yam bean cultivation. Furthermore, the results of this study may help clarify the potential health benefits of yam consumption, which will promote its use in dietary supplements or functional foods.

MATERIALS AND METHODS

Study area: Greenhouse, Department of Biology, Faculty of Science, Mahasarakham University, during October, 2024-February, 2025.

Plant material: Yam bean *Pachyrhizus erosus* (L.) Urban.) seed was sourced from KHP Agro Group.

Seedling preparation: Seeds for analysis of phenolic and flavonoid compounds and antioxidants were prepared by soaking in water at 40°C for three hours. The seeds were then soaked in 5% Sodium Hypochlorite (NaOCl) for 30 minutes and rinsed 2-3 times with distilled water. Seeds were then germinated in petri dishes using the between-paper (BP) method[13], with 20 seeds per dish. Using three layers of tissue paper, 16 mL of sodium chloride solution at concentrations of 0, 25, 50, 75, 100, 150 and 200 mM in each experimental and were kept in a dark place at 25°C for 10 days. Add sodium chloride solution every three days to maintain humidity by spraying the sodium chloride solution.

Hydroponic cultivation under salt stress: Yam bean seeds were prepared by soaking them in warm water at 40°C for three hours. The seeds were then soaked in 5% Sodium Hypochlorite (NaOCl) for 30 minutes and rinsed with distilled water 2-3 times. The cleaned yam bean seeds were then germinated in 1x1x1-inch sponges and kept at 25°C for 10 days. The seedlings were then transplanted using the Deep Floating Technique (DFT)[14]. The plants were grown on 1-inch-thick foam pads placed in three-liter black plastic containers and the roots were immersed in Hoagland's solution[15]. The NaCl solutions at concentrations of 0, 25, 50, 75 and 100 mM were added 10 days after transplanting. Plant samples were collected 45 days after the addition of the NaCl solution for analysis of chlorophyll content and antioxidant activity. The experiment was performed as three replicates in each experimental set.

Pigment quantification: Extraction was performed using 100% acetone from the leaves. The leaves were ground with acetone in a mortar and pestle, a small amount of Magnesium Carbonate ($MgCO_3$) was added and the solution was centrifuged at room temperature for 3-5 minutes at 300-500×g to obtain a clear solution. The absorbance

was measured at 661.6, 644.8 and 470 nm[16]. Equation for calculating absorbance (A): Pigment concentration (C) in µg per mL of extract solution:

Chl a: Ca = 11.24 A661.6-2.04 A644.8

Chl b: Cb = 20.13 A644.8-4.19 A661.6

Total carotenoids (x+c): C(x+c) = (1000 A470-1.90 Ca-63.14 Cb)/214

Preparation of plant material: The extract was prepared by washing the beans with distilled water, drying them at room temperature and then cutting them into small pieces. After that, the plant sample was ground in a mortar and weighed 100 g diluted with 500 mL of 95% ethanol (1:5) stored in a dark place for two hours at room temperature. It was then filtered with cotton wool to remove the residue and filtered with No. 1 filter paper to remove the powder. The extract was evaporated on a hot plate to obtain a viscous extract, which was then dried in a hot air oven for 1-2 days until the mixture was completely dry[17].

Proline content: The proline content was analyzed by collecting yam leaf samples at the position of the 3rd leaf from the shoot and the position of the 5th leaf from the shoot. Approximately 0.1 g of fresh plant samples were weighed, ground with 10 mL of 3% sulfosalicylic acid and filtered with No. 1 filter paper. Then, 2 mL of the filtrate was added, 2 mL of Acid-Ninhydrin and 2 mL of Glacial Acetic acid were added and heated at 100°C for one hour and the reaction was ended in an ice bath at 0°C for 10 minutes. Then, 4 mL of toluene was added to the reaction mixture and shaken for 15-20 seconds to cause the solution to separate. The upper solution was then aspirated and measured for absorbance at 520 nm with a UV-Vis spectrophotometer, using toluene as a blank and a standard proline solution of 1 mg/L. A standard solution was made by serial dilution of the solution and then used to calculate the amount of proline from the standard curve[18].

