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Morpho-Physiology of Mulberry (*Morus* sp.) Plant on Salinity Stress Tolerance

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ABSTRACT

The accession of mulberry (*Morus* sp.) with salinity tolerance unavailable in Indonesia is needed to overcome the problem of marginal land, especially in the saline areas of the country. Therefore, this research aims to determine the accession of salinity tolerance of mulberry from 6 origins in Indonesia based on growth and physiological parameters. The method used was a factorial randomized block design with the first treatment of stem cutting-derived mulberry accession being M1, M2, M3, M4, M5, M6, and M7, while the second was the concentration of NaCl at 0 mM, 40 mM, 60 mM, and 80 mM. The results showed that M2 accession from Bogor was categorized as tolerance to high salt stress, and M1 was classified as sensitive accession. Increasing salt concentration causes a decrease in growth parameters. It also decreased physiological parameters such as leaf and media relative water content while increasing dissolved sugars. Genotypic scores indicate salinity tolerance and the potential number of leaves, while Na^+ content and leaf necrosis percentage showed sodium toxicity in the leaf. Therefore, genotypic score, Na^+/K^+ ratio, and leaf necrosis percentage are the best parameters to select and identify mulberry accession with high salt stress tolerance.

1. Introduction

Abiotic stresses such as drought and salt are the main environmental factors affecting growth and development, decreasing crop production (Aroca *et al.* 2012). Evaporation exceeds rainfall in arid and semi-arid areas, increasing the nutrient concentration of salt in the soil. Furthermore, this causes drought stress in the environment. It is feared that global climate change could trigger the degradation of the world's agricultural land. Salinization is one of the degradations that threatens agriculture's sustainability, which can increase due to human activities. It disturbs 20% of the world's irrigated land (Yeo 1998).

Plants can adapt to osmotic and ionic stress by developing complex mechanisms due to high salt concentrations. Osmotic pressure causes a water deficit in roots and excessive ion toxicity in the leaf, leading to decreased growth (Munns *et al.* 2006; Munns and Gilliham 2015). Shaheen *et al.* (2013) stated that soil salinization would reduce groundwater potential; hence, plants experience osmotic stress, which causes water deficits due to difficulty in water absorption. Accumulating compatible solutes such as dissolved sugars, proline, glycine betaine, and soluble proteins is one of the osmotic regulatory mechanisms (Turan *et al.* 2007; Meguekam *et al.* 2014). Plants can develop complex adaptation mechanisms in response to ionic stress and those used in osmotic stress. Ionic stress is caused by an accumulation of Na^+ ions in plants, typically in the leaf, at levels beyond the threshold that triggers leaf cell death with chlorosis and leaf necrosis, followed by a decrease in cellular metabolic activity, including photosynthesis (Glenn *et al.* 1999). Despite high Na^+ concentrations damaging plants (Parida and Das 2005), some research showed that low salt levels have little effect on growth and

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physiological function. However, it can promote growth in other plants (He *et al.* 2019).

There are comprehensive publications on plant responses to high salt stress and salt tolerance mechanisms (Abdel-Farid *et al.* 2020; Ali *et al.* 2020; Kumar *et al.* 2021; Meguekam *et al.* 2021; Priya *et al.* 2021). Ashraf (1994) reported a significant accumulation of solutes in the form of dissolved sugars, proline, and free amino acids in *Eruca sativa* crops compared to the normal population. The report shows that dissolved sugar is one of the essential components in high salt stress tolerance. Furthermore, salt stress reduces the relative water content (RWC) as ¬in *Pistacia atlantica* Desf. subsp. atlantica (Benhassaini *et al.* 2012), *Eruca sativa* (Hniličková *et al.* 2017), and Medicago sativa (Huihui *et al.* 2021). Water deficit causes decreased leaf turgor, impacting stomata closure and decreasing conductance, and is then one factor limiting the rate of photosynthesis (Chaves *et al.* 2009). Santos *et al.* (2012) stated that increasing the NaCl concentration will reduce the canopy's wet weight on *Eruca sativa* plants. Additionally, Jesus *et al.* (2015) reported that an increase in NaCl concentration would reduce the wet and dry weight of shoots and roots and protein content. However, the proline content increased with changes in enzyme activity in 2 Rocket cultivars (Folha Larga and Cultivada).

Liu *et al.* (2017) showed that mulberry could adapt well to drought, salt stress, waterlogging, and other environmental stressors. Similarly, Yu *et al.* (2013) reported that the Chinese mulberry cultivar Anza 1 was tolerant to 0.5% NaCl concentration. Jhansilakshmi *et al.* (2016) also stated the accession of MI-0476 tolerance to salt stress at 0.4% NaCl concentration with the characters having Na^+ exclusion mechanism, low leaf necrosis, and Na^+/K^+ ratio, as well as high genotypic score. The identification of mulberry accessions with exclusion characteristics of Na^+ and Na^+/K^+ ratio can be used to select high salt stress tolerance (Jhansilakshmi *et al.* 2016).

