

Full Length Research Paper

Effect of salt stress on genetic diversity of *Robinia pseudoacacia* seedlings

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Salt stress is an abiotic stress known to affect plant growth and distribution. In this investigation, this expectation was tested on germination, seedlings' survival and genetic diversity of *Robinia pseudoacacia* (black locust). To do this, seeds of *R. pseudoacacia* collected from a natural population were sown on soil media of different salt concentrations (0.6, 1, 2, 3, 4, 5, 6, 7 and 8 g/kg), after which germination and seedlings survival rates were observed. Subsequently, genomic DNA was isolated from leaf samples for genetic analysis using 10 nuclear SSR primers. The results show that seed germination and seedling survival significantly reduced with increase in salt concentration. Specifically, *R. pseudoacacia* seedlings did not grow on soils with salt concentrations higher than 6 g/kg. As regards the effect of salt stress on genetic diversity of *R. pseudoacacia* seedlings, the overall result from 10 nuclear microsatellite primers revealed an increase in heterozygosity as salt concentration increased, which suggested selection against homozygosity under salt stress. This result further supports the fact that heterozygosity is one of the factors that ensures that tree populations are adaptable to salt stress. Therefore, management of forest tree populations should be geared towards managing genetic diversity in order to engender survival under salt stress.

Key words: *Robinia pseudoacacia*, salt stress, genetic diversity, nuclear microsatellite, adaptability.

INTRODUCTION

Salinity is one of the most severe abiotic stresses affecting plant growth (Wang et al., 2003). It can damage or reduce nearly all functions of the plant (Greenway and Munns, 1980). It interferes with water absorption and enhances accumulation of Na⁺, which lead to imbalance of mineral elements and disturbance of cellular biochemical reactions. The cellular metabolism, physiological, biochemical, as well as photosynthetic activities are all adversely affected by salt stress (Chanyou et al., 2007). In addition, salt stress can also affect seed germination and inhibit elongation of germinated seeds (Greenway and Munns, 1980; Sairam and Tyagi, 2004). Salt stress

as well can affect genetic diversity of plant populations, thereby making plants to adapt to adverse environmental conditions at morphological, physiological and biochemistry and cellular and molecular levels (Amolkumar and Sharma, 2008; Geert-Jan and Pumisitapon, 2008; Ayalew and Maki, 2010). With such adaptation in combination with genetic evolution in certain environment, a plant species can evolve into a specific ecotype with its own genetic diversity.

Reports have shown that forest trees are also prone to salt stress (Geburek, 2000). However, there seem to be limited experimental studies that are explicitly directed at examining the effect of abiotic stress on genetic diversity of forest trees (Allen et al., 1994; Krauss et al., 1998; Hansen et al., 1994). The reason may be due to the longevity feature of forest trees which make evolutionary

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effects to be difficult to track down (Geburek, 2000). Notwithstanding, studying the genetic diversities of different tree populations can reveal their evolutionary histories and information from such studies can be used to analyze their evolutionary potential and trend. In terms of applicability, it can set up foundation for breeding and genetic improvement and can also be of importance in the collection, conservation, evaluation and usage of germplasm resources (Levin et al., 2003; Waples and Gaggiotti, 2006; Masakazu et al., 2010).

Robinia pseudoacacia L. known as black locust is one of the fast-growing broad-leaf tree species in the world. The tree has enormous ecological, economic value and certain salt-tolerant capacity and (Boring and Swank, 1984; Degomez and Wagner, 2001; Gu et al., 2006; Božena et al., 2009). The tree is native to regions with 1,000 to 1,500 mm annual rainfall, yet it is drought-tolerant and survives on as little as 400 mm. Its natural distribution includes the Appalachian and Ozark mountains of the eastern US between 35 to 43° N latitudes (Hanover and Mebrahtu, 1991). Under natural conditions, the species is widely distributed, with good adaptability and rich genetic diversity (Chang et al., 1998; Liesebach et al., 2004; Yang et al., 2004a, 2004b; Nam et al., 2010). The tree is exotic in China with a planting history over 100 years. Its genetic diversity is less than that of American *R. pseudoacacia* (Xie et al., 2001; Gu et al., 2006). Liu et al. (2010) reported that salt stress reduces the germination rate, seedlings survival rate and biomass accumulation of *R. pseudoacacia* seedlings. Hence, the present investigation is to assess the effect of soil salinity on the genetic diversity of *R. pseudoacacia* seedlings.

