*Maejo Int. J. Sci. Technol.* **2018**, *12*(02), 89-100

*Maejo International*
*Journal of Science and Technology*

**ISSN 1905-7873**
Available online at www.mijst.mju.ac.th

***Full Paper***

# Effects of sodium chloride on germination, growth, relative water content, and chlorophyll, proline, malondialdehyde and vitamin C contents in Chinese white radish seedlings (*Raphanus sativus* L. var. *longipinnatus* Bailey)

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*Received: 14 January 2017 / Accepted: 19 April 2018 / Published: 2 May 2018*

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**Abstract:** Effects of sodium chloride (NaCl) on germination, growth, relative water content (RWC), as well as chlorophyll, proline, malondialdehyde (MDA) and vitamin C contents in the seedlings of Chinese white radish specimens *(Raphanus sativus* L. var. *longipinnatus* Bailey) were investigated. The seeds were sown on fine sand culture for 7 days. Fifty mL of NaCl solution at concentrations of 0, 100 and 200 mM were applied to the culture each day during the length of the experiment for 7 days, resulting in the electrical conductivity (using 1:5 soil:water method) of the sand culture being at 0.06-0.07, 0.4-0.8 and 0.8-1.6 dS/m respectively. Specific parameters, viz. germination, growth, RWC, and chlorophyll, proline, MDA and vitamin C contents of the shoots of 7-day-old seedlings, were evaluated. The results show that 100 mM NaCl increases the shoot fresh weight while other parameters show the same tendency when compared to the control. Conversely, 200 mM NaCl significantly decreases germination, growth, RWC and chlorophyll content but increases proline, MDA and vitamin C contents by 132%, 53% and 82% respectively when compared to the control.

**Keywords:** Chinese white radish, *Raphanus sativus* L. var. *longipinnatus* Bailey, salt stress

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## INTRODUCTION

Chinese white radish (*Raphanus sativus* L. var. *longipinnatus* Bailey), which is also referred to as Kaware daikon in Japan, is the vegetable that has the highest per capita rate of consumption within the Brassicaceae family. The seedlings of this vegetable are a cultural favorite due to their unique flavour and taste. In Thailand small seedlings of this plant (10-12 cm high) are widely distributed in supermarkets and broadly consumed. Furthermore, these small seedlings are easy to grow and possess a high nutritional value. Notably, several bioactive compounds are present in high

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amounts in the seedlings of *Raphanus sativus*, such as sinapinic acid esters, flavonoids [1], phenolic compounds [2] and antioxidants [3]. This seems to indicate that the seedlings of this vegetable possess a high potency in terms of their radical scavenging activity, which can provide a protective effect on human health. In addition a particular group of health promoting compounds known as glucosinolates has been found to be present in *R. sativus* sprouts [4-6]. These secondary plant metabolites and their derivatives have been shown to anti-cancer properties by increasing the cellular intrinsic mechanism that deactivates potential carcinogens and reactive oxygen species [1, 4-6].

Salinity is an environmental stress factor and is generally acknowledged as having a negative effect on agricultural production worldwide. However, several studies have identified certain positive effects of salinity. It has been indicated that a concentration of 100 mM of NaCl treatment could enhance the phenolic content and total glucosinolate content of 5-7-day-old *R. sativus* sprouts [2]. Various concentrations of NaCl increased the amounts of phenolic compounds and carotenoids as well as the antioxidant activities in 3-5-day-old *Fagopyrum esculentum* sprouts [7]. A treatment of 50-150 mM of NaCl reduced the growth of *Vigna radiata* seedlings. However, immersing the seeds in 50 mM NaCl for two hr prior to NaCl treatment increased the activities of certain antioxidative enzymes such as superoxide dismutase and catalase in both the roots and shoots of the seedlings [8]. It has been found that the mature Chinese white radish plant could grow under conditions of salinity of 0.4-0.8% [9]. Furthermore, several varieties, strains and cultivars of *R. sativus* have been grown throughout Asia. Previous studies have reported that those varieties are either tolerant or sensitive to NaCl stress [10-13].

