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MORPHO-PHYSIOLOGICAL CHARACTERISTICS, SELECTED MACRONUTRIENT UPTAKE, AND OXIDATIVE STRESS LEVEL OF *Andrographis paniculata* UNDER SALINE CONDITION

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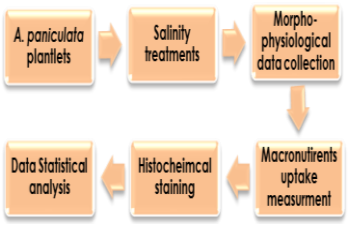
Graphical abstract



Abstract

Andrographis paniculata (Hempedu Bumi) is an important therapeutical herb that tends to be affected by salinity stress, leading to reduction of plant productivity and growth. This study aimed to study the effects of salt stress on the morpho-physiology, selected macronutrients uptake, as well as intensity and localization of reactive oxygen species in *A. paniculata*. Symptoms of salt toxicity were observed on *A. paniculata* including dehydration and browning of leaves. Na^+ content in plants was significantly increased as NaCl concentration increases, while K^+ content was significantly reduced. For Ca^{2+} and Mg^{2+} content, no significant difference was detected between control and plants treated with 92.40 mM NaCl. Histochemical staining showed that the accumulation of superoxide in 41.10 mM salt-treated leaves was higher than the control, whereas no differences were found for hydrogen peroxide. *A. paniculata* appeared to be affected by saline condition.

Keywords: *Andrographis paniculata*, salinity stress, reactive oxygen species, nutrients uptake

1.0 INTRODUCTION

Medicinal plants are being investigated extensively worldwide to find alternative remedies and health approaches free from side effects caused by synthetic chemicals. About 25% of commonly used medicine contains phytochemicals and 80% of the world population used medicinal plants as the first line of primary health care [1]. The total global market of medicinal plants and related products was US$60 billion in 2003 with a double digit growth [2], which is expected to be US $500 billion by 2050 [3]. *Andrographis paniculata* that belongs to the family Acanthaceae family possesses many pharmacological properties. Currently, this plant is in high demand and its bioactive compound (andrographolide) has been commercialized. Good quality dried leaves cost US$5.0 per kg and the purified andrographolide (major bioactive compound of *A. paniculata*) from specialist chemical suppliers could be sold as much as US$ 100,000 per kg [4]. Thus, this plant needs to be grown commercially.

Salinity stress adversely affects plant growth, development and productivity. It also alters the production of certain metabolites [5]. It is estimated that approximately 1128 million hectares (Mha) of lands worldwide is salt-affected [6]. In Malaysia, salinity problem is spread around most of the regions of the country, especially at the coastal regions that account for a total of 4.58 Mha of salt-affected land [7]. Salinity stress produces composite stresses and plants suffer osmotic stress or water deficit, ionic imbalances, ionic toxicity, oxidative stress, decrease in CO_2 assimilation and susceptibility to injury [8; 9]. All of these factors have negative effects on the growth and development of plants. Firstly, plants face osmotic stress (a rapid phase) and growth starts to reduce immediately due to sudden increase in salinity stress

surrounding the root zone of plants. Secondly, after long time exposure of plants to salinity stress, plants suffer from ionic toxicity (a slower phase) due to the accumulation of Na^+ in the old leaves of plants. This phase causes a significant reduction of productivity because of leaf death. In this case, photosynthesis capacity of stressed plants were reduced and cannot provide required energy for the growth and development of young leaves, ultimately further reduction in growth rate [10; 11]. Excessive accumulation of Na^+ in cytoplasm disrupts K^+ nutrition and inhibits activity of many enzymes [12]. Therefore, the required level of macro and micro nutrients accumulation from soils altered and there is the possibility that many nutrient interactions in salt stressed plants may have important consequences to growth.

A. paniculata negatively responses to salinity stress where certain morphological and physiological traits were suppressed [13; 14; 15]. The seedlings could not survive at 12 dSm^{-1} of NaCl [14]. Since there is a need to use plants of the same genetic material and suitable treatments without plants substrate interactions to improve the understanding of response of *A. paniculata* to salinity stress, this study was initiated. In this study, morphological and physiological responses, uptake of selected nutrients, and oxidative stress level of *A. paniculata* were elucidated.

2.0 EXPERIMENTAL

2.1 Plant Propagation

Vegetative propagation through stem-cutting was applied in this study. Healthy apical shoots with 3 nodes and 6-8 fully expanded leaves were cut from a healthy growing *A. paniculata*. The microcuttings were moistened with water and dipped in rooting medium supplemented with α-naphthalene acetic acid (NAA; Sri Products, Malaysia) prior to inoculation into peat moss (Peltacom, Poland) in the planting tray. Water was sprayed on peat moss once daily to moisten it.