MATERIALS AND METHODS

Planting procedure

About 1000 kg seeds of *R. pseudoacacia* used for the study were procured from the Hebei Forest Bureau, China. To reduce bias, the 1000 kg collected from local farmers (1 kg from each farmer) across Hebei province in China, were thoroughly mixed together. Seeds were thereafter sterilized with 0.5% mercury (II) chloride (HgCl₂) for 20 min, and thereafter washed in water at 80°C. Next, the seeds were soaked in water at 60°C for 1 h and afterward removed and left to cool to room temperature. Subsequently, the seeds were soaked in water for 24 h. Afterward, the seeds were sown into pots containing sowing media of different saline concentrations (salt composed of NaCl, 80%; CaCO₃, 10%; MgSO₄, 5%; K₂SO₄, 5%) of 0.6, 1, 2, 3, 4, 5 and 6 g/kg) in such a way that each pot with a specific saline concentration contained 200 seeds, thereby making the experiment to be a completely randomized design. Thus, each pot with a specific saline concentration was replicated thrice. The pots which were 30 cm in diameter and 30 cm in height contained sowing media (15 kg each) that were prepared with salt-rich soil (14 g/kg) from Yanshan Cangzhou (38°19'N 116°52'E) and soil with low salt content (0.6 g/kg) from Baoding, China (38°51'N 115°29'E). The rationale behind preparing the sowing media with soil mixture from natural sources is to mimic field

condition. In order to avoid elution of salt ions by rainfall, the pots were placed under a humidified propagator. Tending management, such as weeding, pest control and irrigation, was also conducted.

Measurement of germination and survival rate

From a previous study (Liu et al., 2010), it appeared that seeds can germinate and grow into seedlings within 10 days of sowing. Therefore, germination rate of the seeds were estimated on the tenth day after sowing. Afterward, seedlings mortality rate were estimated every tenth day within a period of 30 days.

DNA isolation and PCR amplification

Leaf samples were collected from 30 young *R. pseudoacacia* representing each saline concentration (0.6, 1, 2, 3, 4, 5 and 6 g/kg) for extraction of genomic DNA. The procedure for the DNA isolation was according to CTAB protocol (Clarke, 2009). Based on the recommendation of Lian and Hogetsu (2002) and Chunlan et al. (2004), 21 nuclear SSR primers pairs were screened for polymorphism out of which 10 pairs with high polymorphism, clear bands and good reproducibility were selected. The selected primers used for the PCR amplification are shown in Table 1.

PCR amplifications were carried out in a total volume of 10 µL, with 2 µL template DNA, 1.1 µL of 10 × PCR buffer, 0.2 µL of 2.5 mM dNTPs, 0.5 µL of each 10 pM primer, 5.6 µL of ddH₂O and 0.1 µL of 5U Taq DNA polymerase (Promega, Madison, USA). The PCR was performed in a Biometra T3 Thermocycler (Biometra GmbH, Göttingen, Germany) under the following conditions: 95°C for 10 min; 35 cycles of 95°C for 1 min, 53 to 58°C for 50 s, 72°C for 50 s; 72°C for 7 min. The PCR product underwent vertical electrophoresis (Bio-Rad) in a 5% denaturing polyacrylamide gel and silver staining.

Data analysis

Germination and survival rate of the seedlings were transformed before subjecting them to analysis of variance (ANOVA) based on the recommendation of Akindele (1996). Post-mortem analysis was carried out using Duncan's multiple range test (DMRT). These analyses was carried out using the Statistical Package for Social Scientist (SPSS) Version 18.0.

In analyzing fragments from PCR amplification, size of alleles (bp) was detected with MK DL2000 Lambda marker DNA. The average number of alleles per locus (*A*) was computed by dividing the total number of alleles observed at all gene loci by the total number of gene loci. Average observed heterozygosity (*H_o*), which is the proportion of all heterozygous genotypes across all loci was also estimated. In addition, effective number of alleles (*N_e*) (Equation 1), fixation index (*F*) (Equation 2) and expected heterozygosity (*H_e*) (Equation 3) were also estimated. Estimation of these genetic parameters was carried out using SAS 9.2. Additionally, the relationship among *R. pseudoacacia* seedlings of different salt concentrations was analyzed by means of a cluster analysis using the UPGMA (unweighted pair group method using arithmetic average) dendrogram based on Nei's genetic distances (1978). The UPGMA was constructed using the program NTSYS-pc ver. 2.0 (Rohlf, 1998).

$$N_e = \frac{1}{\sum (p_i)^2} \quad (\text{Morgante et al., 1994}) \quad (1)$$

Table 1. Primers for SSR analysis in this study.

