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Response of King of Bitters (*Andrographis paniculata* Nees.) seedlings to salinity stress beyond the salt tolerance threshold

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Abstract

Salt tolerance threshold is an important factor for screening salt tolerant plants in the selection process. The objectives of present study were to determine the salt tolerance threshold and to evaluate the 32 accessions of *Andrographis paniculata* for salt tolerance. Two experiments were carried out for this purpose. In first experiment, 40-day-old seedlings were grown at different salinity levels (control, 4, 8, 12 and 16 dSm^{-1}) on Hoagland medium and compared at various exposure times (0, 5, 10, 15 and 20 days). The results indicated that salinity, exposure time and their interactions had significant effect on the morphologic and agronomic traits. The relative growth rate (RGR) during exposure time was significantly decreased in two stages. The initial decrease happened five days after salt exposure, mainly due to osmotic stress, while the secondary reduce occurred 15 days after salt exposure because of ion stress. The results revealed no significant differences among accessions based on salt tolerance index in first ten days. All the studied morphological traits were decreased with increasing salinity levels. Finally, it was realized that 12 dSm^{-1} salinity for a period of 15 days can be considered as the salt tolerance threshold for *A. paniculata* seedlings. In the second experiment 32 different accessions were evaluated under salinity stress. All measured Agro-morphological traits; chlorophyll content, K^+ and Ca^{2+} content were significantly decreased with increasing salinity levels, while proline and Na^+ content increased. Under salinity stress, tolerant accessions could accumulate higher proline, K^+ and Ca^{2+}, and lower Na^+ content than sensitive accessions. Consequently, the results suggest that proline, Na^+, K^+ and Ca^{2+} are the most effective indicators for salinity tolerance screening in *A. paniculata*.

Keywords: *Andrographis paniculata*, Ion content, Proline, Salinity stress, Salt tolerance, Salt tolerance threshold.
Abbreviations: A_accession, AP_*Andrographis paniculata*, CHLO_chlorophyll, ET_exposure time, MLL_mean of leaf length, MLW_mean of leaf width, NL_number of leaves, RDW_root dry weight, RFW_root fresh weight, RL_root length, S_salinity level; SDW_shoot dry weight, SFW_shoot fresh weight, SL_shoot length, STI_salt tolerance index, TDW_total dry weight.

Introduction

Soil salinity is one of the major environmental stress factors that causes many adverse effects on growth and productivity of plants. Soils are known as saline when the EC is 4 dSm^{-1} or more, which generates an osmotic pressure (Munns and Tester, 2008). Soil salinity is a global issue and more than 6% of land of the world is salt affected. Productivity of plants in arid and semiarid regions of the world is very low due to soil salinity (Ashraf et al., 2002; Munns, 2002; Munns and Tester, 2008). Salt tolerance threshold is a critical parameter for measuring plant salt tolerance, which is generally known as the relative yield response to increasing salinity in root zone; exposed to saline water (Maas, 1990). The salinity tolerance threshold is a specific target for improving plant salt stress tolerance. To understand the salt tolerance mechanisms, it is necessary to understand whether the growth and yield reduction is due to osmotic effect of salinity, or the toxic effect of certain ions accumulated within the plant. The reduction in growth occurs in two specifics phases. The first, phase (osmotic phase) is a rapid response, where the growth starts to decrease immediately after the salt concentration around the roots increased. This will identify the threshold level of plant. The salinity threshold level is approximately 40 mM NaCl (~ 4 dSm^{-1}) for most plants. The second salinity response (ion toxicity phase) is slower, which is normally due to the accumulation of Na^+ in the old leaves. The latter response causes a significant reduction of yield, due to the leaf death. In this case the photosynthetic capacity of the plant will no longer be able to provide the required carbohydrate of the young leaves, which causes further reduction in growth rate (Maggio et al., 2007; Munns and Tester, 2008). In many salt sensitive plants, a major part of the growth inhibition is caused by excessive Na^+ (Jaleel et al., 2007). The high sodium disturbs the potassium (K^+) nutrition, when sodium is accumulated in the cytoplasm and inhibits