Mulberry is the most important plant in sericulture. Mulberry leaves were used to feed silkworms, and the leaves contain compounds with medicinal properties of secondary metabolites. However, the content of these compounds tends to increase under salinity stress conditions. Mulberry plant that is tolerant to high salinity stress is unavailable in Indonesia, so studying their tolerance level in salt stress environmental conditions is necessary.

2. Materials and Methods

2.1. Plant Materials and Experimental Design

The plant material used in this research came from 6 origins consisting of 7 accessions, including M1, M2, M3, M4, M5, M6, and M7, as shown in Table 1.

The stem cuttings from a 1-year-old mulberry mother plant were planted in plastic cups until rooted, then transferred to pots with a diameter and height of 30 and 20 cm. Furthermore, the medium comprises soil and manure (1:1 (w/w)). Plants were pruned to the same height of ±100 cm before treatment. A total of 24 selected plants of each accession with a height of 90-100 cm, 4 to 6 shoots, and 13 to 17 leaves per shoot were utilized for treatment. They were arranged using a factorial group design consisting of 2 treatments. The first was a mulberry accession consisting of 7 levels, namely M1, M2, M3, M4, M5, M6, and M7, while the second was the concentration of NaCl which consisted of 4 levels, including 0 mM, 40 mM, 60 mM, and 80 mM. The salinity treatment was performed using surface watering with l L of Hoagland's solution containing 100 mM NaCl daily until it reached the desired concentration. Control plants were watered daily with l L of Hoagland's solution without NaCl. Finally, each treatment had 3 replications.

Table 1. Mulberry plant accession codes and origins

Accession code	Origin
M1	SULI-01 belongs to the Forestry Research and Development Agency of Bogor Regency, West Java
M2	Sukamantri Sub-District, Bogor Regency, West Java
M3	Sukamantri Sub-District, Bogor Regency, West Java
M4	Muktiharjo Sub-District, Pati Regency, Central Java
M5	Asem Bagus Sub-District, Situbondo Regency, East Java
M6	Negara Sub-District, Jembrana Regency, Bali
M7	Bili-Bili Sub-District, Gowa Regency, South Sulawesi

2.2. Measurement of Plant Growth

The growth parameters observed include plant height, number of shoots, stem diameter, leaf area, shoot and root dry weight, and the number of leaves. Measurement was undertaken for 16 weeks after weeks after treatment (WAT) or after the electrical conductivity (EC) concentration was reached (40 mM, 60 mM, 80 mM). The EC NaCl solution was measured using a conductivity meter (Mediatech) with 1:2 (soil: water, w/v). The plant height was calculated from the soil surface to the plant growing point. Furthermore, the number of leaves on each branch emerging from the main stem was counted at 16 WAT. The diameter of the stem was measured using a caliper at the height of 5 cm from the soil surface. The leaf area was determined using ImageJ software (version 1.44v), with the mature leaf at the 5th position from the shoot tip. The results of measuring the growth parameters were obtained from an average of 3 plants with each accession of mulberry plants in every replication. In addition, to shoot and root dry weight, the plant biomass was measured at harvest (28 WAT). Leaves, stems, and roots were put into different envelopes and dried in an oven at 70°C for 4 days (leaves), 5 days (roots), and stems (7 days) until a constant dry weight was attained.

2.3. Measurement of Leaf and Media Relative to Water Content

The leaf's relative water content (RWC) was determined according to the method of Barrs and Weatherley (1962). The mature leaf at the 5th position from the tip of the shoot was collected at 16 WAT. A total of 10 samples were obtained using a cork borer with a diameter of 1 cm. They were weighed to obtain fresh weight (FW), after which hydration was conducted for 24 hours in a petri dish to determine the saturated weight (SW) previously dried using tissue paper. Furthermore, the leaf samples were oven-dried at 80°C to obtain dry weight (DW) for 48 hours. The results of the leaf RWC measurements were obtained from an average of 3 plants in each mulberry plant accession in the replications. The following equation was used to calculate the leaf RWC:

$$\text{Leaf RWC (\%)} = (FW - DW) / (SW - DW) \times 100\%$$

The media RWC was determined gravimetrically using ±100 g soil samples (top, middle, and bottom). The soils were weighed to obtain fresh weight (FW), then dried in an oven at 80°C for 48 hours to obtain dry weight (DW). The following equation was used to calculate the media RWC:

$$\text{Media RWC (\%)} = (FW - DW) / FW \times 100\%$$

2.4. Measurement of Soluble Sugar

Leaf samples were collected from 3 mulberry plants of each accession in every replication to measure dissolved sugar content. A mature leaf still actively photosynthesizing was collected at the 5th position from the tip of the shoot at 16 WAT. The samples were dried using an oven at 80°C for 48 hours to reach a constant dry weight. The dried leaf was then ground into powder and was analyzed for its dissolved sugar content using the Luff-Schoorl method (Eviati and Sulaeman 2009).