The salt-tolerant capacities depend on many factors such as plant parts, plant age, planting site, variety, strain and cultivar. The plants that can tolerate levels of excessive salinity stress are identified by a higher growth capability and positive changes to certain physiological parameters such as chlorophyll content and photosynthetic rate [10-13]. However, the small seedlings of the 7-day-old Chinese white radish plant (the variety grown in Thailand) have not been thoroughly investigated for potency and salt tolerance. The objective of this study is to investigate the effects of NaCl at different concentrations on the germination, growth, relative water content (RWC) and chlorophyll content, as well as proline, malondialdehyde (MDA) and vitamin C contents in Chinese white radish sprouts. The results will provide fundamental knowledge on the Chinese white radish sprouts in terms of salt tolerance and may assist researchers in achieving a practical way of enhancing the health-promoting compounds of this food-plant.

## MATERIALS AND METHODS

### Plant Materials

Seeds of the Chinese white radish plant were purchased from Jia Tai Company, Bangkok. Fine sand was firstly washed 3 times with tap water and dried at 105°C for 24 hr. One litre of the dried sand was placed in a 26x30-cm plastic box. Two hundred mL of NaCl solution were administered to the sand box. After immersing the seeds in distilled water for 18 hr, 20 complete seeds (turgid and non-lean) were collected and planted in the box. Three boxes of the radish were used for each concentration of NaCl (0, 100 and 200 mM). Fifty mL of the same concentration of the NaCl solution were sprinkled onto the sand every day. On the first and 7$^{th}$ day of cultivation, electrical conductivity (EC) of the sand culture was measured. Normal seedlings that had a shoot and root length longer than 2 cm and that presented 2 leaves were selected for analysis as described below.

**Electrical Conductivity (EC) of Sand Culture**

EC of the sand culture was measured according to the 1:5 (soil: water) method of the Land Development Department [14] and Rayment and Higginson [15]. The suspension of sand culture was prepared by weighing 10 g of the sand culture in a flask and adding 50 mL of distilled water. The suspension was shaken at 160 rpm for 2 hr to dissolve soluble salts and was then filtered through filter paper (No. 5) to determine EC by Oakton ECTestr11+.

**Germination and Seedling Growth**

Normal seedlings were carefully separated and counted. The percentage of germination was calculated: germination (%) = (germinated seedlings x 100) / total of planted seeds.

The lengths were measured from where the stem met the ground to the end of the youngest shoot in order to determine shoot length and then to the longest root for root length. The shoot and root parts were then immediately cut to determine shoot and root fresh weight.

**Relative Water Content (RWC)**

RWC was determined according to the procedures of Barrs and Weatherley [16] and Roger [17] with slight modifications. The shoot part of the plant was excised for RWC assay. After shoot fresh weight (FW) determination, this shoot part was floated in distilled water for 3.5 hr. The turgid samples were quickly blotted dry prior to determination of turgid weight (TW). After that, the shoot part was oven-dried at 80°C for 24 hr and the shoot dry weight (DW) was recorded. RWC was calculated: RWC = (FW-DW/(TW-DW) *100.

**Chlorophyll Content**

Chlorophyll content was determined according to the modified method of Chappelle et al. [18]. The shoot parts of the seedlings (0.5 g) were soaked in 10 mL of dimethyl sulfoxide and incubated at room temperature in the dark for one night. The absorbance of the extract was spectrometrically determined at 648 and 664 ηm. The chlorophyll content was calculated using the following formulas:

Chlorophyll a content = 12.25 $A_{664}$ – 2.79 $A_{648}$

Chlorophyll b content = 21.50 $A_{664}$ – 5.10 $A_{648}$

**Proline Content**

Proline content was determined following the methods of Bates et al. [19] and Ghoulam et al. [20] with slight modifications. The seedling shoots (0.3 g) were pestled in 5 mL of 40% methanol. One mL of the extract solution was mixed with 1 mL of acid-ninhydrin reagent. The solution was boiled at 100°C to catalyse the reaction. After 1 hr of boiling, the reaction was instantly stopped with ice. Five mL of toluene was added to the solution and it was shaken well. The absorbance of the upper phase was spectrometrically measured at 528 ηm. The proline content in the seedlings was calculated by comparison with a standard curve of proline.

**Malondialdehyde (MDA) Content**

The content of MDA, an end-product of lipid peroxidation, was determined according to the modified methods of Heath and Packer [21] and Velikova et al. [22]. The shoot sample was homogenised and reacted with trichloroacetic acid and 2-thiobarbituric acid. The amount of the resulting complex (red pigment) was calculated from the extinction coefficient of 155 mM.cm$^{-1}$.