the activity of many enzymes (Jaleel et al., 2007). The deleterious effects of salinity on plant growth is attributed to the decrease in osmotic potential of the growing medium (osmotic stress), specific ion toxicity (salt stress) and nutritional imbalances or combination of all these factors. Disruption of potassium (K^+) nutrition, can be a result of inorganic ion (Na^+, Cl^-, and K^+) and compatible organic solute (soluble carbohydrates, amino acids, proline, betaines, etc) accumulations (Hasegawa et al., 2000; Ashraf, 2004; Luo et al., 2005), while salt tolerant plants (halophytes) can tolerate high salt concentrations (Parvaiz and Satyawati, 2008). These factors alter various biochemical and physiological responses in plants and cause adverse effects on photosynthesis, growth and development (Ashraf and Sarwar, 2002; Serraj and Sinclair, 2002; Munns and James, 2003). Adaptation to saline environment mainly depends on the ability of plants to exclude salt (Hasegawa et al., 2000). This adaptation is generally associated with osmotic adjustment by using some osmotic regulators, such as potassium, soluble sugars, proline and glycine betaine (Hasegawa et al., 2000; Munns, 2005). Understanding of the effects of salinity stress on plant growth and the ways by which plants can tolerate salt stress condition could provide important information for selection of salt tolerant plants (De Vos et al., 2010). The ability of different plant species to withstand salinity stress is different and depends on several interacting variables including climatic conditions, developmental stages, salinity levels and exposure times (Munns, 2002; Zhu, 2002). Some medicinal and aromatic plants have been reported to tolerate salinity stress (Ashraf and Orooj, 2006), which indicated the potential of growing medicinal plants on saline areas. Medicinal plants needs to grow commercially due to the increasing demand of traditional medical systems and pharmaceutical industry, but soil salinity and pollution are serious threats to growth of medicinal plants (Qureshi et al., 2005). Thus, it is important to explore the salt tolerance capacity of potential medicinal plants such as *A. paniculata.* The *A. paniculata* is an important medicinal plant commonly known as "Kalmegh or King of Bitters" belongs to the family Acanthaceae (Rajpar et al., 2007; Gomathinayagam et al., 2009). The aerial parts such as leaves have been used in traditional medicines for the treatment of common cold, fever, sore-throat, and diarrhea (Saralamp et al., 1996; Valdiani et al., 2012). The importance of the plant lies in its diterpenoids, of which andrographolide is the main compound with immuno-stimulant (Puri et al., 1993), antipyretic, anti-flammatory, and anti diarrhea properties (Gupta et al., 1993). Some other related diterpenoids constituents are 14-deoxy-11,12-didehydro-andrographolide, neoandrographolide, and andrographiside that possess various medicinal properties (Lattoo et al., 2008). The plant is an erect annual herb with a dark-green quadrangular stem; lanceolate and pinnate leaves; small and white flowers; linear-oblong capsules; and tiny yellowish brown seeds (Jiang et al., 2009). It grows abundantly in tropical climates (Lattoo et al., 2008). Our understanding of the quantitative effects of salinity levels and exposure times on important salt tolerance parameters like threshold (EC-dSm^{-1}) and slope (% per dSm^{-1}) values have not been determined for *A. paniculata* seedlings yet. There is no clear index for identifying salinity tolerance in plants (Ashraf, 1994). Yield is the direct criterion to assess the salinity stress response, but the salt tolerance mechanisms are complex and need to consider multi parameters like proline level, antioxidants and related ion contents. The objectives of the present study were to investigate the effects of salinity on morphological and physiological traits of 32 accessions of *A. paniculata*. The assessment of the salt tolerance and identification of tolerant accessions and their capability of growth and reproduction under saline environment will help to develop better lines to grow on salt affected lands with little yield reductions.

Results

Effects of salinity levels and salinity exposure times on growth indices of A. paniculata

The results showed that salinity levels (S), exposure times (ET) and their interactions significantly affected growth indices ($p<0.01$). The interaction of salinity levels × accession (S × A) was not significant in terms of relative growth rate (RGR). The salinity levels and exposure times significantly affected accessions (Table 1). Among the accessions the highest RGR (0.09 g/g/day) was observed in accession No. 11249, whereas the lowest (0.08 g/g/day) with the same condition belonged to accession No. 11216. In all accessions morphological traits and RGR decreased under salinity condition. The first day, when leaves started to fall, was observed on the 10^{th} day at 16 dSm^{-1} and the 15^{th} day at 12 dSm^{-1} salinity levels. The RGR in high salinity levels (12 and 16 dSm^{-1}) at 20 days after applying salinity stress were 0.002 and -0.027 g/g/day, respectively (Fig 1). As shown in Fig 1, the greatest decrease in RGR was observed at high salinity (16 dSm^{-1}) after 20^{th} days of stress. The mean of RGR over the salinity levels for exposure times varied from 0.03 (20 days) to 0.13 (5 days). Among the exposure times, 5 days salt exposure showed the highest RGR followed by 10, 15 and 20 days exposures, while among the salinity levels there were significant difference between 0.11 to 0.03 (control and 16 dSm^{-1}). Based on the RGR, two linear functions with different slopes were identified. The first phase was at initially stage of exposure time (after 5 days) and the second slope was after 15 days of exposure time (Fig 1). This might be either due to the effect of osmotic stress in the initial period of stress and specific ion toxicity (salt stress) or long stress time at the end of period. No significant difference was detected between the accessions for salt tolerance index (STI) at 5 and 10 days after salinity stress; however, the accessions showed significant differences ($p<0.01$) at 15 and 20 days after salinity stress (Table 2). Furthermore, the results revealed that the STI at 15 and 20 days after salinity stress was significantly affected by the interaction of S × A. The mean of STI over the salinity for exposure times varied from 91.65% (5 days) to 63.07% (20 days), in which the salinity levels were from 100% (control) to 61.17% (16 dSm^{-1}), respectively. Overall , 12 dSm^{-1} salinity level and 15 days salt exposure was the best salinity level in respect to STI, followed by 4, 8 and 16 dSm^{-1} and 5, 10 and 20 days. The slope of the salt tolerance index showed 7.17% decrease in 4 dSm^{-1}, 9.61% in 8 dSm^{-1}, 13.57% in 12 dSm^{-1} and 15.87% in 16 dSm^{-1} after 20 days exposure time (Fig 2). This might be due to the effect of high salinity level and long stress time.

The changes on dry weight and STI under salinity condition

Salinity levels affected the all measured morphological traits of *A. paniculata* seedlings (Table 3). Total dry weight results indicated that growth was negatively correlated to the substrate concentration of NaCl ($p < 0.01$). The seedlings grown at the low levels of NaCl (control) reached relatively higher total dry weights and did not show the toxicity symptoms; however, the total dry weight was significantly

Table 1. Variance analysis of effects of different stress times and salinity levels on the measured characteristics of six accessions of *A. paniculata.*

Source	df	Mean Square					
		STI	RGR	NL	SL	RL	TDW
Exposure time (ET)	3	14094.07**	0.14**	2.92**	2.54**	0.89**	1.54**
Salinity level (S)	4	16539.46**	0.04**	13.47**	9.20**	5.61**	0.33**
Accession (A)	5	251.15**	0.00**	0.84**	2.87**	0.41**	0.08**
ET × S	12	1237.72**	0.01**	1.05**	0.22**	0.60**	0.07**
ET × A	15	106.30**	0.01**	0.11**	0.03**	0.13**	0.01**
S × A	20	28.09**	0.00^{ns}	0.08**	0.04**	0.11**	0.00**
ET × S× A	60	11.41*	0.00^{ns}	0.05**	0.01^{ns}	0.04**	0.00^{ns}

**, * and ns, referred to 1%, 5% and not significant, respectively. STI: salt tolerance index (%), RGR: relative growth rate (g/g/day), NL: number of leaves, SL: shoot length (cm), RL: root length (cm) and TDW: total dry weight (g).