Phenolic content: Using the Folin-Ciocalteu Method[19], 200 µL of the extracts from the germinated seeds, leaves and roots of yam bean were added to 500 µL of 10% Folin-Ciocalteu. The extract was shaken and left at room temperature for five minutes. Then, 800 µL of 7.5% $NaHCO_3$ was added. The mixture was mixed and left at room temperature for 30 minutes. The absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 765 nm using ethanol as the blank (three replications). Gallic acid was used as a standard for comparison. A 1 mg/mL standard was prepared and the absorbance was measured at various wavelengths. The absorbance of the extract was analyzed for phenolic compounds. All were compared with a standard curve of gallic acid in the form of milligrams equivalent of gallic acid per gram of extract (mg GAE/g extract) to calculate the total phenolic compounds (y = 5.1645x+0.1596; R2 = 0.981) expressed in milligrams equivalent of gallic acid per gram of extract (mg GAE/g extract).

Flavonoid content: Following Prommuak *et al.*[19], 500 µL of the extracts from sprouted seeds, leaves and roots of yam bean were mixed with 1,500 µL of 95% ethanol. Then, 100 µL of 10% $AlCl_3$ and 100 µL of 1M potassium acetate were added. The volume was adjusted to 5,000 µL with distilled water and stored at room temperature for 30 minutes. The absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 415 nm, using deionized water as the blank (three replications). Quercetin was used as a standard solution for comparison, prepared at 1 mg/mL. The absorbance was measured at various wavelengths and the absorbance of the extracts was analyzed for total flavonoid content against a standard curve. The quercetin content was expressed in milligrams of quercetin equivalent per gram of extract (mg QE/g extract). The total flavonoid content (y = 7.4122x+0.0099; R2 = 0.9999) was calculated in milligrams of quercetin equivalent per gram of extract (mg QE/g extract).

Antioxidant activity: Antioxidant activity was assayed using the DPPH method[20]. A 100 µM DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) solution was prepared in methanol. Then, pipette 20 µL of the extract and add 180 µL of DPPH solution to a 96-well plate. Incubate in the dark at room temperature for 30 minutes and measure the absorbance using a microplate reader at a wavelength of 517 nm. Methanol was used as the blank. Three replicates were performed, using vitamin C (ascorbic acid) as a standard for comparison. To prepare the standard, a 1 mg/mL standard was diluted using serial dilutions and the percent inhibition was calculated.

$$\text{Percent inhibition} = \frac{AB - AE}{AB} \times 100$$

where, AB represents the absorbance of the blank and AE represents the absorbance of the extract.

A graph was created to show the relationship between the percent antioxidant activity (free radical inhibition) and the sodium chloride concentration for each experiment and

used to calculate the concentration from a standard solution of vitamin C (ascorbic acid). The absorbance value was used to calculate the percentage of inhibition of the free radicals (% inhibition).

Experimental results analysis: Data were analyzed using basic statistics using Microsoft Excel, including mean ($\bar{x}$), percentage (%), standard deviation (SD), One-way ANOVA and Duncan's Multiple Range Test (DMRT) at a 95% confidence level using the SPSS statistical software[21].

RESULTS

When the plants received salt for 45 days, they died, so there are no data for the 100 mM concentration of NaCl. Chlorophyll content analysis revealed that increasing salt concentrations decreased the chlorophyll and carotenoid contents. A 0 mM showed the highest chlorophyll a, chlorophyll b and total chlorophyll contents, at 22.26, 8.21 and 4.72 mg/mL, respectively. A 75 mM showed the lowest chlorophyll a, chlorophyll b and total chlorophyll contents, at 16.98, 5.87 and 3.89 mg/mL, respectively, representing decreases of 23.72, 28.50 and 17.58% compared to the control. In terms of carotenoid content, it was found that at 0 mM, the carotenoid content was the highest, which was 13.40 mg/mL and at 75 mM, the carotenoid content was the lowest, which was 10.42 mg/mL, which was a 22.24% decrease compared to the control. Proline analysis results revealed the highest proline content at 75 mM NaCl, at 6.18 µg/g fresh weight. This was followed by 50, 25 and 0 mM, with proline contents of 5.93, 5.63 and 4.90 µg/g fresh weight, respectively (Table 1). It can be seen that the proline content at NaCl concentration of 75 mM was increased from the control group by 20.71%.