### Vitamin C Content

Vitamin C content was determined using the 2,6-dichlorophenolindophenol dye method as described by AOAC [23] and Deepa et al. [24] with minor alterations. Fresh seedling shoots (1 g) were pestled in 10 mL of 3% meta-phosphoric acid. The extract solution was then centrifuged at 3000g for 20 min. at 4°C. The supernatant (2 mL) was titrated against standard 2,6-dichlorophenolindophenol. Vitamin C content was calculated using standard ascorbic acid.

### Cultivation Condition and Data Analysis

Experiments were performed in a room at a temperature of 32±3°C under 2,500±500 lx light intensity and at 70±5% relative humidity at the Department of Biology, Faculty of Science, Chiang Mai University, Thailand from 1 August 2013 to 1 June 2014. All experiments were conducted following a completely randomised design. All data obtained were exposed to the one-way ANOVA test and the differences were analysed by the least significant difference (LSD) test. Each data point is presented as the mean average of three replicates. Comparisons with P values of $< 0.05$ were considered significantly different.

## RESULTS AND DICUSSION

The EC of the sand culture using the 1:5 (soil:water) method is shown in Table 1. Generally, if the EC measured by this method is higher than 0.15, the soil is considered saline [14, 25]. As is shown in Figure 1 and Table 1, seed germination of Chinese white radish plants was inhibited by more than 80% when subjected to 200 mM NaCl, while it was not significantly inhibited when 100 mM NaCl was administered. Furthermore, this concentration did not affect shoot length, but did increase shoot fresh weight when compared to the control. Conversely, 200 mM NaCl had a significant effect on seedling growth by reducing shoot length by 33% and shoot fresh weight by 57% of the control. This high concentration was also found to reduce the root length and root fresh weight by 56% and 33% respectively. Extreme NaCl concentration in the soil causes multiple negative effects to plant growth and development. Dissolved NaCl in the root zone causes significant negative osmotic potential that reduces the soil-water potential. This is also categorised as an abiotic factor that is known as a form of osmotic stress [26].

0 mM NaCl 100 mM NaCl 200 mM NaCl

**Figure 1.** Chinese white radish seedlings subjected to different NaCl concentrations over a period of 7 days (scale bar of 1 cm)

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**Table 1.** Effects of NaCl on seed germination, growth and some physiological parameters of shoots of 7-day-old Chinese white radish seedlings

| | NaCl concentration | | | F-test |
|---|---|---|---|---|
| | **0 mM** | **100 mM** | **200 mM** | |
| EC of sand culture[1] (dS/m) | 0.06-0.07 | 0.4-0.8 | 0.8-1.6 | |
| Germination[2] (%) | 93.75[3] ± 6.29a (100[4]) | 83.75 ± 14.36a (89) | 17.50 ± 2.89b (19) | * |
| Shoot length (cm) | 9.61 ± 0.19a (100) | 8.95 ± 1.13a (93) | 3.19 ± 0.29b (33) | * |
| Root length (cm) | 5.56 ± 0.49a (100) | 5.25 ± 0.28a (94) | 3.14 ± 0.73b (56) | * |
| Shoot fresh weight (g) | 0.23 ± 0.01b (100) | 0.31 ± 0.02a (135) | 0.13 ± 0.01c (57) | * |
| Root fresh weight (g) | 0.03 ± 0.00a (100) | 0.03 ± 0.00a (100) | 0.01 ± 0.01b (33) | * |
| Relative water content (%) | 92.62 ± 4.90a (100) | 88.76 ± 1.79a (96) | 51.91 ± 5.29b (56) | * |
| Chlorophyll a content (µg/g fresh weight) | 274.13 ± 13.00a (100) | 273.60 ± 1.00a (100) | 24.88 ± 11.20b (9) | * |
| Chlorophyll b content (µg/g fresh weight) | 117.85 ± 10.00a (100) | 119.89 ± 3.80a (102) | 14.86 ± 12.00b (13) | * |
| Chlorophyll a+b content (µg/g fresh weight) | 391.98 ± 21.80a (100) | 393.49 ± 4.80a (100) | 39.74 ± 17.20b (10) | * |
| Proline content (µg/g fresh weight) | 0.31 ± 0.06b (100) | 0.38 ± 0.08b (123) | 0.72 ± 0.15a (232) | * |
| MDA content (ηmol/g fresh weight) | 7.00 ± 0.7b (100) | 6.50 ± 0.3b (93) | 10.70 ± 0.6a (153) | * |
| Vitamin C content (mg/g fresh weight) | 8.62 ± 1.36b (100) | 8.62 ± 0.68b (100) | 15.67 ± 0.68a (182) | * |

**Notes**

[1] EC by 1:5 (soil:water) method was measured on the $1^{th}$ and $7^{th}$ day of cultivation respectively.