Table 2. Analysis of variance for effects of salinity concentrations on salinity tolerance index of six accessions of *A. paniculata* different stress times separately.

Source	df	Mean square of STI			
		5 days	10 days	15 days	20 days
Salinity (S)	4	1052.6**	2190.4**	4633.1**	12376.5**
Accession (A)	5	14.3 ^{ns}	30.4 ^{ns}	443.8**	81.6**
S× A	20	7.9 ^{ns}	10.8 ^{ns}	34.9**	8.7**

Statistical significance is indicated by ** (P<0.01),* (P<0.05) and ns (no significant).

reduced at higher levels of salinity (12 dSm^{-1}), indicating the symptoms of salt toxicity as growth depression. The main concentration of NaCl that significantly reduced the dry weight by 48.11% (compared to control) was 12 dSm^{-1}. Based on STI some accessions such as 11314, 11228, 11329 and 11249 were showed the high tolerance, and some accessions like 11266, 11306, 11216 and 11264 the lowest. The STI of tolerant accession (11249) were found to be higher (63.01%) than the sensitive accession (11266) (21.15%) in high salinity level (Table 4).

Chlorophyll and proline content

Proline and chlorophyll content were significantly influenced by salinity at P < 0.01 (Table 5). The NaCl treatment decreased the chlorophyll content, but increased proline; however, the response was variable. The result showed that there were significant differences among salinity levels in terms of proline and chlorophyll content. Based on obtained result there were significant differences among accessions in term of chlorophyll content, while there were no significant differences among accessions in term of proline (Table 5). However, tolerant accessions were produced high chlorophyll and proline content (Fig. 3a).

Na^+, K^+ and Ca^{2+} content

There were significant differences among salinity levels and different accessions (Table 5). Salt treatment increased sodium (Na^+) in all accessions compared with control group. In the high salinity level, tolerant accessions such as 11314, 11228, 11329 and 11249 were showed significantly less Na^+ than other accessions (11266, 11306, 11216, and 11264). Accession number 11329 had the lowest Na^+ accumulation (26.13) in high salinity level, while accession number 11266 had highest Na^+ accumulation (42.39) (Fig 3b). All of the accessions showed a decreased K^+ content upon salt treatment. Potassium contents of accession numbers 11314, 11228, 11329, 11249 were found to be higher than accession 11266, 11306, 11216, 11264 in the salt medium. The Calcium (Ca^{2+}) accumulation in different accessions decreased with salt treatment. The decrease in tolerant accessions was lower than sensitive accessions (Table 4). The K^+/Na^+ ratios of tolerant accessions in salted medium were lower than sensitive accessions in non-salinity medium. The K^+/Na^+ ratios were significantly different among the accessions. In salted media, the highest K^+/Na^+ ratio was found in accession number 11329 (0.116) and the lowest was recorded in 11266 (0.064) (Fig 3c), on the other hand, Na^+/Ca^{2+} ratios of all accessions in salted medium were higher than those cultured in control medium. Under the salinity conditions, tolerant accessions had lower Na^+/Ca^{2+} than sensitive (Fig 3d). The correlation between most measured traits was significant at p<0.01 (Table 6 and Fig. 4). Interestingly, some traits like proline, Na^+, K^+, Ca^{2+}, K^+/Na^+ and Na^+/Ca^{2+} concentrations were highly correlated to STI, while others were not. The correlation between STI and Na^+ and Na^+/Ca^{2+} were significant and negative, while a positive correlation was determined between STI and proline, K^+, Ca^{2+} and K^+/Na^+ concentrations under salt stress. The correlation between STI and many other traits such as SL, RL, NL, SFW, SDW, RFW, RDW and chlorophyll exhibited low significances. The UPGMA cluster analysis of the 32 *A. paniculata* accessions based on the measured traits under salinity conditions, produced four clusters. The cluster 1 (red cluster) involved nine tolerant accession. The cluster 2 (green cluster) contained seven semi-tolerant accessions, where as cluster 3 (orange cluster) indicated two semi-sensitive accessions and finally the cluster 4 (blue cluster) consisted 14 sensitive accessions (Fig 5). The distance between leader and joiner accession in cluster analysis based on all measured traits was 5.99.

Discussion

Plant growth is one of the major agricultural indices of salt stress tolerance as indicated by different studies (Munns, 2002). In order to identify salt tolerance threshold of *A. paniculata* seedlings, growth indices like NL, SL, RL, SFW, RFW, SDW, RDW, STI and RGR were recorded at different concentrations of NaCl and various durations of exposure times. The results indicated that applied NaCl inhibited the plant growth, and led to a decrease in dry weight in salted medium as compared to control medium. This may be related to the effect of salt stress, which resulted in the limitation of water absorption and biochemical processes. Likewise, many researchers reported that dry weight in salinity condition was

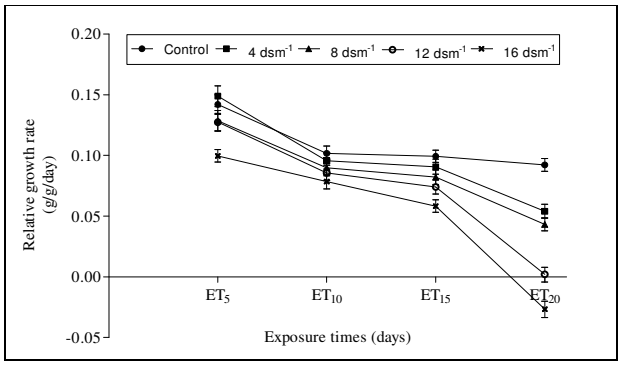

Fig 1. Interaction effect of salinity levels and exposure times on relative growth rates (RGR) of six accessions of *A. paniculata*. The five days exposure time showed highest RGR, while at 20 days exposure times seedlings did not tolerate 12 and 16 dSm^{-1} salinity levels and RGR was at lowest. Vertical bars represent S.E. for three samples.