2.2 Salinity Treatment

The 14 days-old plantlets of *A. paniculata* were transferred into the Hoagland solution [16] for 3 days adaptation prior to salinity treatment. After that, the plants were treated with different concentrations of NaCl (0, 20.55, 41.10, 66.75 and 92.40 mM; [17]) in Hoagland solution with three replicates each. The plants were then placed under shade in a completely randomized design.

2.2 Morpho-physiological Characteristics

After 4 days salinity exposure, morphological appearances of *A. paniculata* were observed and photographed. Then, all plants were harvested and data on plant height (PH), root length (RL), leaf length (LL) and width (LW), total fresh weight (FW), total dry weight (TDW) (after drying at 60 °C for 3 days) and salt tolerance index (STI) were collected.

2.3 Determination of Selected Macronutrient Contents in *A. paniculata*

Dried plants were ground into fine powder with liquid nitrogen, weighed and then digested with concentrated HNO_3 (65%) in 60 ^{0}C water bath. After a clear solution was obtained, the samples were diluted to 1% HNO_3 and filtered with 0.45 µm membrane filter. The selected macronutrients contents were measured (in ppm) using atomic absorption spectroscopy [18].

2.4 Histochemical Staining of *A. paniculata* leaves

Superoxide ($O_2^{\bullet-}$) was visually detected by histochemical staining with nitro blue tetrazolium (NBT) [19] and hydrogen peroxide (H_2O_2) was visually detected by using 3,3'-Diamino-benzidene (DAB) solutions [20] in the leaves of *A. paniculata*. For superoxide, three leaves from each replicates were immersed in 1 mg/mL solution of NBT in 10 mM phosphate buffer (pH 7.8) and left under fluorescent light for 2 h at room temperature. Meanwhile for hydrogen peroxide, another set of leaves were excised and immersed in freshly prepared 10 mM phosphate buffer (pH 7.8) containing 1 mg/mL DAB (pH 3.8) for 8 h under direct fluorescent light at room temperature. After both treatments, the leaves were immersed in absolute ethanol for overnight and photographed.

2.5 Statistical Analysis

The data were analysed using SPSS statistical software, and analysis of variance (ANOVA) was used to compare the statistical difference based on Post Hoc LSD multiple comparisons of $p \leq 0.05$. Results were presented as means and standard error of the mean (SEM).

3.0 RESULTS AND DISCUSSION

3.1 Morpho-physiological Response of *A. paniculata* Towards Salinity Stress

Figure 1 shows the morphological response of *A. paniculata* on day 4 of treatment with NaCl and severity index of salinity effects on *A. paniculata* is shown in Table 1. Symptom of osmotic stress and ion toxicity including dehydration, necrosis, wilting, chlorosis, and abscission of leaves can be seen on plant treated with NaCl concentrations higher than 41.10mM, in comparison to the control. The leaves of plants treated with 20.55 mM NaCl remain turgor similar with control. The finding is similar to a report that shows salinity effects on plants include dehydration, which can be observed by leaf rolling, and shedding (wilting) [21]. Additionally, leaves treated with 66.75

and 92.40 mM appeared severely dehydrated and burned with large area of necrosis. Na^+ toxicity will cause leaf to dehydrate and died, while Cl^- toxicity lead to dryness and burn on leaves [22]. The width and length of *A. paniculata* leaves decreased, which resulted by salt toxicity. Additionally, prolonged exposure lead to ion toxicity which inhibits development of new leaves (cell division), stomatal closure (photosynthesis activities) and eventually died of old leaves [23; 24]. This correlated with previous study where salinity also affected the morphology of several medicinal plants such as *Thymus vulgaris*, *Majorana hortensis*, *Mentha pulegium*, and peppermint which include reduced leaves area and abscission [25]. However, a previous study observed that abscission of leaves only happened in plant subjected to 143.70 and 193.40 mM of NaCl on the 15th and 10th day after exposure to salt, respectively [26]. Although this study used similar plant as Talei *et al*. [26], cultivar within a particular species also have different capabilities in tolerating salinity stress [27]. Moreover, irrigation method, composition of saline medium, climatic conditions, exposure time [28; 29], salt concentration, plants species, and plant growth stage [26] also play roles in plant response to saline condition. Table 2 shows physiological responses of *Andrographis paniculata* treated with different NaCl concentrations on day 4. The fresh weight of *A. paniculata* exposed to salt concentrations was significantly decreased, in comparison to the control. Similarly, the root length was significantly decreased at 41.10, 66.75, and 92.40 mM NaCl, as compared to control. The water uptake is less when root conductivity is low, resulted in water deficit when plant transpirational rate is more than the water absorption rate [28]. Ion toxicity (Na^+ and Cl^-) happens when root tissues reach to toxicity level [30] and root continuously uptake Na^+ and Cl^- to shoot tissue by transpirational flux in order to maintain plant water status [31]. Therefore, salinity stress directly affected the growth of the root [32]. This phenomenon further explains the significant differences at 41.10, 66.75, and 92.40 mM, as compared to control for leaf length and leaf width.