[2] Appearance of normal seedlings with primary root longer than 1 cm and at least 2 primary leaves

[3] All data are means of three replications ± standard deviation.

[4] Numbers in the brackets indicate per cent of control in each parameter row.

* Significant difference at $p < 0.05$

Means with different letters in a row of the same parameter are considered significantly different by least significance difference test.

In addition, high accumulation of sodium ions and chloride ions in the soil results in the ion toxicity for the plant cells. Osmotic stress and ion toxicity are described as the primary effects of NaCl stress [26]. In the present study hyper-salinity was exhibited at a concentration of 200 mM, which sharply inhibited seed germination as well as the early stages of the seedling growth of Chinese white radish plants (Table 1). This can be attributed to osmotic stress or to specific ion toxicity [27]. However, a prior inhibitory effect has been documented [28]. A high accumulation of NaCl can result in greater negative values of soil-water potential. This condition can lead to inadequate water uptake, which may result in a reduced level of activation of the hydrolytic enzymes [26]. Previous studies have also reported that high levels of salinity induce both a reduction in seed germination and a delay in the initiation of germination [29, 30].

The RWC of the 100-mM-NaCl-treated seedlings was found to be similar to that of the untreated seedlings; by contrast, it was clearly decreased among the 200-mM-NaCl-treated seedlings when compared to the untreated plants. The content of chlorophylls a and b in the seedlings that had been exposed to 100 mM NaCl weas found to be similar to that in the untreated seedlings. However, the chlorophylls content in the 200-mM-NaCl-treated seedlings was drastically decreased to 9-13% of that of the untreated seedlings (Table 1).

Osmotic stress is typically an important primary effect in plants that have been exposed to salinity stress [26]. In the present study a higher accumulation of 200 mM NaCl might reduce the free energy of water in the sand culture leading to a reduction in RWC in the seedlings of the Chinese white radish plant. The salt stress results in a reduction of RWC and this has been reported among both halophytes and non-halophytes [29, 31-33]. Previous studies have revealed that the hyper-accumulation of sodium ions disturbs potassium ion absorption by root cells and a decrease in the potassium ion/sodium ion ratio in the plant cells ensues [33-35]. Potassium is an important nutrient element and plays a major role in photosynthesis; for example, it controls the stomatal movement [36] and stimulates chlorophyll synthesis [37]. The drastic reduction of chlorophyll contents in Chinese white radish seedlings treated with 200 mM NaCl in this study may be attributed to a reduction in potassium ions and water in the cells. Significant decreases in chlorophyll content under extreme salinity stress in this study are in agreement with the results of previous studies involving *Phaseolus vulgaris*, *Vigna subterranea*, *Azolla microphylla* and *A. caroliniana* [35, 38-39].

Proline, MDA and vitamin C contents in the shoot of Chinese white radish seedlings were insignificantly changed by 100 mM NaCl when compared to the untreated specimens. However, the induction of those compounds was remarkably observed in the shoot seedlings that had been subjected to 200 mM NaCl (Table 1). Proline can play a crucial role as an osmoprotectant under extreme stress conditions, primarily in droughts and under conditions of soil salinity stress [26]. Furthermore, it has been reported that this compound may function as a scavenger by eliminating reactive oxygen species (ROS) [40] by serving as a signal molecule that regulates reproductive development [41], and as an energy sink to regulate the redox potential [42] in plant cells. Based on the findings of the present study, increased levels of proline accumulation in the Chinese white radish plants which were subjected to stress by 200 mM NaCl did not correspond to the extent of their seedling growth or development (Figure 1 and Table 1). This hyper-accumulation of proline might result from a symptom of salt injury rather than from a protective function of the plant cells [43]. Particularly, the salt-sensitive plants consistently accumulate higher amounts of proline when compared to the tolerant plants [44-46].