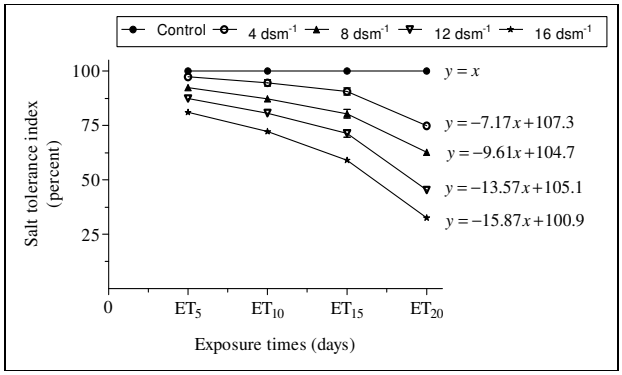

Fig 2. Interaction effect of salinity levels and exposure times on salt tolerance index (STI) of six accessions of *A. paniculata*. The control showed the highest STI, while 16 dSm^{-1} salinity levels was the lowest. The rate of STIs in different salinity and exposure times were different.

less than that in control medium (Munns, 2002; Taffouo et al., 2004; Parida and Das, 2005; Rajpar et al., 2007). One of the important parameter for determining salt tolerant plants under salinity is the biomass differences among plant species (Koca et al., 2007; Maggio et al., 2007; Dombrowski et al., 2008). The inhibition of plant growth under salt stress could be due to either reduction of osmotic on water absorption or extreme accumulation of ions, known as specific ion effect (Ashraf and Sarwar, 2002; Munns and James, 2003; Taffouo et al., 2004). Low level of salinity facilitated morphological traits in *A. paniculata*, similar to the results of Rajpor et al. (2007), while high salinity levels (16 dSm^{-1}) suppressed them. While tolerant accessions such as 11329 produced 44 % less dry weight in high salinity level, the sensitive accessions such as 11266 produced 78 % less dry weight compared to control medium. Same result was obtained in tolerant plants like sugar beet and moderately salt tolerant cotton (Greenway and Munns, 1980). Several researchers have reported similar differences between sensitive and tolerant genotypes on different plant species (Munns, 2002; Taffouo et al., 2004; Rajpar et al., 2007). The slope of the salt tolerance index showed approximately 9% decrease per salt exposure and salinity level (1.8% per day and 2.25 per dSm^{-1}). This may be due to the effect of high salinity level and long exposure time. Based on relative mass, the salt tolerance threshold is represented by two different linear slopes. During the initial period of salinity stress, seedlings were unable to acclimate to conditions due to osmotic reduction in water availability. 15 days after exposure time, seedlings were not able to withstand the salinity stress due to the accumulation of Na^+, which known as specific ion effect that significantly reduces the plant growth. A change in slope within the declining region of the response function to salinity was observed for PH, RL, NL, RDW and SDW at approximately 20 days after salinity stress. Most accessions of *A. paniculata* were able to tolerate maximum up to 12 dS m^{-1} of salinity (NaCl) and could be maintained for more than 15 days at seedling stage. At 16 dSm^{-1}, the leaves began to fall after 10 days at seedling stage. An increased biomass is usually recognized as a general response to salinity and may improve salinity tolerance by restricting the instability of toxic ions to the shoots and roots, which consequently delays the onset of the tolerance threshold. The overall results suggested 12 ds m^{-1} salinity and 15 days of stress as a salt tolerance threshold of *A. paniculata* seedlings. The ability of different plant species to tolerate salinity stress is different and depends on several interacting variables such as salinity levels and exposure times. In the present study, the seedlings responses in terms of STI were similar in the early stages of salt exposure time (5 and 10 days), but increasing exposure times significantly affected the seedlings. Therefore, to evaluate plant species to tolerate salinity stress, it is necessary to consider salt tolerance threshold. One of the most important mechanisms of tolerant plants under salinity condition is the accumulation of compatible solutes such as proline. In this study tolerant accessions showed high accumulation of proline. Likewise, many researchers reported the role of proline in osmotic adjustment, protecting cell structure and its function in many crops (Desingh and Kanagaraj, 2007; Koca et al., 2007; Turan et al., 2007; Fischer, 2011). The result of the present study showed that NaCl treatment caused an increase in Na concentration, and a decrease in K^+ and Ca^{2+} concentration in all accessions. This is in agreement with findings of others (Munns et al., 2002; Teakle et al., 2010). However, the increase in Na^+ concentration in sensitive accession like 11266 was higher than tolerants like 11329 and the decrease in K^+ and Ca^{2+} concentration in sensitive was higher than in tolerant accession. This situation caused the tolerant accessions to have the higher K^+/Na^+ ratio and a lower Na^+/Ca^{2+} ratio compared to the sensitive ones. These ion ratios were reported to be associate with the relatively salt tolerant genotypes in many species (Dvoak et al., 1994; Perez-Alfocea et al., 1996). A high K^+/Na^+ ratio in the plant cytoplasm is essential for normal cellular functions of plants. The Na^+ competes with K^+ uptake and may block the K^+ specific transporters under salinity. By considering this point that all growth indices decreased with increasing salinity levels, it could be concluded that 12 dSm^{-1} salinity and 15 days stress are the salt tolerance threshold of this species at seedling stage. In this study, STI and correlated traits such as proline, Na^+, K^+, Ca^{2+} concentrations and K^+/Na^+ and Na^+/Ca^{2+} ratios appeared to determine salinity tolerance in *A. paniculata* seedlings and cluster analysis based on these traits indicated high similarity among accessions in each cluster. However, among the 32 studied accessions, numbers 11314, 11228, 11329 and 11249 are considered to being the most tolerant and 11266, 11306, 11216 and 11264 the most sensitive accessions.