In this study, extracts were obtained from germinated seeds, stems, leaves and roots of yam bean. It was found that when the plants were exposed to salt for 45 days at a concentration of 100 mM, the plants died, making it impossible to collect leaves and roots for extraction. The extractable amount in each treatment ranged from 5.70 to 11.80%. The net weight of the extract was then calculated. The total net weight of the extract from sweet potato pea seedlings, leaves and roots in the control group was 20.78%, while in the groups receiving NaCl at concentrations of 25, 50 and 75 mM, the net weights were 19.91, 23.46 and 28.23%, respectively. The extraction results are shown in Table 2.

Table 1: Chlorophyll contents, carotenoid contents and proline contents in yam bean leaves under different sodium chloride concentrations

	Chlorophyll contents (µg/mL)				
NaCl concentration (mM)	A	B	Total	Carotenoid contents (mg/mL)	Proline contents (µg/g FW)
0	22.26±1.46^{a}	8.21±1.03^{a}	4.72±3.18^{a}	13.40±1.19^{a}	4.90±0.22^{a}
25	18.62±3.02^{ab}	6.97±1.21^{ab}	4.17±0.40^{ab}	11.58±1.47^{ab}	5.63±0.23^{a}
50	18.72±2.25^{ab}	6.59±0.86^{ab}	4.04±0.44^{b}	11.16±1.27^{ab}	5.93±1.28^{a}
75	16.98±0.83^{b}	5.87±0.11^{b}	3.89±0.27^{b}	10.42±0.51^{b}	6.18±0.70^{a}

Different letters, a and b, in vertical direction indicate statistically significant differences ($p<0.05$)

Table 2: Net weight percentage of extracts from leaves, roots and germinated seeds of yam bean under different sodium chloride concentrations

NaCl (mM)	Plant part	Weight of plant used (g)	Extract weight (g)	Yield (%)
0	Seedling	1	0.07	7.2
	Leaf	10	0.79	7.88
	Root	2	0.11	5.70
Total	20.78			
25	Seedling	1	0.06	6.1
	Leaf	10	0.70	6.99
	Root	5	0.34	6.82
Total	19.91			
50	Seedling	1	0.06	6.1
	Leaf	10	0.74	7.43
	Root	4	0.40	9.93
Total	23.46			
75	Seedling	1	0.09	9.2
	Leaf	8	0.58	7.23
	Root	2	0.24	11.80
Total	28.23			
100	Seedling	1	0.06	6.4
150		1	0.09	9.1
200		1	0.10	9.9

Table 3: Phenolic and flavonoid compounds from seedling, leaves and roots of yam bean under different sodium chloride concentrations

Plant part	NaCl (mM)	Total phenolic compounds (mg/g extract)	Total flavonoid compounds (mg/g extract)
Seedling	0	7.00±0.02^{f}	2.57±0.03^{c}
	25	10.83±0.30^{c}	3.15±0.11^{b}
	50	11.00±0.21^{c}	5.02±0.03^{a}
	75	9.60±0.0.06^{d}	2.12±0.03^{e}
	100	8.29±0.24^{e}	2.39±0.08^{d}
	150	12.03±0.07^{b}	2.13±0.00^{e}
	200	12.90±0.11^{a}	1.85±0.07^{f}
Leaf	0	4.40±0.07^{d}	23.21±0.09^{c}
	25	20.63±0.03^{b}	27.01±0.28^{b}
	50	22.82±0.18^{a}	38.35±0.32^{a}
	75	15.28±0.10^{c}	20.11±1.12^{d}
Root	0	11.76±0.29^{b}	1.26±0.00^{a}
	25	15.22±0.12^{a}	1.29±0.03^{a}
	50	7.89±2.03^{c}	0.57±0.00^{d}
	75	4.76±0.43^{d}	0.49±0.03^{c}