MDA is a highly reactive compound that is present as a result of the lipid peroxidation of polyunsaturated fatty acids [47]. This process is classified as a non-enzymatic autoxidation process caused by ROS. MDA is considered an oxidative damage marker at the cellular level. Several stresses such as those associated with salinity and high temperatures are mostly attributed to the enhancement of excessive ROS production in response to oxidative stress, which directly causes the peroxidation of membrane lipids [26, 48-49]. Treatments of 200 mM NaCl in this study led to a significant increase in the MDA content of Chinese white radish seedlings, indicating that the shoot cells suffered membrane damage due to the peroxidation of lipids. These results agree with those of previous studies, in which hyper-salinity caused severe lipid peroxidation in *Vigna radiata*, *Phaseolus vulgaris*, *Saccharum officinarum* and *Triticum aestivum* [8, 35, 50-51] and in halophytic plants such as *Cakile maritima*, *Sesuvium portulacastrum* and *Limonium stocksii* [52-54]. However, Hameed et al. [55] reported small increases in MDA content being correlated with the growth induction of the halophyte *Suaeda fruticosa*.

Vitamin C or ascorbic acid is a small molecule that is known to act as an antioxidant in many plants. This substance is involved in ROS detoxification when plants suffer from extreme environmental stresses such as salinity, drought and high temperatures [26]. Ascorbic acid can terminate the toxicity of hydrogen peroxide by converting it to water [56-57]. Conversely, it can also be changed to hydroxyl radical whose harmful effects disrupt the structure of certain cell components such as proteins and phospholipids [58]. Ascorbic acid is also related to the regeneration of another important non-enzymatic antioxidant, α-tocopherol [59]. Moreover, ascorbic acid has been known to play a fundamental role in plant growth and development such as in cell division, the expansion of the cell wall and other developmental processes [60-62]. The results of the present study show that the ascorbic acid content of the seedlings was significantly higher when treated with 200 mM NaCl compared with the control, while it was found to be invariable when treated with 100 mM NaCl. Previous findings also showed that ascorbic acid content increases in halophytes under NaCl stress. Halophytic plants *Limonium stocksii* and *Sphaerophysa kotschyana* accumulated ascorbic acid when they were subjected to 150-300 mM NaCl [54, 63]. Furthermore, a reduction in ascorbic acid content under salinity stress conditions has been found in non-halophytic plants such as *Aloe vera*, *Triticum aestivum* genotypes HD 2687 and HD 2009 and *Arabidopsis* [64-66]. A higher accumulation of ascorbic acid might be a mechanism for the alleviation of cell damage caused by salt stress conditions.

Based on the findings of the present research study, the 7-day-old seedlings of Chinese white radish plants could grow normally in sand culture (EC 0.4-0.8 dS/m) when treated with 100 mM NaCl. This condition could promote the growth of these plants by increasing shoot fresh weight. The seedlings seemed to be tolerant and preferred a low NaCl concentration. Even though a high EC (0.8-1.6 dS/m) of the sand culture from 200 mM NaCl was found to be too toxic, the lower concentration of NaCl (100 mM) may actually be beneficial to these seedlings. Sodium ions can replace potassium ions in functioning as an osmoregulator as well as supporting nitrate uptake in plant root cells [67]. Further investigation on the effects of low NaCl concentrations on the seedlings of Chinese white radish plants should therefore be undertaken.

## CONCLUSIONS

NaCl of 100 mM giving a sand-culture EC of 0.4-0.8 dS/m did not affect seedling germination, growth, RWC, or chlorophyll, proline, MDA and vitamin C contents of the shoots of Chinese white radish seedlings. In contrast, NaCl of 200 mM giving an EC of 0.8-1.6 dS/m for the sand culture resulted in adverse effects by decreasing seedling germination, growth, RWC and chlorophyll content, while increasing proline, MDA and vitamin C contents.

**ACKNOWLEDGEMENTS**

The authors are very grateful for the assistance of Miss Nathakan Na Chiangmai, Miss Nutkamol Masepan and Miss Onjira Srisuk who served as our assistant researchers. We also acknowledge Mr. Russell Kirk Hollis for editing this manuscript. This research was supported by CMU Mid-Career Research Fellowship Programme, Chiang Mai University, Thailand.

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