Table 3. Analysis of variance for salinity levels on morphological characteristics of 32 accessions of *A. paniculata.*

Source	df	Mean square						
		NL	SL	RL	SFW	RFW	SDW	RDW
Salinity (S)	3	2.34**	6.98**	1.70**	1.53**	0.12**	0.40**	0.03**
Accession (A)	31	0.43**	0.89**	0.38**	0.49**	0.05**	0.06**	0.01**
S × A	93	0.10^{ns}	0.14^{ns}	0.14^{ns}	0.05^{ns}	0.01^{ns}	0.002^{ns}	0.001^{ns}
CV%		9.3	14.4	17.5	26.9	28.8	13.6	25.7

Table 4. Effects of salinity levels on salt tolerance index, chlorophyll, proline and K^+ content and Na^+/Ca^{2+} ratio in 32 accession of *A. paniculata* (Mean values ±standard error).

Accession	Mean values ±standard error				
	STI (%)	CHLO (µg.g^{-1} FW)	Proline (µmol/g FW)	K^+ (%)	Na^+/Ca^{2+}
11266	53.9±10.0 ^{a}	37.4±0.5 ^{abc}	0.017±0.003 ^{a}	2.99±0.07 ^{abcdef}	31.7±2.3 ^{m}
11306	61.8±7.1 ^{ab}	37.6±1.1 ^{abcd}	0.030±0.004^{b}	2.91±0.08^{abc}	24.8±1.4 ^{ghijkl}
11216	61.9±8.1 ^{bc}	39.9±0.9 ^{cdefj}	0.022±0.005^{ab}	2.85±0.09 ^{a}	26.3±2.1 ^{jkl}
11264	63.9±6.9 ^{bcd}	37.5±0.9 ^{abc}	0.030±0.007^{b}	2.93±0.07 ^{abcd}	23.1±1.4 ^{defghij}
11265	65.5±6.9 ^{bcd}	39.0±1.0 ^{abcdefj}	0.028±0.007^{ab}	2.87±0.07 ^{ab}	27.7±1.3 ^{l}
11317	67.7±6.8 ^{bcde}	41.5±0.9^{j}	0.025±0.003^{ab}	3.12±0.06 ^{fghijk}	21.0±1.3 ^{bcdef}
11313	68.2±7.0 ^{bcde}	37.6±0.5 $_{abcd}$	0.035±0.008^{b}	3.05±0.07 ^{cdefgh}	26.1±1.6 ^{ijkl}
11328	69.1±7.4 ^{bcdef}	37.3±0.5 ^{abc}	0.028±0.004^{ab}	3.03±0.06 ^{cdefg}	24.3±1.5 ^{ghijk}
11159	69.2±6.9 ^{bcdef}	40.4±0.9 ^{defj}	0.028±0.003^{ab}	3.10±0.04 ^{efghij}	20.6±1.7 ^{bcde}
11238	70.1±6.3 ^{bcdef}	37.1±0.7 ^{abc}	0.026±0.005^{ab}	3.08±0.07 ^{defghij}	25.3±1.0 ^{hijkl}
11204	70.2±8.0 ^{bcdef}	39.0±1.3 ^{abcdefj}	0.032±0.005^{b}	3.13±0.06 ^{fghijk}	23.5±1.3 ^{defghij}
11301	70.3±5.8 ^{bcdef}	41.3±0.8 ^{fj}	0.025±0.003^{ab}	3.06±0.07 ^{cdefgh}	23.3±1.8 ^{defghij}
11325	70.4±7.1 ^{bcdef}	38.0±1.0 ^{abcde}	0.028±0.005^{ab}	3.08±0.07 ^{defghij}	25.3±1.2 ^{hijkl}
11304	70.8±7.8 ^{bcdef}	37.9±0.9 ^{abcde}	0.032±0.004^{b}	3.09±0.07 ^{defghij}	27.1±1.3 ^{kl}
11245	71.1±6.6 ^{bcdef}	39.2±0.9 ^{bcdefj}	0.029±0.004^{ab}	3.01±0.05 ^{bcdef}	25.4±1.1 ^{hijkl}
11179	71.2±7.3 ^{bcdef}	38.6±0.9 ^{abcdefj}	0.024±0.002^{ab}	3.05±0.08 ^{cdefgh}	20.5±1.6 ^{bcd}
11348	71.6±7.6 ^{bcdef}	36.3±0.8^{a}	0.026±0.004^{ab}	2.95±0.09 ^{abcde}	22.4±1.5 ^{cdefgh}
11339	72.2±7.3 ^{bcdef}	38.0±1.4 ^{abcde}	0.023±0.004^{ab}	3.08±0.07 ^{defghij}	23.7±1.4 ^{efghijk}
11303	75.2±5.8 ^{bcdef}	38.9±0.8 ^{abcdefj}	0.027±0.005^{ab}	3.13±0.07 ^{fghijk}	24.4±1.5 ^{ghijk}
11349	75.5±6.3 ^{bcdef}	40.0±1.1 ^{cdefj}	0.026±0.004^{ab}	3.08±0.03 ^{cdefghi}	21.7±1.2 ^{cdefg}
11323	75.7±7.4 ^{bcdef}	38.7±0.6 ^{abcdefj}	0.025±0.004^{ab}	3.08±0.06 ^{cdefghi}	25.6±1.3 ^{hijkl}
11345	75.8±7.7 ^{bcdef}	37.6±1.1 ^{abc}	0.028±0.005	3.13±0.07 ^{fghijk}	23.8±1.4 ^{fghijk}
11347	75.9±6.1 ^{cdef}	38.3±0.7 ^{abcde}	0.023±0.004^{ab}	3.06±0.06 ^{cdefghi}	22.8±1.2 ^{defghi}
11231	76.6±4.9 ^{def}	38.2±1.1 ^{abcde}	0.029±0.004^{b}	3.08±0.06 ^{defghij}	24.1±1.1 ^{fghijk}
11340	77.1±5.4 ^{def}	40.1±0.5 ^{efj}	0.029±0.005^{ab}	3.12±0.07 ^{efghij}	18.3±0.8 ^{ab}
11352	77.2±6.9 ^{def}	38.3±0.7 ^{abcde}	0.024±0.004^{ab}	3.13±0.04 ^{fghijk}	21.0±1.1 ^{bcdef}
11218	77.5±6.5 ^{def}	40.0±0.8 ^{cdefj}	0.031±0.005^{b}	3.12±0.07 ^{efghij}	22.9±1.4 ^{defghi}
11176	77.5±7.1 ^{def}	40.0±0.8 ^{cdefj}	0.0260.005^{ab}	3.21±0.05 ^{hijk}	19.5±1.1 ^{abc}
11249	77. ±6.6 ^{def}	40.0±0.9 ^{cdefj}	0.027±0.005^{ab}	3.26±0.06 ^{jk}	24.0±0.8 ^{fghijk}
11329	80.5±5.3 ^{ef}	40.7±1.0 ^{efj}	0.027±0.004^{ab}	3.20±0.06 ^{ghijk}	17.3±0.9 ^{a}
11228	81.9±6.4 ^{ef}	38.4±1.2 ^{abcdef}	0.032±0.005^{b}	3.24±0.12 ^{hjk}	20.6±1.0 ^{bcde}
11314	83.9±5.9 ^{f}	36.3±1.2^{ab}	0.026±0.004^{ab}	3.31±0.14 ^{k}	21.7±1.5 ^{cdefg}