Locus	Repeat sequence	Sequence of primer(5'-3')	Annealing Temperature (°C)	Allele size (bp)	No. of allele	H_o	H_e	GenBank accession no.
Rops04	(AC) ₁₀	GTCTAATTCAC TTTTCTCACGAG GGACACCACCRAAATTCTACC	56	105 - 110	3	0.056	0.538	AB075030
Rops05	(AC) ₂ GC(AC) ₇	TGGTGATTAAGTCGCAAGGTG GTTGTGACTTGTACGTAAGTC	56	120 - 138	8	0.800	0.859	AB075031
Rops06	(GT) ₃ ACA(GT) ₁₁	CTAAGGAGGTGCTGACCCTC TTAATCTGTGATGGGACACTG	56	117 - 144	7	0.556	0.816	AB075032
Rops08	(CA) ₈ TA(CA) ₃	TTCTGAGGAAGGGTTCCGTGG GTTAAAGCAACAGGCACATGG	56	191 - 205	6	0.778	0.683	AB075033
Rops15	(CT) ₂₀	GCCCATTTTCAAGAATCCATATATTGG TCATCCTTGTTTTGGACAATC	54	112 - 254	43	0.950	0.910	AB120731
Rops16	(CT) ₁₃	AACCCTAAAAGCCTCGTTATC TGGCATTTTTTGAAGACACC	56	195 - 223	15	0.889	0.910	AB120732
Rops18	(AC) ₈	AGATAAGATCAAGTGCAAGAGTGTAAG TAATCCTCGAGGGAACAATAC	54	135 - 219	13	0.856	0.845	AB120733
Rp102	(GA) ₁₂	CCAAATCTCAAATGTGCTAAGTAGC ACTTGGGCTATGGTATTGCA	58	205 - 211	4	0.333	0.489	AB353928
Rp106	(GT) ₉	AAACTGAATTATATCCCTTTACGGC GCATATATCCACCAGATACCCG	56	143 - 154	5	0.821	0.750	AB353929
Rp109	(AG) ₁₇	GAGGAATCACAAAACCGTTTGG TGGGATTTGAGAGAGTGGTGGTG	56	136 - 160	10	0.795	0.797	AB353930

$$F = 1 - \frac{H_o}{H_e} \quad (\text{Wright, 1951}) \quad (2)$$

$$H_e = 1 - \sum (p_i)^2 \quad (\text{Nei, 1973}) \quad (3)$$

Where, p is the frequency of the i th allele.

RESULTS

Survival rates at different saline concentrations

The seeds' germination rate and survival rate of seedlings are shown in Table 2. The result

indicates that rate of germination and survival significantly decreased with the increase in salinization throughout the period of assessment. Rates of germination and survival reduced gently as salt concentration increased from 0.6 to 3 g/kg. However, the rates dropped remarkably as salt concentration increased from 4 to 8 g/kg. In fact,

Table 2. Germination and survival rate of *R. pseudoacacia* under different regimes soil concentration.

Salt concentration (g/kg)	Germination rate (%)	Survival rate (%)			Overall survival rate (%)
	10 Days	20 Days	30 Days	40 Days	
CK	96 ^a	93	92	92	92.3 ^a
1.0	95 ^a	90	90	90	91.3 ^b
2.0	94 ^a	84	83	82	83.2 ^c
3.0	88 ^b	81	74	74	76.3 ^d
4.0	78 ^c	58	49	49	52.0 ^e
5.0	66 ^d	44	34	34	37.3 ^f
6.0	38 ^e	27	17	17	20.3 ^g
7.0	17 ^f	0	0	0	0.0 ^h
8.0	8 ^g	0	0	0	0.0 ^h

Days indicate days after seed sowing; CK, control (0.6 g/kg); values with different alphabets are significantly different from each other at 0.05 level of significance.