Moreover, plants compartmentalize ions into tissues in order to protect cell normal functions such as growth and metabolism [31]. Mechanisms of ion regulation varies in plants in either reduce Na^+ uptake or vacuole compartmentalization of Na^+ [33]. Usually, Na^+ and Cl^- ion were compartmentalized in older leaves which later resulted in salt toxicity in plants including decrease in leaves photosynthetic area and leaves death [34]. In this study, the plant height and dry weight of *A. paniculata* were unaffected by salinity treatments. In agreement with the present findings, the plant height of *Salvia miltiorrhiza* was unchanged when exposed to NaCl concentrations of 25, 50, 75 and 100 mM for 30 days [35]. Similar trend was also found on olive tree where its growth was unaffected by salinity stress [36]. However, the findings obtained in the present study were quite distinct compared to Talei *et al*. [26] that used *A. paniculata* as well. According to the authors, the plant growth and dry weight were significantly affected by salt. This may be due the different experimentation procedure using *A. paniculata* for salinity stress treatment. For example, in the present study, plants from the stem cutting were used, whereas Talei *et al*. [26] grew plants from seeds.

Moreover, Talei *et al*. [26] suggested that salt threshold level for *A. paniculata* was at 143.70 mM at 15 days of salt exposure, and treatment of salts in less than 10 days did not affect the salt tolerance index (STI) of seedlings unless prolonged the times of exposure. However, Table 1 shows that STI was reduced exponentially as salt concentrations increased. The difference between the findings of Talei *et al*. [26] and the present study could also due to different source of plant materials and accession. Furthermore, they use both Hoagland solution and jiffy media which may have interacted with each other and thus gave different results. The taste and aroma of spices grown are greater under arid and semi-arid conditions caused by water stress and high light intensities than central Europe [37]. This is because under stress condition, plants regulate the synthesis of their secondary metabolites for survival [38]. In a study, three different ecotypes of *Ocimum basilicum* L. (Rajan, Khanewal, and Multan) were used to study their capability of tolerating salt stress [39]. The study found that Rajan is more salt sensitive than Khanewal and Multan in response to salt stress as the plants tend to inhibit the root and shoot growth. This shows that even cultivars within the same species responses differently under salt stress.

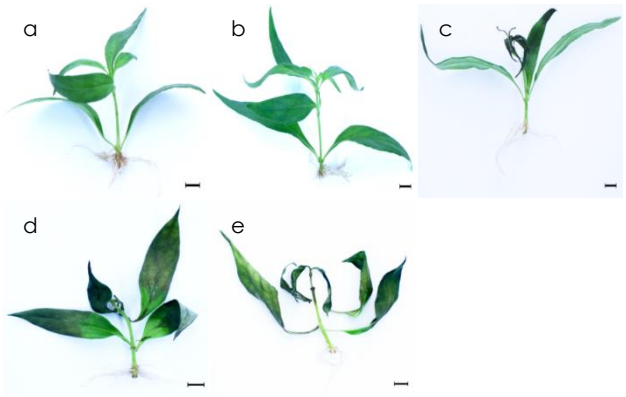


Figure 1 Symptoms of osmotic stress and NaCl toxicity on *A. paniculata* subjected to 4 days of NaCl treatments: (a) control, (b) 20.55 mM, (c) 41.10 mM, (d) 66.75 mM and, (e) 92.40 mM. Scale bar: 1 cm

Table 1 Severity index of *Andrographis paniculata* on day 4 of NaCl treatment

NaCl (mM)	BL	BLT	BST	Wilting	Dehydration	LA
0	-	-	-	-	-	-
20.55	-	-	-	-	-	-
41.10	+	+	√	+	+	-
66.75	++	++	√	++	++	√
92.40	+++	+++	√	+++	+++	√

+ Mild (0-33 %), ++ Moderate (34-68 %), +++ Severe (69-100%). Indication: Yes: √, No: -, Brownish leaves: BL, Burnt leaves tips: BLT, Burnt shoot tips: BST, and leaves abscission: LA.