2.5. Measurement of Tolerance Index

According to Jhansilakshmi *et al.* (2016), the genotypic score was calculated by multiplying the leaf yield response index and the tolerance index using the following formula:

$$\text{Leaf yield response index (LYRI)} = \frac{\text{leaf yield of genotype in stress}}{\text{mean leaf yield of all genotypes in stress}}$$

$$\text{Tolerance index (TI)} = \frac{\text{leaf yield of genotype in stress}}{\text{leaf yield of genotype in non-stress}}$$

$$\text{Genotypic score} = \text{LYRI} \times \text{TI}$$

$$\text{\% Necrosis} = \frac{\text{leaf area covered by a brown burnt like area}}{\text{total leaf area}}$$

2.6. Measurement of Na^+ and K^+ Content

Samples of dry powder from the leaf at position 5th from shoots collected at 16 WAT were used to analyze Na^+ and K^+ contents, which were calculated using the atomic absorption spectrophotometry (AAS) method (Eviati and Sulaeman 2009).

2.7. Statistical Analysis

Data analysis was performed with SPSS software (version 24.0; SPSS Inc.; Chicago, IL, USA). Differences between the means of dry shoot weight, dry root weight, plant height, leaves number, shoot number, stem diameter, leaf area, leaf RWC, media RWC, and soluble sugar were tested at the 5% probability level using Duncan's new multiple range test.

3. Results

3.1. Plant Growth of Morus

Growth parameters, including plant height, number of shoots, stem diameter, leaf area, and wet and dry weight of shoots and roots, were not affected by the interaction between treatments ($p>0.05$). However, it was separately influenced by a single factor (accessions) ($p<0.05$). Table 2 shows the various growth parameters affected by different accessions.

The M3 accession had the highest plant height and was significantly different ($p<0.05$) from the others. Meanwhile, M6 had the highest number of shoots but did not differ significantly from M5 and M7. The M2 accession had the highest stem diameter but was not significantly different from M3 and M4. Furthermore, M4 had no significant difference in leaf area compared to M1 and M2. Table 3 shows the biomass affected by different mulberry accessions.

The highest root/shoot ratio of 0.79 was shown by the M7 accession, while M5 indicated the lowest root/shoot ratio (Table 3). The root and shoot ratio of the M7 accession had no significant difference from other accessions. According to Table 4, plant height, the number of shoots, stem diameter and leaf area were affected ($p<0.05$) by NaCl concentration.

Plant height, the number of shoots, stem diameter, and leaf area have the lowest values at 80 mM NaCl concentration. Table 5 shows that mulberry accession biomass was affected by NaCl concentration. NaCl concentration of 80 mM had

Table 2. Growth of different mulberry accessions at 16 WAT

Accession	Plant height (cm)	Number of shoots	Stem diameter (cm)	Leaf area (cm^2)
M1	117.92^{d}	3.50^{cd}	1.65^{b}	135.27^{a}
M2	177.67^{b}	4.25^{b-d}	2.02^{a}	115.90^{ab}
M3	201.83^{a}	4.17^{b-d}	1.91^{a}	89.69^{bc}
M4	149.92^{c}	3.17^{d}	2.01^{a}	146.45^{a}
M5	171.08^{bc}	4.83^{a-c}	1.56^{b}	67.06^{c}
M6	159.00^{bc}	5.83^{a}	1.65^{b}	29.86^{d}
M7	165.08^{bc}	5.17^{ab}	1.68^{b}	88.32^{bc}

Means values with different superscript letters in the same column denote significant ($p<0.05$) differences between groups

Table 3. Biomass of different mulberry accessions at 28 WAT

Accession	Shoot wet weight (g)	Root wet weight (g)	Shoot dry weight (g)	Root dry weight (g)	Root/shoot ratio
M1	172.17^{cd}	160.76^{c}	79.54^{cd}	53.74^{c}	0.68^{a}
M2	236.34^{b}	256.15^{b}	117.60^{b}	88.33^{b}	0.75^{a}
M3	354.99^{a}	303.02^{a}	158.26^{a}	106.58^{a}	0.67^{a}
M4	208.55^{bc}	210.24^{b}	93.26^{bc}	64.11^{c}	0.69^{a}
M5	152.46^{cd}	153.84^{c}	77.26^{cd}	48.47^{c}	0.63^{a}
M6	139.12^{d}	140.12^{c}	59.86^{d}	46.86^{c}	0.78^{a}
M7	167.28^{cd}	208.48^{b}	77.88^{cd}	61.38^{c}	0.79^{a}

Means values with different superscript letters in the same column denote significant ($p<0.05$) differences between groups

Table 4. Mulberry plant growth at different NaCl concentrations at 16 WAT

Concentration of NaCl (mM)	Plant height (cm)	Number of shoots	Stem diameter (cm)	Leaf area (cm^2)
0	171.90^{a}	4.76^{a}	2.05^{a}	143.11^{a}
40	167.38^{a}	5.00^{a}	1.88^{b}	100.29^{b}
60	171.19^{a}	4.24^{ab}	1.73^{b}	79.88^{bc}
80	142.38^{b}	3.67^{b}	1.47^{c}	61.03^{c}