Table 3. Genetic diversity of *R. pseudoacacia* under different salt concentrations.

Concentration of salt (g/kg)	<i>A</i>	<i>A_e</i>	<i>H_o</i>	<i>F</i>
CK	3.1	2.49	0.58	-0.01
1	3.0	2.49	0.58	-0.07
2	3.0	2.23	0.53	-0.05
3	3.0	2.47	0.64	-0.13
4	2.9	2.55	0.65	-0.22
5	3.0	2.32	0.69	-0.33
6	2.9	2.32	0.70	-0.30

CK, control (0.6 g/kg).

almost the whole seedlings were dead at 7 to 8 g/kg saline concentration. There was an overall reduction in survival rate between the 10th and 30th day of assessment, while the survival rate was stable between the 30th and 40th day after sowing.

Zymogram characteristics of amplification of SSR primers

Clear and stable electrophoretic bands were visualized after electrophoresis with PAA gel. Genotypes and gene frequency of alleles were determined based on these clear bands and afterward, genetic parameters were estimated. Average number of alleles per locus was 3.2, while intraspecific polymorphism across the loci was 100%.

Effects of salt stress on the genetic diversity of *R. pseudoacacia* population

Pooled genetic diversity estimates across all loci are presented in Table 3, while locus by locus diversity measures are shown in Figure 1. The pooled data shows that as salt concentration increased from 0.6 to 6.0 g/kg, only

subtle reductions were observed in the number of allele per locus (*A*) and number of effective allele (*A_e*) as they varied from 3.1 to 2.9, and from 2.4961 to 2.3193, respectively. However, the observed heterozygosity *H_o* increased from 0.5833 to 0.7067, while the expected heterozygosity *H_e* slightly reduced from 0.5773 to 0.5411. Similarly, fixation index (*F*) also decreased with increase in salt concentration. In addition, the difference between observed and expected heterozygosity (*H_o* – *H_e*) increased as salt concentration increased (Figure 2), thus indicating heterozygote surplus and elimination of homozygotes as salt concentration increased. As regard locus by locus assessment, there was no apparent variation in the allelic diversity as salt concentration increased, except at Rop 06 and Rop 15 where number of allele slightly reduced from three to two, and five to four, respectively. At most loci, there was an overall increase in *H_o* and decrease in *F* with increase in salt concentration, although this was not the case at Rop 15 loci.