Table 2 Physiological responses of *Andrographis paniculata* treated with different NaCl concentrations on day 4. Different letters denote significant difference within column

NaCl (mM)	PH (cm)	RRL (cm)	RLL (cm)	RLW (cm)	FW (mg)	DW (mg)	STI (%)
0	3.62±0.15^{a}	1.36 ±0.33^{a}	0.06±0.03^{a}	0.04±0.04^{a}	0.43±0.03^{a}	52±6.83^{a}	100
20.55	3.15 ±0.28^{a}	0.91±0.19^{a}	0.07±0.01^{a}	0.03±0.03^{a}	0.36±0.03^{b}	52±6.87^{a}	83
41.10	2.98 ±0.16^{a}	0.16±0.06^{b}	-0.29±0.08^{b}	-0.12±0.02^{b}	0.31±0.02^{b}	53 ±8.27^{a}	72
66.75	3.12 ±0.14^{a}	0.13 ±0.03^{b}	-0.35±0.12^{c}	-0.25±0.06^{c}	0.24±0.03^{c}	50 ±5.64^{a}	55
92.40	3.25 ±0.25^{a}	0.13 ±0.02^{b}	-0.35±0.10^{bc}	-0.27±0.04^{c}	0.21±0.01^{c}	51±7.87^{a}	49

Plant height: PH, Relative root length: RRL, Relative leaf length: RLL, Relative leaf width: RLW, Fresh weight: FW, Dry weight: DW, Salt tolerant index: STI.

3.2 Nutrients Uptake Ability

High accumulation of Na^+ and Cl^- results in imbalance nutrients uptake including potassium (K^+), calcium (Ca^{2+}), phosphorus (P), magnesium (Mg^{2+}), manganese (Mn^{2+}), and nitrate (NO_3^-), leading to deficiency of plant nutrient [28; 22; 40]. This is due to the competition between ions or osmotic potential at the root surface. Usually, Na^+ ions uptake increases during salt stress condition, while K^+ and Ca^{2+} ions uptake decreases [41]. As shown in Table 3, as expected, the higher the NaCl treatment, the higher the Na^+ was taken into the plants ($p < 0.05$). The current study was supported by Talei *et al.* [15] and Rajpar *et al*. [14], where the Na^+ increased as the concentrations of NaCl increases. In a situation where Na^+ exists in abundance, the channels on the root hairs were forced to absorb more Na^+ into the plants. This condition leads to the accumulation of cytotoxic Na^+ inside the plants [42].

Nevertheless, K^+ content of 41.10 mM and 92.40 mM NaCl-treated plants was significantly lowered than the control ($p < 0.05$). In a previous study on *Chenopodium quinoa* and *A. paniculata* the concentration of K^+ was reduced generally in higher salt treatment compared to control [42, 43]. In the presence of high Na^+ ions, K^+ transporters have high affinity toward Na^+ compared to K^+. This is due to the similarity between potassium and sodium in terms of the hydrated ionic radii [44]. Hence, the competition at the binding sites increases between K^+ and Na^+ and thus, the influx of Na^+ became higher than K^+.

On the other hand, the uptake of Ca^{2+} was not significantly different from the normal condition ($p > 0.05$), except for 20.55 mM NaCl treatment. According to Talei *et al.* [15], NaCl treatment increased the Na^+ accumulation in *A. paniculata*, but decreased the uptake of K^+ and Ca^{2+} concentrations. Similarly results were reported for *A. paniculata* [42] and in *Matricaria Chamomilla* [45]. Ca^{2+} bind loosely to the transporter compared to the Na^+. Therefore, in higher Na^+ concentrations, Ca^{2+} has lower capacity to be absorbed into the plants. Meanwhile, Mg^{2+} uptake in salt-treated plants (except 92.40 mM) was higher than the control ($p < 0.05$). In contrast, Rajpar *et al*. [42] states that as the Exchangeable Sodium Percentage (ESP) levels increases, the Mg^{2+} concentration was significantly lower from the control. The contradicting results may attribute to different plant sources, length of exposure and treatment methods. It was also possible that K^+ shares the same transporter with Na^+, but not with Ca^{2+} and Mg^{2+}. Since Ca^{2+} and Mg^{2+} used different transporter, Na^+ uptake into the plants did not affect the uptake of Ca^{2+} and Mg^{2+}.