Means values with different superscript letters in the same column denote significant ($p<0.05$) differences between groups

Table 5. Mulberry plant biomass at different concentrations of NaCl at 28 WAT

Concentration of NaCl (mM)	Shoot wet weight (g)	Root wet weight (g)	Shoot dry weight (g)	Root dry weight (g)
0	305.10^{a}	139.90^{a}	257.86^{a}	93.81^{a}
40	206.49^{b}	96.15^{b}	222.02^{b}	69.80^{b}
60	213.63^{b}	94.55^{b}	208.13^{b}	63.94^{b}
80	92.45^{c}	48.63^{c}	130.62^{c}	40.72^{c}

Means values with different superscript letters in the same column denote significant ($p<0.05$) differences between groups

the smallest value and was significantly different ($p<0.05$) from others for plant height, stem diameter, and wet and dry weight of shoots and roots.

The number of mulberry leaves was affected by the interaction between accessions and NaCl concentration ($p<0.05$). The mean leaf count for the NaCl concentrations in all mulberry accessions was reduced, as shown in Figure 1. Additionally, the number of leaves from each accession showed the least amount at 80 mM NaCl concentration (1.33, 28.67, 20.67, 7, 23, 9.33, 46.33 leaves number in M1, M2, M3, M4, M5, M6, and M7, respectively). Figure 2 shows leaf reduction in M1 accession treated with NaCl.

Leaf RWC is one of the physiological parameters in response to osmotic stress. It was influenced by accession ($p<0.05$). Furthermore, M6 showed the highest leaf RWC (95.52%) and was significantly different from M4, as shown in Figure 3.

The NaCl concentration affected the leaf RWC ($p<0.05$). It is inversely proportional to the leaf RWC. Figure 4 shows a fairly sharp decrease in the 80 mM NaCl treatment, which showed the lowest leaf RWC and was significantly different from the control.

The media RWC was affected by the interaction of accession and NaCl concentration ($p<0.05$) (Figure 5). M2, M4, and M5 accessions showed a similar pattern, namely an increase in the value of the media at 40 mM NaCl concentration, which decreased.

3.2. Soluble Sugar in *Morus* Leaf

Dissolved sugar in mulberry leaf was affected by accession ($p<0.05$), as shown in Figure 6. M4 accession showed the highest dissolved sugar content (4.0%) but did not differ significantly from M3 and M6 accession.

Dissolved sugar was also affected by NaCl concentration, as shown in Figure 7. Similarly, the

Figure 2. Reduced leaves in one replication of M1 accession treated with NaCl at 16 WAT

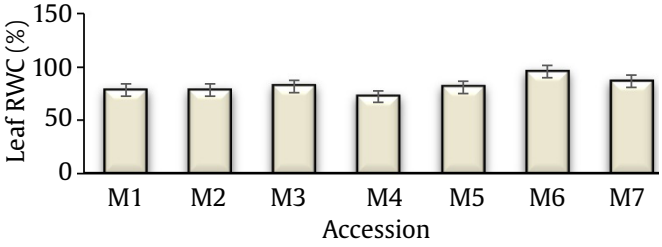

Figure 3. Leaf RWC on different mulberry accessions at 16 WAT

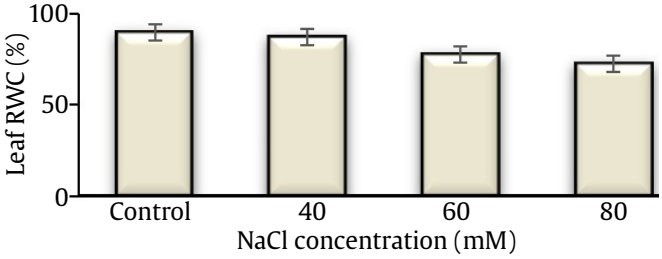

Figure 4. The relative water content of 16 WAT mulberry leaves at different NaCl concentrations