Different letters, a and b, in vertical direction indicate statistically significant difference ($p<0.05$)

Table 4: Antioxidant activity from seedling, leaves and roots of yam bean under different sodium chloride concentrations

Plant part	NaCl (mM)	Inhibition (%)
Seedling	0	10.77±1.09^{a}
	25	6.96±1.07^{bc}
	50	6.40±0.70^{bcd}
	75	7.84±1.00^{b}
	100	4.93±1.80^{cd}
	150	6.77±1.12^{bcd}
	200	4.48±2.70^{d}
Leaf	0	4.85±1.92^{c}
	25	8.98±1.10^{b}
	50	9.49±0.66^{b}
	75	23.77±1.35^{a}
Root	0	9.38±2.28b^{c}
	25	13.30±1.18^{a}
	50	11.34±0.72^{ab}
	75	7.49±44^{c}
Vitamin C		0.04

Different letters, a and b, in vertical direction indicate statistically significant difference ($p<0.05$)

The experiments revealed the highest phenolic content in the seedling fraction at a concentration of 200 mM, with 12.90 mg/g of gallic acid equivalents per gram of extract. The lowest phenolic content was found in the control, with 7.00 mg/g of gallic acid equivalents per gram of extract. The highest phenolic content in the leaf fraction at a concentration of 50 mM was found at 22.82 mg/g of gallic acid equivalents per gram of extract. The lowest phenolic content was found at a concentration of 0 mM, with 4.34 mg/g of gallic acid equivalents per gram of extract. In the root part, it was found that at the concentration of 25 mM, there was the highest phenolic compounds, which was 15.22 mg/g equivalent of gallic acid per gram of extract. The least amount was at the concentration of 75 mM, which had phenolic compounds at 4.76 mg/g equivalent of gallic acid per gram of extract. The experiment revealed that the flavonoid content of the seedling fraction at a concentration of 50 mM was the highest, at 5.02 mg/g quercetin equivalent per gram of extract. The least was at a concentration of 200 mM, at 1.85 mg/g quercetin equivalent per gram of extract. The highest flavonoid content of the leaf fraction at a concentration of 50 mM was 38.35 mg quercetin equivalent per gram of extract. The least was at a concentration of 75 mM, at 20.11 mg quercetin equivalent per gram of extract. In the root part, it was found that at the concentration of 25 mM, there was the highest amount of flavonoid compounds, which was 1.29 mg/g quercetin equivalent per gram of extract. The least amount was at the concentration of 75 mM, which had flavonoid compounds at 0.49 mg/g quercetin equivalent per gram of extract (Table 3).

The study found that the seedling extract at a concentration of 0 mM exhibited the best antioxidant activity, with a free radical scavenging percentage of 10.06. The least antioxidant activity was found at 200 mM, with a free radical scavenging percentage of 4.48. The leaf extract at a concentration of 75 mM exhibited the best antioxidant

activity, with a free radical scavenging percentage of 79.60. The least antioxidant activity was found at 0 mM, with a free radical scavenging percentage of 4.85. For the root extract fibers, the 25 mM concentration had the best antioxidant activity, with a free radical inhibition percentage of 13.30, while at 75 mM it had the least antioxidant activity, with a free radical inhibition percentage of 7.49 (Table 4).

DISCUSSION

The amount of photosynthetic pigments in the leaves revealed that the group without the sodium chloride solution had the highest chlorophyll and carotenoid contents. Increasing sodium chloride concentrations resulted in a decrease in the chlorophyll and carotenoid contents. From the test for the amount of pigments used in the photosynthesis process in leaves, it was found that when the concentration of sodium chloride increased, the amounts of chlorophyll a, chlorophyll b, total chlorophyll and carotenoids decreased. The decrease in the amount of these pigments used in the photosynthesis process may be due to the toxicity of the salt[22,23]. Stress due to oxidation causes plant cell degeneration and changes in cytoplasmic function, causing the leaves to have reduced chlorophyll and carotenoid contents under salt stress[24]. Under salt stress, a major process affected in plants is photosynthesis. Increasing salinity levels decrease the rate of carbon fixation and results in a decrease in photosynthetic pigments, chlorophyll and carotenoids, the levels of which are indicators of photosynthetic activity. Several studies have indicated the effect of salinity on pigments[25].