Cluster analysis of seedlings of *R. pseudoacacia* population under salt stress

The average genetic distances of treatments of saline

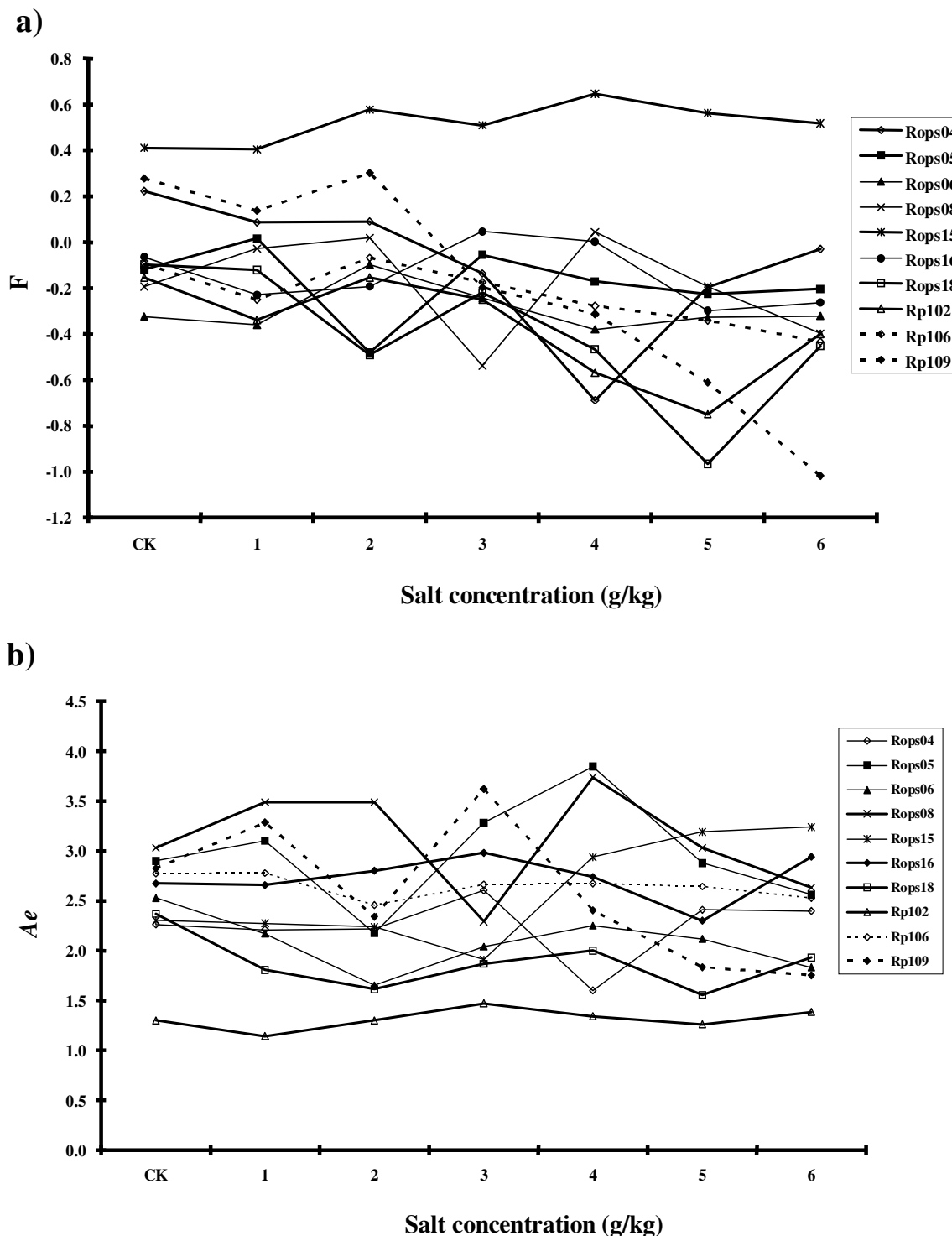


Figure 1. Effect of salt concentration on genetic diversity (a) fixation index F , (b) effective number of allele A_e , (c) observed heterozygosity H_o , (d) number of alleles A .

soils are depicted by cluster tree diagram in Figure 3. The cluster analysis shows that at genetic distance of 0.07, the 7 treatments clustered into 3 groups; (1) treatment

under salt concentrations from 0.6 to 3.0 g/kg, (2) population under the salt concentration of 4.0 g/kg and (3) treatment under salt concentrations from 5.0 to 6.9 g/kg.