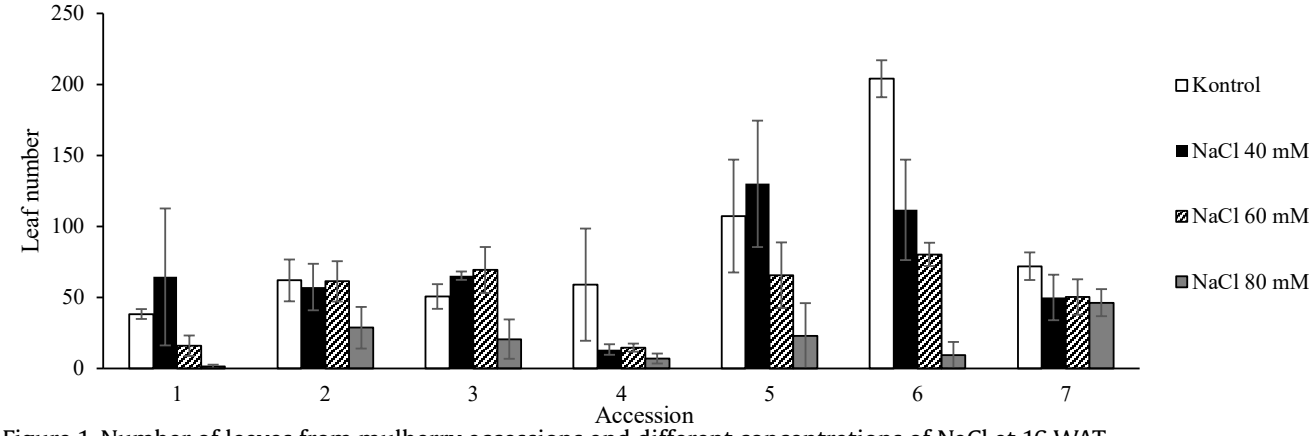

Figure 1. Number of leaves from mulberry accessions and different concentrations of NaCl at 16 WAT

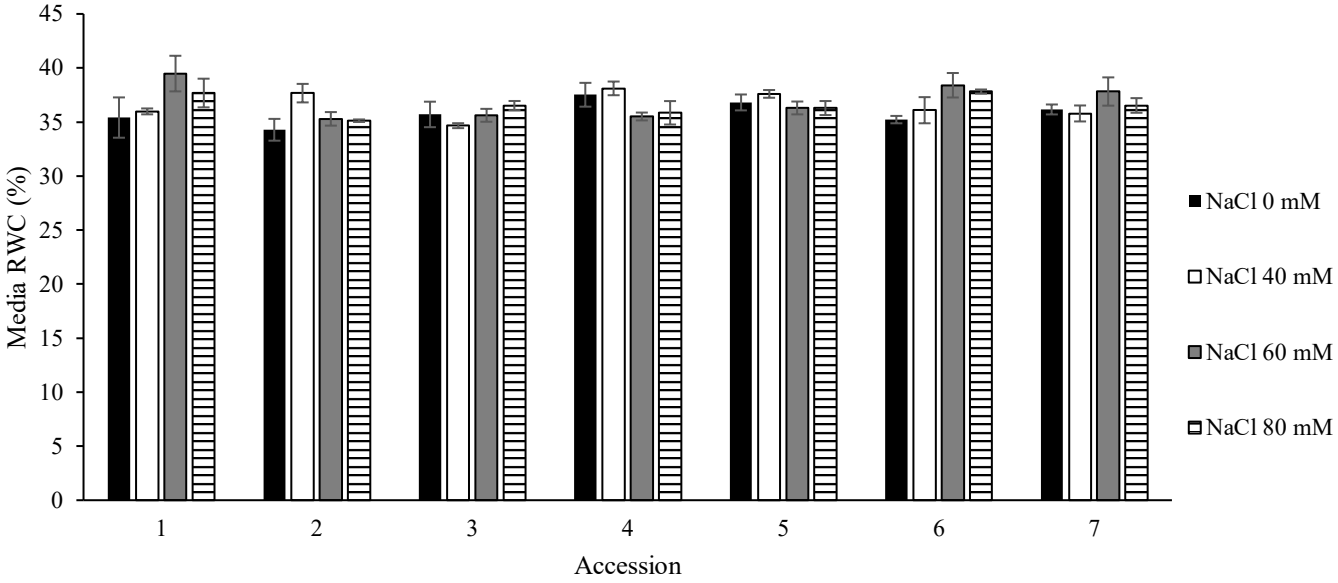

Figure 5. The relative water content of media on different accessions of mulberry and NaCl concentration

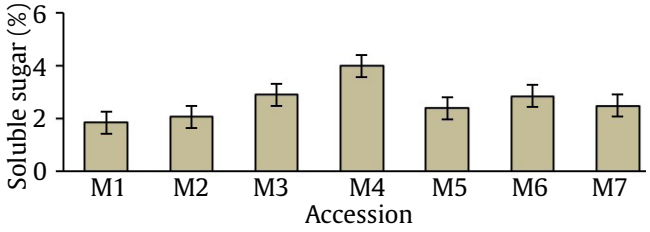

Figure 6. Dissolved sugar in different mulberry accession leaves in 16 WAT

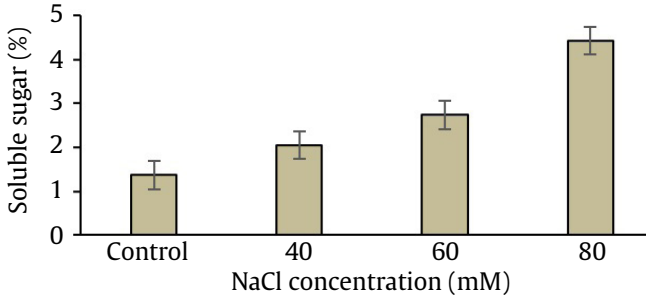

Figure 7. Dissolved sugar in different accession mulberry leaf at NaCl concentration at 16 WAT

dissolved sugar content increased with the NaCl concentration. The highest content in the leaf was 4.43% at 80 mM NaCl concentration and significantly different from others ($p<0.05$).