Different letters indicate significant difference between the values of pairs of treatment inthe same column at P < 0.01 according Duncan's multiple comparisons.

Table 5. Analysis of variance for salinity levels on measured plant characteristics of 32 accessions of *A. paniculata.*

Source	df	Mean square								
		TDW	STI	CHLO	Proline	Na^+	K^+	Ca^{2+}	K^+/Na^+	Na^+/Ca^{2+}
Salinity(S)	3	0.42**	162.7**	99.0**	1.93**	8.43**	0.21**	0.12**	0.07**	1381.0**
Accession (A)	31	0.07**	2.2**	21.5**	0.02^{ns}	0.83**	0.01**	0.01**	0.00**	100.6**
S× A	93	0.00^{ns}	0.7^{ns}	8.5^{ns}	0.03^{ns}	0.06^{ns}	0.00^{ns}	0.00^{ns}	0.00^{ns}	9.3^{ns}
CV%		13.79	11.0	7.4	10.62	2.84	4.26	6.70	14.41	3.0

Statistical significance is indicated by ** (P<0.01),* (P<0.05) and ns (no significant). TDW (g), STI (%), CHLO (µg.g^{-1} FW), Proline (µmol/g FW), and Na^+, K^+, Ca^{2+} (%).

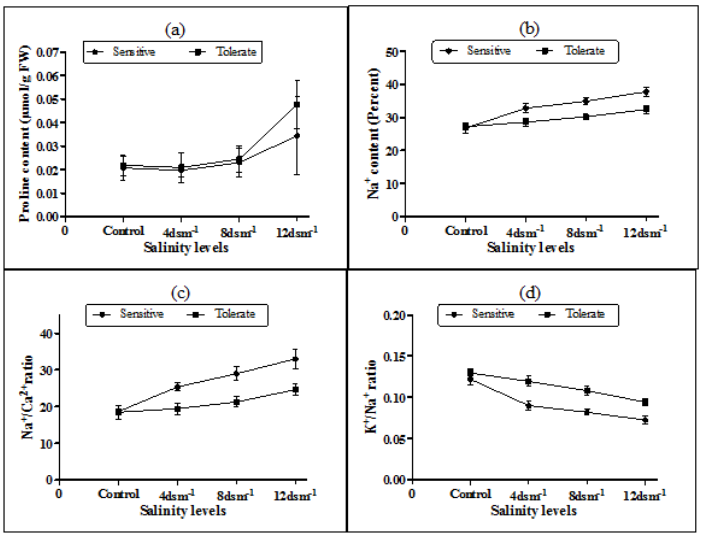

Fig 3. The effects of salinity levels on trend of proline (a), Na^+ content (b), Na^+/Ca^{2+} ratio (c) and K^+/Na^+ ratio (d) in tolerant and sesitive accessions of *A.paniculata*. Increasing salinity levels led to increase in proline and Na^+ content and Na^+/Ca^{2+} ratio and decrease in K^+/Na^+ ratio. Vertical bars represent SE for three samples.