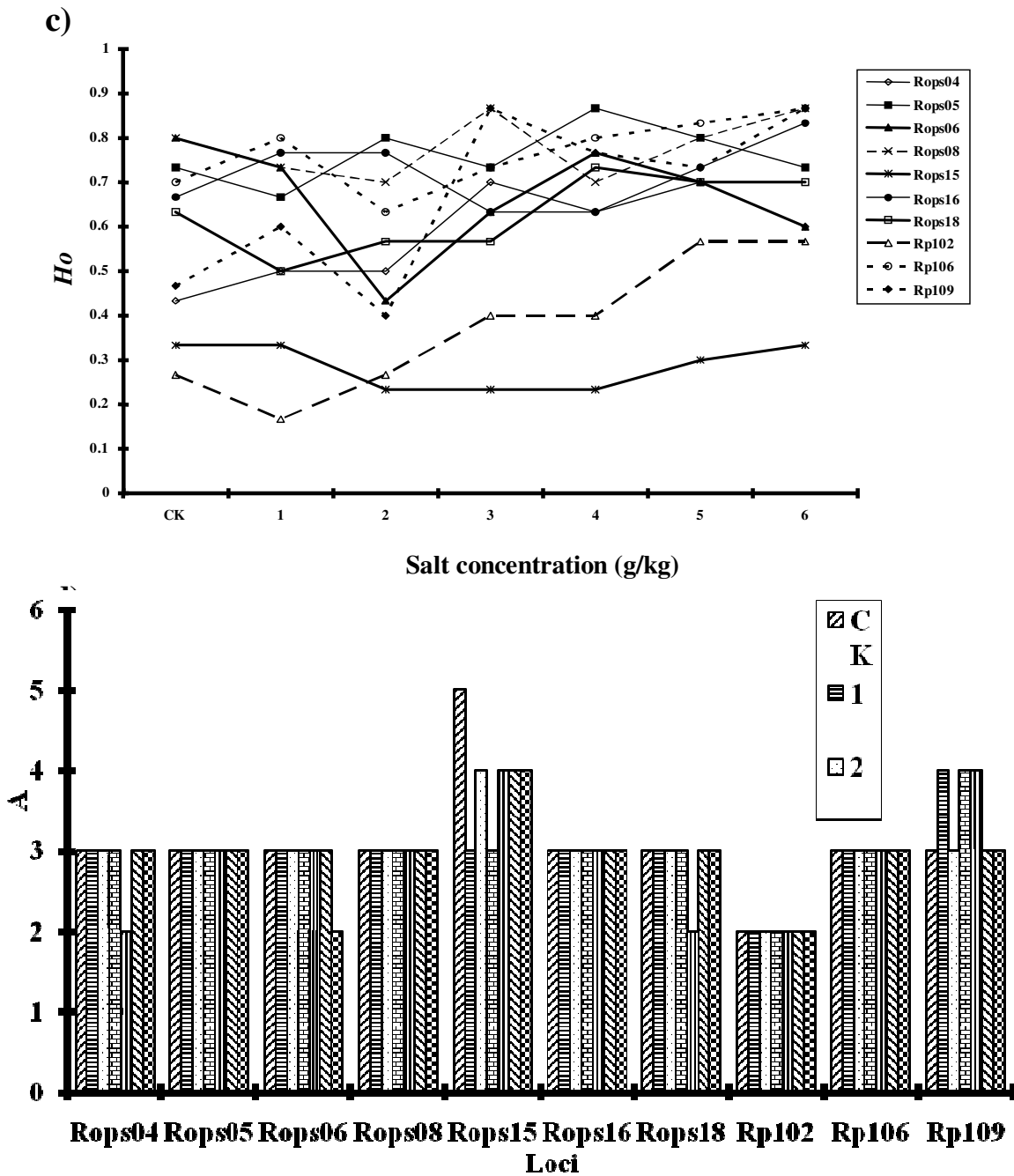


Figure 1. Contd.

DISCUSSION

Salt stress is one of the manifold environmental stresses that affect plants' survival (Abogadallah et al., 2010). Therefore, to study effects of salt stress on genetic diversity of forest tree populations in this study, an experimental situation in a controlled environment was chosen in which *R. pseudoacacia* seedlings were raised

on soils with varying regimes of salt concentration. This experimental condition was adopted because of the difficulty of using natural stands. In addition, the study was carried out in a controlled environment in order to eliminate effects of other stress factors such as water and lack of nutrient. Another essential requirement for the study was application of a genetic marker like nuclear SSR marker because it can directly reveal the differences