3.3. Measurement of Tolerance Index

The number of leaves parameter is needed to calculate the tolerance index, leaf yield response index, and genotypic score. Table 6 showed that the highest genotypic score at 80 mM NaCl concentration was discovered in M7 accession to be 1.53. Meanwhile, the lowest genotypic score percentage at 80 mM NaCl concentration was observed in the M1 accession to be 0.0. It exhibited a decrease in genotypic score with increasing NaCl concentration.

3.4. Na^+ and K^+ Content in *Morus* Leaf

Na^+ accumulation in the leaf of all mulberry accessions at 80 mM NaCl concentration ranged from 0.5-2.13%, as shown in Table 7. The Na^+/K^+ ratio of the M5 accession was not significantly different from M3 and M7 accessions.

3.5. Leaf Necrosis

The percentage of leaf necrosis was affected by accession ($p<0.05$), as shown in Figure 8. M6 accession had the highest percentage of leaf necrosis at 43.33%, significantly different from M2 accession at 14.17%. The proportion of leaf necrosis in the M6 accession was not considerably different from M1 and M4 accessions ($p>0.05$). The percentage of leaf necrosis was affected by different accessions of the mulberry leaf ($p<0.05$) that improved with increasing NaCl concentration, as shown in Figure 9.

4. Discussion

Mulberry growth was calculated by measuring plant height, the number of shoots, stem diameter, leaf area, shoot and root dry weight, and the number of leaves. The parameters decreased with increasing NaCl

Table 6. Tolerance index, leaf number response index, and the genotypic score of mulberry accession in response to salinity stress

Accession	Number of leaf/plant				Tolerance index			Leaf number response index			Genotypic score		
	0 mM	40 mM	60 mM	80 mM	40 mM	60 mM	80 mM	40 mM	60 mM	80 mM	40 mM	60 mM	80 mM
M1	38.33	64.33	16.00	1.33	1.68	0.42	0.03	0.92	0.31	0.07	1.54	0.13	0.00
M2	62.00	57.33	61.33	28.67	0.92	0.99	0.46	0.82	1.20	1.47	0.75	1.19	0.68
M3	50.67	65.33	69.33	20.67	1.29	1.37	0.41	0.93	1.36	1.06	1.20	1.86	0.43
M4	59.00	13.33	14.67	7.00	0.23	0.25	0.12	0.19	0.29	0.36	0.04	0.07	0.04
M5	107.33	130.00	65.67	23.00	1.21	0.61	0.21	1.85	1.29	1.18	2.24	0.79	0.25
M6	204.00	111.67	80.33	9.33	0.55	0.39	0.05	1.59	1.57	0.48	0.87	0.62	0.02
M7	72.00	50.00	50.33	46.33	0.69	0.70	0.64	0.71	0.99	2.38	0.49	0.69	1.53
Average	84.76	70.28	51.09	19.48									

Table 7. Na^+/K^+ ratio as a salinity stress response in 7 mulberry accessions

Accession	Na+ content (%)				K+ content (%)				Na+/K+ ratio			
	0 mM	40 mM	60 mM	80 mM	0 mM	40 mM	60 mM	80 mM	0 mM	40 mM	60 mM	80 mM
M1	0.23^{gh}	0.94^{c-f}	1.00^{c-e}	1.18^{cd}	3.12^{i-j}	4.63^{c-e}	5.70^{ab}	5.90^{a}	0.07^{e-h}	0.22^{b-f}	0.18^{c-h}	0.20^{c-g}
M2	0.05^{h}	0.52^{e-h}	0.74^{c-g}	1.00^{c-e}	4.12^{c-h}	4.26^{c-g}	4.50^{c-f}	4.80^{cd}	0.02^{gh}	0.12^{e-h}	0.17^{c-h}	0.21^{c-f}
M3	0.30^{gh}	0.16^{gh}	1.18^{cd}	2.13^{a}	3.34^{h-j}	3.74^{e-j}	4.79^{cd}	5.00^{bc}	0.08^{e-h}	0.04^{f-h}	0.24^{b-f}	0.43^{a}
M4	0.04^{h}	0.16^{gh}	1.13^{cd}	1.30^{bc}	2.97^{j}	3.16^{ij}	3.14^{ij}	5.88^{a}	0.01^{h}	0.05^{f-h}	0.39^{ab}	0.22^{b-f}
M5	0.23^{gh}	0.38^{f-h}	1.75^{ab}	1.80^{ab}	2.80^{j}	3.15^{ij}	3.90^{d-i}	4.00^{d-i}	0.08^{e-h}	0.13^{d-h}	0.44^{a}	0.46^{a}
M6	0.64^{d-h}	0.15^{gh}	0.25^{gh}	0.50^{e-h}	3.19^{h-j}	3.25^{h-j}	3.30^{h-j}	3.50^{g-j}	0.20^{c-g}	0.05^{f-h}	0.08^{e-h}	0.14^{d-h}
M7	0.14^{gh}	0.26^{gh}	1.10^{c-e}	1.10^{c-e}	3.67^{f-j}	3.64^{f-j}	3.30^{h-j}	3.60^{f-j}	0.04^{f-h}	0.07^{e-h}	0.31^{a-d}	0.31^{a-d}

Means values with different superscript letters in the same column denote significant ($p<0.05$) differences between groups.