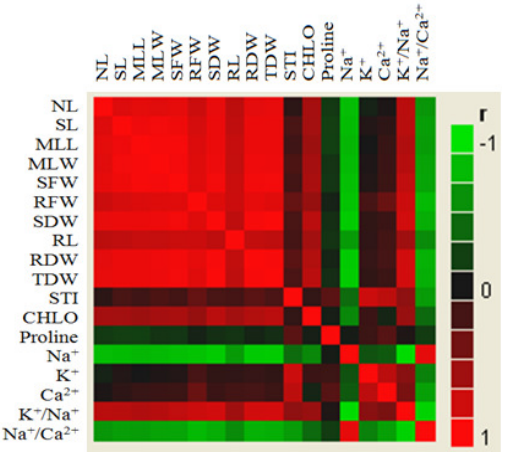

Fig 4. Correlations between 32 accessions for 18 morphological, physiological and biochemical traits. The strength and direction of the correlations among the different traits are indicated by the color (red indicates positive correlations while green indicates negative correlations, and the shading represents the strength of the correlation).

Materials and methods

Plant materials and chemicals

A total of thirty-two accessions of *A. paniculata* seeds were used in this study. The seeds were provided by Agro Gene Bank, Universiti Putra Malaysia, Serdang, Selangor, Malaysia (Table 7). The chemicals used for the treatments were of analytical grade obtained from Fisher Scientific (Leicestershire, UK).

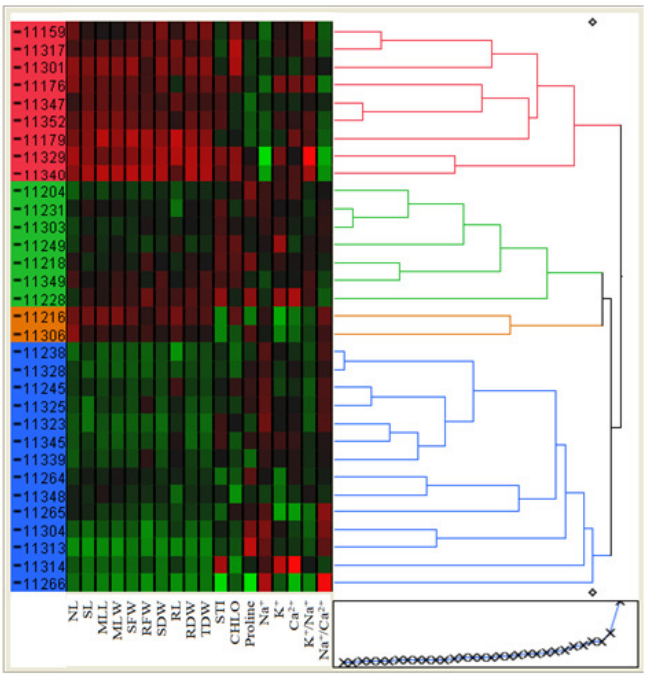

Fig 5. Dendrogram generated by UPGMA clustering method of 32 accessions of *A. paniculata* based on all measured traits under salinity condition. Red color refers to the high value of the studied traits while, green color refers to the low value. The shading represents the strength of the trait, in which the bright colors have higher values than those shadows. The indicator box under the dendrogram demonstrates the number of accessions and the cutting point designates the number of clusters.

Seed germination conditions

The seeds were surface sterilized with 10% sodium hypochlorite (NaClO) solution for 10 minutes (Talei et al., 2011) and thoroughly rinsed with distilled water. The seeds were then sown in 15 cm diameter Petri dishes containing filter papers moistened with uniform volume of sterile water. The Petri dishes were sealed with parafilm to prevent any water loss and then incubated in growth chamber at controlled temperature, light and humidity (light/dark regime of 14/10h at 28-30 ^{o}C, relative humidity 60-75%). The seedlings at two leaves stage were transferred into the jiffy media. Seedlings of each accession at six-eight leaves stage were grown in different concentration of saline water in separate trays. Each seedling was irrigated once a day with Hoagland nutrient solution with different concentration of NaCl.

Experimental technique

Two experiments were carried out as follows. The first experiment was to identify the salt tolerance threshold and second experiment was to determine salt tolerant accession of medicinal plant of *A. paniculata* during 2010. Since there has been insufficient study on salt tolerance threshold in *A. paniculata* first experiment was carried out with a split split-plot based on the Randomized Complete Block Design (RCBD) with three factors and three replicates. The three

Table 6. Phenotypic correlation coefficient (r) among measured traits in 32 accessions of *A. paniculata* under salinity conditions.

	NL	SL	MLL	MLW	SFW	RFW	SDW	RL	RDW	TDW	STI	CHLO	Proline	Na^+	K^+	Ca^{2+}	K^+/Na^+	Na^+/Ca^{2+}
NL	1																	
SL	.78**	1																
MLL	.79**	.93**	1															
MLW	.76**	.90**	.96**	1														
SFW	.84**	.91**	.94**	.91**	1													
RFW	.50**	.57**	.55**	.55**	.65**	1												
SDW	.61**	.70**	.69**	.64**	.71**	.40**	1											
RL	.48**	.60**	.62**	.61**	.58**	.47**	.43**	1										
RDW	.63**	.70**	.71**	.67**	.76**	.63**	.82**	.46**	1									
TDW	.62**	.71**	.70**	.66**	.73**	.43**	1.0**	.44**	.85**	1								
STI	.31**	.45**	.38**	.31**	.33**	.12*	.61**	.30**	.47**	.60**	1							
CHLO	.50**	.45**	.48**	.50**	.49**	.28**	.33**	.34**	.39**	.35**	.17**	1						
Proline	-.15**	-.25**	-.19**	-.17**	-.14**	-.19**	-.23**	-.23**	-.21**	-.23**	-.29**	0.04	1					
Na^+	-.36**	-.45**	-.43**	-.39**	-.42**	-.20**	-.67**	-.28**	-.53**	-.66**	-.53**	-.14**	.21**	1				
K^+	.15**	.29**	.21**	.16**	.20**	.23**	.39**	.17**	.30**	.38**	.55**	0.02	-.24**	-.36**	1			
Ca^{2+}	.17**	.28**	.23**	.20**	.21**	.24**	.36**	.19**	.27**	.36**	.49**	-0.02	-.19**	-.38**	.80**	1		
K^+/Na^+	.33**	.43**	.39**	.34**	.38**	.22**	.68**	.26**	.54**	.67**	.60**	.11*	-.25**	-.93**	.61**	.54**	1	
Na^+/Ca^{2+}	-.34**	-.46**	-.43**	-.39**	-.41**	-.26**	-.65**	-.30**	-.51**	-.64**	-.63**	-.11*	.24**	.90**	-.62**	-.72**	-.91**	1