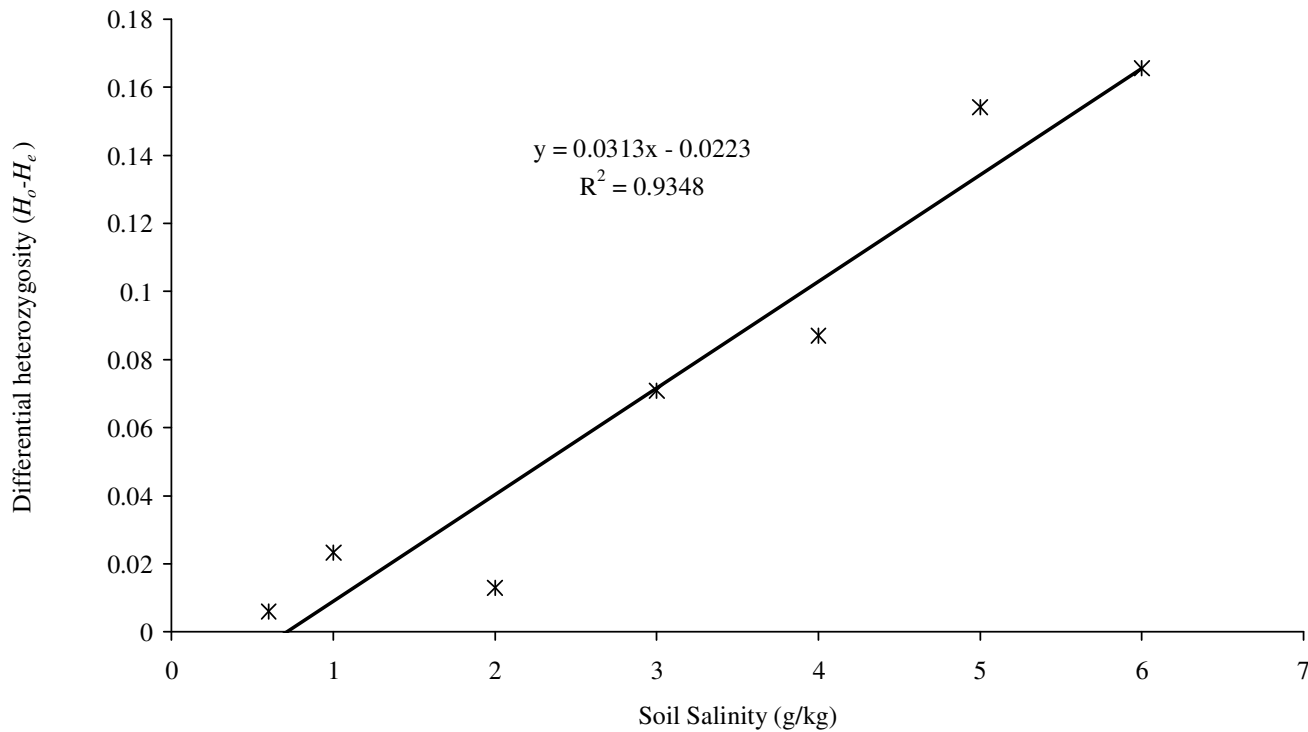


Figure 2. Relationship between soil salinity and deferential heterozygosity.

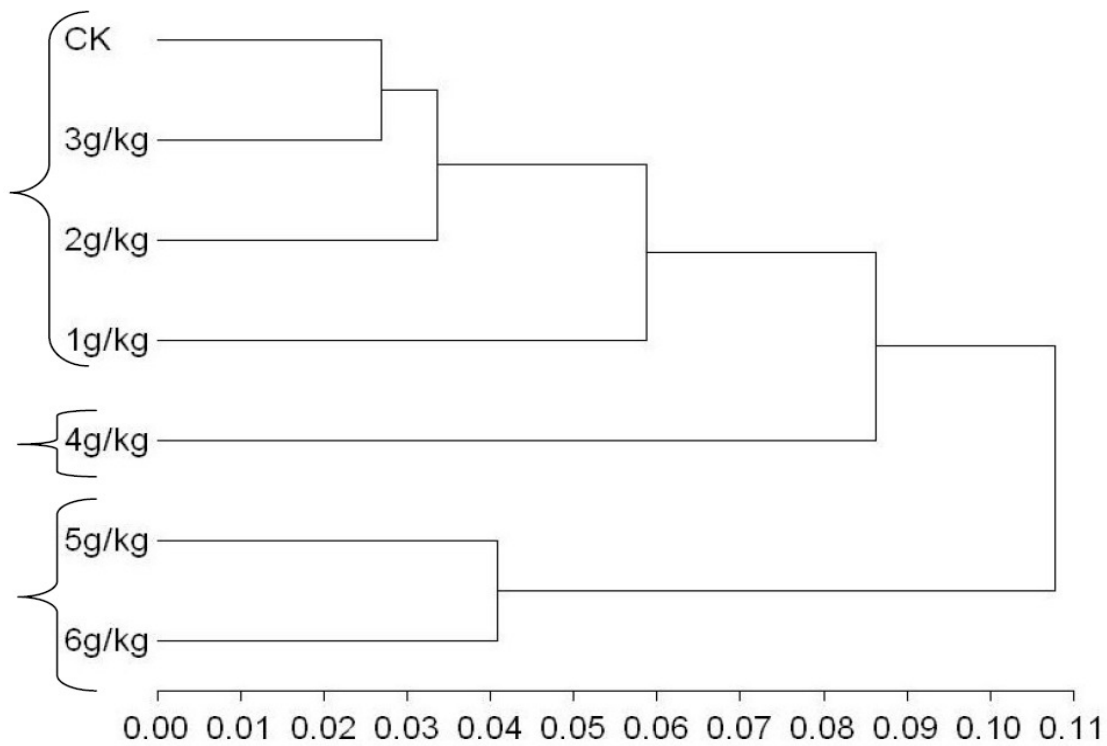


Figure 3. Cluster diagram based on SSR pair wise genetic distances among treatments of *R. pseudoacacia* under different salt concentrations. CK, control (0.6 g/kg).