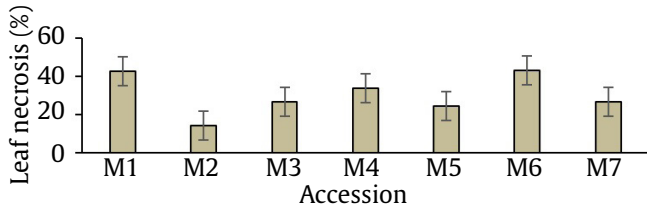

Figure 8. Leaf necrosis percentage of different mulberry accession in 16 WAT

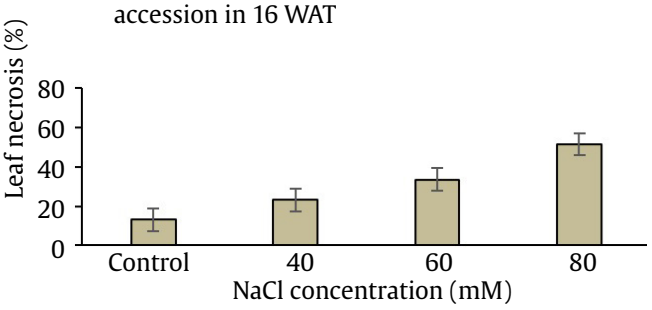

Figure 9. Percentage of leaf necrosis at different mulberry accession in 16 WAT at different NaCl concentrations

concentration, as shown in Tables 4 and 5. The effect was quite severe at the highest salt concentration of 80 mM. Furthermore, the worst damage was experienced at the highest salt concentrations in wheat plants (Sairam *et al.* 2002) and green beans (Mekhaldi *et al.* 2008) as a result of the water deficit due to the relatively high solute concentration in the soil. NaCl reduces the plant's ability to take water due to decreased potential, which decreases plant growth. Acosta-Motos *et al.* (2017) stated that lowering osmotic and water potential and hydraulic conductivity will reduce water availability, thereby triggering nutrient imbalance. Decreased plant growth can also be caused by excessive salt entering the leaf through transpiration, injuring the cells (Meguekam *et al.* 2021).

The roots or shoots ratio of M7 and M2 at 0.79 and 0.75, respectively, was the highest, as shown in Table 3. According to Kapoor *et al.* (2020), the result of carbon assimilation was allocated more for root growth than the canopy to maintain the water absorption capacity of plants under drought-stress conditions; as a result, decreasing plant growth. Plant growth yield is closely related to biomass, namely dry weight. Furthermore, the M3 accession has the best wet and dry weight of shoots and roots, followed by the M2 accession, as shown in Table 3.

Physiological changes related to the ability to retain water in the leaf due to high solute concentrations were observed in the leaf RWC values, as shown in Figure 3. The low leaf RWC value in the M4 accession could be due to the larger leaf area of the M4 accession, which was 146.45 cm^2 compared to others. It is therefore thought to result in high transpiration, as shown in Table 2. Meanwhile, the high value in the M6 accession,

as shown in Figure 3, could be due to the low leaf area of 29.86 cm^2, as presented in Table 2, presumably resulting in low transpiration. The high transpiration rate in broad leaves, such as M4 accession, and water deficit caused by salt treatment can lead to low leaf RWC values, as shown by the accessions. The leaf area of the M2 accession was not significantly different from M4, as indicated in Table 2. Figure 3 shows that M2 accession maintained its water status, implying tolerance to salinity stress.

Furthermore, it was similarly reported by Gan *et al.* (2021) that salinity tolerance mulberry (Jisang3) did not decrease leaf RWC, while the sensitive accession (Guisangyou12) reduced significantly. According to Figure 4, increased NaCl concentration decreases the leaf RWC value. A reasonably sharp decrease was shown in the 80 mM NaCl treatment. Similar results were reported by El-Bassiouny and Bekheta (2005), Wang *et al.* (2012), and Hand *et al.* (2017) on wheat, pepper, and Iris lacteal, respectively. Increasing NaCl concentration decreased osmotic potential in salt tolerance rocket plant "Astro" and reduced leaf RWC value to a concentration of 200 mM NaCl (Hniličková *et al.* 2017).

In addition to leaf RWC, the media values can be used to examine the tolerance ability of mulberry plants to salt stress. M2, M4, and M5 accessions showed a decrease in the value of media RWC with increasing NaCl concentration. Meanwhile, M1, M3, M6, and M7 accessions exhibit development with increasing NaCl concentration. Benhassaini *et al.* (2012) and Munns (2002) stated that salinity reduces the rate of water transport toward the canopy, thereby causing a decrease in the growth rate and changes in metabolism.