The significant correlation is indicated by ** (P<0.01) and * (P<0.05).

Table 7. List of 32 different accessions of *Andrographis paniculata* from different states of Malaysia.

No	Accession number	State	Vernacular name	No	Accession number	State	Vernacular name
1	11159	Selangor	Tutup Bumi	17	11328	Pahang	Hempedu Ular
2	11176	Selangor	Tutup Bumi	18	11345	Pahang	Hempedu Ular
3	11179	Selangor	Tutup Bumi	19	11347	Pahang	Hempedu Ular
4	11204	Negeri Sembilan	Hempedu Bumi	20	11303	Kelantan	Lidah Ular
5	11216	Negeri Sembilan	Hempedu Bumi	21	11329	Kelantan	Lidah Ular
6	11218	Negeri Sembilan	Hempedu Bumi	22	11339	Kelantan	Lidah Ular
7	11228	Negeri Sembilan	Hempedu Bumi	23	11340	Kelantan	Lidah Ular
8	11231	Negeri Sembilan	Hempedu Bumi	24	11349	Kelantan	Lidah Ular
9	11238	Negeri Sembilan	Hempedu Bumi	25	11304	Johor	Hempedu Bumi
10	11241	Negeri Sembilan	Hempedu Bumi	26	11306	Johor	Hempedu Bumi
11	11245	Negeri Sembilan	Hempedu Bumi	27	11325	Johor	Hempedu Bumi
12	11249	Negeri Sembilan	Hempedu Bumi	28	11352	Johor	Hempedu Bumi
13	11264	Perak	Akar Cerita	29	11314	Terengganu	Lidah Ular
14	11266	Perak	Akar Cerita	30	11317	Terengganu	Lidah Ular
15	11301	Pahang	Hempedu Ular	31	11323	Terengganu	Lidah Ular
16	11313	Pahang	Hempedu Ular	32	11348	Terengganu	Lidah Ular

factors evaluated in the experiment were: different salinity levels (control, 4, 8, 12 and 16 dSm^{-1}) in the main plots, six different accessions (11179, 11216, 11241, 11249, 11264 and 11329) in the sub-main plots, and five different exposure times (Control, 5, 10, 15 and 20 days). Therefore, there were 30 treatment combinations for each exposure time. Each seedling was irrigated once a day with five different concentrations of NaCl. After the end of each exposure times, all seedlings were harvested, and the data on morphological traits including; shoot and root length (SL & RL), number of leaves (NL), fresh and dry shoot weight (SFW and SDW), fresh and dry root weight (RFW and RDW) after drying at 68ºC for 48 hours were measured. The relative growth rate (RGR) was evaluated according to Benincasa (1988), and the salt tolerance index (STI) was calculated according to Baci et al. (2003) as follows:

$$RGR = \frac{Ln\ DM_2 - Ln\ DM_1}{t_2 - t_1}$$

$$STI = \frac{TDW\ at\ S_x}{TDW\ at\ S_1} \times 100$$

Where, DM_1 is the initial total (shoot + root) dry weight, DM_2 the final total dry weight, (t_2 - t_1) the difference in time interval between two samplings, TDW= total dry weight, S_1 = control treatment, and S_x = saline treatment. Second experiment was carried out with a split-plot based on Randomized Complete Block Design (RCBD) with two factors and three replicates. The factors were four different concentration of saline water (control, 4, 8, and 12 dSm^{-1}) in main plot and 32 accessions in sub main plots. Each seedling was irrigated once a day with four levels of saline water. After every three applications of saline water, seedlings were again irrigated with normal Hoagland nutrient solution. After 15 days of salinity exposure, all seedlings were harvested and data on morphological and physiological traits including; SL, RL, NL, SFW, SDW, RFW, RDW, chlorophyll, proline and Na^+, K^+ and Ca^{2+} content were measured and salt tolerance index (STI) was calculated as described earlier .

Determination of proline and chlorophyll content

Proline was determined by the ninhydrin method described by Bates et al. (1973) on spectrophotometer (Shimadzu UV-1201). Total chlorophyll (chlorophyll a + b) was extracted in 80% (v/v) aqueous acetone and absorption was measured on a Shimadzu UV-1201 spectrophotometer at 645 and 663 nm (Arnon, 1949).

Determination of ion content

Sodium, potassium and calcium content in aerial parts of the plant were determined by using an Analyst 5100, Perkin Elmer instrument, USA described by Miller (1998). Nitrogen was determined by the digestion method described by (AOAC, 1990) by using an automated ion analyzer (Lachat instrument, Wisconsin, USA).

Statistical analysis

At first, the raw data were tested for normality distribution by using the SAS software No. 9 and the square and log transformed data were then analyzed for measured traits using analysis of variance and the Duncan's multiple range test ($P < 0.01$). The graph pad prism software No. 5 was used for drawing the graphs and JMP software was used for correlation and UPGMA cluster analysis.

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