Table 3 Selected macronutrients determination in different NaCl concentrations. Different letters denote significant difference within column

NaCl (mM)	Na^+	K^+	Ca^{2+}	Mg^{2+}
0	0.48±0.01^{a}	6.05±0.25^{a}	8.76±0.05^{a}	0.59±0.00^{a}
20.55	0.83±0.08^{b}	5.83±0.28^{ac}	6.85±0.16^{b}	0.74±0.01^{bc}
41.10	1.31±0.01^{c}	4.43±0.07^{bc}	9.72±0.23^{a}	0.67±0.01^{c}
66.75	1.95±0.20^{cd}	4.59±0.17^{abc}	8.89±0.56^{a}	0.76±0.02^{b}
92.40	2.14±0.16^{d}	3.86±0.31^{b}	9.26±0.25^{a}	0.59±0.02^{a}

3.3 Detection of Superoxide ($O_2^{\bullet-}$) and Hydrogen Peroxide (H_2O_2)

Oxidative stress level in plants could be indicated by $O_2^{\bullet-}$ and H_2O_2. As shown in Figure 2, more dark blue spot representing $O_2^{\bullet-}$ were observed when niroblue tetrazolium (NBT) was reduced to formazan on leaves of plant treated with 41.10 mM of NaCl compared to control and those treated with 20.55 mM NaCl. This indicates that the treated plants were under a higher level of salt stress than the controls. These $O_2^{\bullet-}$ was found at petiole and veins. However, no obvious dark

blue spot was observed on leaves of plants treated with 66.75 and 92.40 mM of NaCl. This is likely that plants grew under such NaCl concentrations were severely dehydrated and cell death has occurred. The intensity of H_2O_2, on the other hand, was found to be similar between control and salt-treated plants (Figure 2). H_2O_2 was detected when H_2O_2 react with DAB and then polymerized to form brown precipitate on the leaves. $O_2^{\bullet-}$ is short-lived (1µs) while H_2O_2 is longer-lived (1 ms). ROS-induced oxidative damage is triggered in plants as the secondary effects resulted from primary effects (osmotic stress and ion toxicity) under salt stress [33; 46; 47]. High production of ROS interrupts normal homeostasis activity in plant cells [25; 32]. When plants close their stomata under high salinity, photosynthetic rate reduces as CO_2 is limited and therefore accumulate $O_2^{\bullet-}$ in the chloroplast [33]. ROS consequently oxidize the plant's chlorophyll and cause lipid peroxidation [48]. In order to survive under adverse environmental condition, plants have antioxidative capability to counteract with excessive ROS level, such as by increasing the level of superoxide dismutase, catalase, and peroxidase to scavenge the excessive ROS.

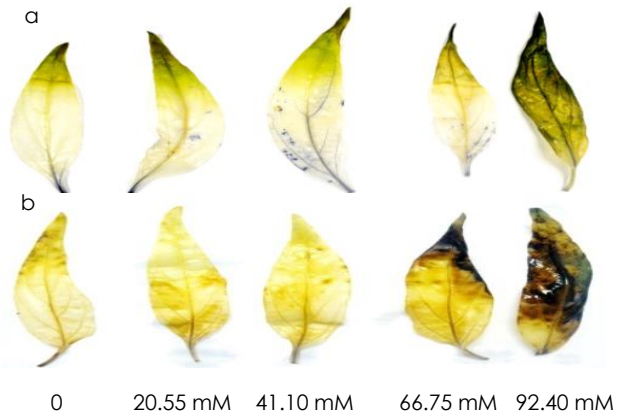


Figure 2 Histochemical staining of (a) superoxide and (b) hydrogen peroxide in *Andrographis paniculata* leaves treated with different concentrations of NaCl for 4 days

4.0 CONCLUSION

A. paniculata is a salt-sensitive plant and salinity treatment adversely affects its growth and development. Generally, the higher the saline concentration, the severe the symptoms of salinity stress observed on plants. Salt toxicity can be seen on *A. paniculata* starting from 41.10 mM up to 92.40 mM of NaCl treatment including leaves necrosis, abscission, inhibition of root growth, and reduction on fresh weight. In contrast, the plant height and dry weight were unaffected by NaCl treatments. NaCl treatments also affect selected nutrients uptake in *A. paniculata*. The amount of Na^+ increases significantly while K^+ decreased significantly as the NaCl concentrations increases. A higher amount of $O_2^{\bullet-}$ was spotted on leaf treated with 41.10 mM of NaCl, indicating a higher level of oxidative stress. Thus, this study provides additional information on *A. paniculata* response towards salt stress in term of morpho-physiological characteristics and nutrients uptake changes as well as the intensity of reactive oxygen species by using plant with similar genetic material.

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