The regulation of soluble sugar content in the leaf was associated with decreased growth under salt-stress conditions. Azcon-Bieto' (1983) reported that the decreased rates of carbon assimilation and reduced yields were associated with carbohydrate accumulation in many species. Additionally, Singh (2004) stated that large soluble sugar accumulation decreased the cell osmotic potential and reduced turgidity in tolerant genotypes. The tolerant varieties always accumulate more dissolved sugars in the leaf and developing tissues than the sensitive counterpart (Watanabe *et al.* 2000). This was also reported by Gandonou *et al.* (2011), stating that the sugarcane tolerant cultivar (CP66-346) accumulated more soluble sugar in the leaf than the sensitive counterpart (CP65-357). The same results were reported in sunflower (Ashraf and Tufail 1995) and Populus (Watanabe *et al.* 2000). Figure 6 showed that M4 accession had the highest soluble sugar content but did not differ significantly from M3 and M6 accessions.

The increase in dissolved sugars also occurred with a rise in NaCl concentration, as shown in Figure 7. The highest dissolved sugar content of 4.43% was observed at 80 mM NaCl concentration and was significantly different. An increase in the accumulation of dissolved sugars in the leaf along with a rise in NaCl concentration was also reported by Gandonou *et al.* (2011); Benhassaini *et al.* (2012); Leite da Silva *et al.* (2021) in *Saccharum* sp., *Pistacia atlantica*, and *Physalis angulata* L. plants, respectively. When exposed to salt stress, plants accumulate compatible osmolytes such as proline and dissolved sugars (Zhu 2002). Furthermore, the dissolved sugars serve as an osmolyte to balance the potential water equilibrium due to the high accumulation of ions in the cells (Taiz *et al.* 2015); hence, cells are not prone to plasmolysis due to salt stress.

The initial selection of tolerance to salinity can also be seen from the high genotypic score. M7 accession showed a high genotypic score at 80 mM NaCl concentration, followed by M2, M3, and M5 accessions, as shown in Table 6. In addition to genotypic scores, biomass is the primary determinant of plant productivity. Ashraf and Mcneilly (1987) reported that plant biomass variation concerning salinity is thought to provide the best way for the initial selection process for tolerance genotypes based on the evaluation of special characteristics and characters. According to Table 3, the shoot and root biomass of M2 was higher than the M7 accession. Therefore, the M2 accession was more tolerant to salinity stress than the M7 accession.

The critical determinant of tolerance to salinity stress can be identified based on a low Na^+ strategy (Flowers and Yeo 1995). The accumulation of this ion in the leaf of all mulberry accessions at a concentration of 80 mM NaCl ranged from 0.5-2.13%, as shown in Table 7. The M2 accession contains 1% Na^+ at 80 mM NaCl concentration. Furthermore, the low ion content in the leaf of MI-0763 and MI-0246 mulberry accessions at 80 mM NaCl concentration indicated a relatively high Na^+ exclusion mechanism (Jhansilakhsmi *et al.* 2016).

The accumulation of Na^+ is related to its toxicity in the leaf, which causes necrosis. Leaf necrosis in each accession varied between 14.17-43.33%, as shown in Figure 8. The M6 accession had the highest necrosis percentage, resulting in a pretty high leaf decrease,

as presented in Figure 1. M2 accession has a relatively low Na^+ content; hence, it does not cause toxicity. This was indicated by the lowest leaf necrosis percentage in the M2 accession compared to other accessions, as shown in Figure 8. According to Jhansilakshmi *et al.* (2016), mulberry plants harvested for their leaf (foliage crop), the parameters of the number of leaves, and the percentage of necrosis are methods for the initial selection of salinity tolerance. Despite having a high percentage of leaf necrosis, some sensitive accessions may have high biomass. Table 3 showed that M1 accession also had a moderately high shoot dry weight, despite having moderately severe leaf necrosis, as presented in Figure 8. This could be due to the increased leaf area in sensitive accessions such as M1 and M4, as demonstrated in Table 2, which affected the biomass. The accumulation of Na^+ or Cl^- concentrations in the leaf generally results in a burn-like area (Zhu 2002). Jhansilakshmi *et al.* (2016) reported that salt accumulation was higher in the leaf than at the root tips. High salinity also causes leaf cell injury and inhibits growth (Tuteja 2007). Furthermore, the concentrated salt in old leaves causes them to die faster (Munns *et al.* 2006).

According to the growth and physiological parameters in Figures 1-9 and Tables 1-7, M2 accession from Bogor was categorized as tolerant to high salt stress, while M1 was classified as sensitive accession. Genotypic score, Na^+/K^+ ratio, and leaf necrosis percentage are the best parameters to select and identify mulberry accessions tolerant to high salt stress while considering biomass. Therefore, this research provides a new perspective on integrating tolerance characteristics into appropriate breeding programs to increase crop productivity in saline soils based on the differential response of mulberry accession to salinity.

For further research, the concentration of NaCl could be increased up to 150 mM using mulberry accessions categorized as tolerant. NaCl concentration more than equal to 150 mM is categorized as very high salinity.

Conflict of Interest

The authors declare no conflict of interest.

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