The leaf proline content revealed higher proline content in the group treated with sodium chloride solution than the group without sodium chloride solution. The group treated with the sodium chloride solution at 25 mM had the highest proline content in the leaves, which was a 20.71% increase over the control. The proline content of the leaves was found to be higher in the group treated with sodium chloride solution than in the group not treated with the sodium chloride solution. When plants are stressed, they produce more proline because proline enhances stress tolerance by lowering the osmotic potential of cells below the extracellular water potential, thus enabling plants to absorb water better. In addition, the synthesis of proline from glutamate helps maintain the proper NADPH/$NADP^+$ balance in chloroplasts, which can prevent chloroplast damage and help plants survive under stress conditions. According to the study of Zhang and Becker[26], it was found that the amount of proline accumulated in leaves depends on the age of the leaves as when plants are exposed to salt, old leaves have a higher salt content than young leaves. As a result, old leaves produce more proline than young leaves to prevent damage to the leaf membrane, which helps the plants to survive[27].

As salt concentration increased, phenolic compounds also increased in seedlings. In leaves and roots, phenolic compounds were highest at NaCl concentrations of 50 mM and 25 mM, respectively. Similar to the flavonoid content, when plants were exposed to salt at 50 mM NaCl, both seedlings and leaves had the highest flavonoid content. Plants under salt stress tend to have increased ROS free radical scavenging activity. Plants produce phenolics and flavonoids to protect against ROS damage. It was also found that at 50 mM, plants adapted well due to the production of both compounds in high amounts. However, at too high a salt concentration (75 mM), plants were damaged by stress because at 100 mM, plants died. Increasing NaCl concentrations caused an increase in leaf necrosis, phenolic compounds and flavonoids, suggesting that phenols and flavonoids play an important role in plant defense mechanisms against the toxic effects of NaCl. Phenolic compounds exhibit antioxidant activity by generating hydrogen atoms and inactivating free radicals. Flavonoids can also act as antioxidants to scavenge ROS and protect plants from oxidative damage. Therefore, increasing the content of phenols and flavonoids can enhance the antioxidant activity of plants[28]. Under abiotic stress, phenolic acids and flavonoids, a group of plant secondary metabolites, are increased in plants acting as stress-protective agents, which help plants cope with environmental constraints[29,30]. When plants were exposed to salt for 45 days, antioxidant activity was increased in the leaves at a 75 mM NaCl concentration, as well as in the roots at 25 mM and 50 mM concentrations. The relationship between antioxidant capacity and plant salinity tolerance has been observed in many plant species, including salinity-tolerant glycophytes[31].

CONCLUSION

Increasing NaCl concentrations decreased chlorophyll and carotenoid contents, but increased proline content in leaves. Phenolic compounds in seedlings and leaf antioxidant activity were also increased. Plants exposed to 50 mM NaCl showed the highest flavonoid contents in seedlings, leaves and roots. Plants have adapted to accumulate certain substances when under tress in an attempt to protect the plant from death.

SIGNIFICANCE STATEMENT

This study discovered the differential biochemical responses of yam bean to salt stress, including reduced chlorophyll and carotenoids, increased proline accumulation and enhanced antioxidant activity, that can be beneficial for improving salinity tolerance in leguminous crops. By revealing how yam bean adapts to varying NaCl concentrations, this study will help researchers uncover the critical areas of plant stress physiology and biochemical adaptation that many researchers were not able to explore. Thus, a new theory on salinity resilience mechanisms in yam bean may be arrived at.

ACKNOWLEDGMENT

This research project was financially supported by Mahasarakham University, Thailand.

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