among DNA in gene groups, which are not influenced by environment and interaction of genes. The marker has the advantage of being co-dominant and stable under environmental influence, and it has been widely applied in genetic studies (Greuk et al., 2008; Khoshnood et al., 2010). Due to the great specificity of nuclear SSR markers, the first step when applying such technique is to find suitable SSR primers (Zane et al., 2002; Lian et al., 2006) and for this study, SSR primers specifically designed for *R. pseudoacacia* were selected to analyze the effect of salt stress (Lian and Hogetsu, 2002; Chunlan et al., 2004).

Ordinarily, analyzing effect of salt stress on genetic diversity using a neutral marker like SSR can be difficult due to the fact that selection induced by environmental stress like salt stress would primarily act on single gene loci (Finkeldey and Hattmer, 2007). However, changes in genetic diversity at putatively neutral gene loci can still be used to monitor selective processes particularly when there appears to be associations between selected loci and marker loci (Finkeldey and Ziehe, 2004). This explanation is supported by results from comparison of genetic structures at isozyme gene loci between tolerant and sensitive *Fagus sylvatica* in environmentally stressed forests (Ziehe et al., 1999), and viability selection at early stages of *Platypodium elegans* (Hufford and Hamrick, 2003) using nuclear SSR gene loci, thereby suggesting selective response of trees to environmental stress.

Concerning the effect salinity on seed germination and seedling survival of *R. pseudoacacia*, the result in this study is in accordance with earlier report of Liu et al. (2010). There was a reduction in seed germination rate as well as seedling survival rate as soil salinity increased. In fact, at the 20th day of assessment, all seedlings growing on soil media 7 and 8 g/kg salt had died, which strongly suggests that regeneration of *R. pseudoacacia* may be difficult on salt-rich soils. The likely reason for reduction in seed germination and seedling growth as soil salinity increased may be due to the fact that salinity affects seed germination and growth of plants adversely by disrupting the physiological and metabolic processes occurring in plant body (Afzal et al., 2006; Rashid et al., 2004). Specifically, salt do impose osmotic stress and ion toxicity due to high concentration of sodium and chloride, as well as nutrient ion imbalance due to high level of Na⁺ and Cl⁻ which reduces the uptake of K⁺, NO⁻, PO₄ etc. (Nawaz et al., 2010), and as a result inhibit seed germination and seedlings plant growth (Jamil et al., 2006; Basalah, 2010). Though it has been noted that trees can still tolerate salinity to a certain extent when compared with agricultural field crops (Niazi et al., 1985; Ali et al., 1987), our result further confirms that seed germination, growth and survival of trees are still sensitive to salinity at their seedling stage (Ramoliya and Pandey, 2002; Yokota, 2003; Parsons, 2004).

Salt tolerance can control genetic variation among

provenances, as it was observed in *Eucalyptus camaldulensis* (Eldridge et al., 1994). Considering the effect of salinity on the genetic diversity of *R. pseudoacacia* seedlings based on the pooled data from all the 10 SSR primers, it is important to recall that each salt treatment contained the same number of seeds at the beginning of the experiment. Thus, adverse effect of increase in soil salinity on germination and seedlings' survival of *R. pseudoacacia* was obvious (Table 2). From the pooled data, it appeared that there was only a subtle reduction in allelic diversity from low-salt soil to salt rich soil, but there was overall increase in heterozygosity as regimes of salt concentration increased. Ordinarily, increase in heterozygosity as salt concentration increased gives impression that raising soil salinity level could be a way of increasing genetic diversity in tree populations, but that was not the case. By and large, it is intriguing that surviving *R. pseudoacacia* seedlings in our study appeared to be more genetically diversified at most SSR loci on salt-rich soils than low saline soils. In related studies, similar observations at isozyme gene loci were reported for different tree species, such as, *Picea abies*, *Pinus sylvestris*, *F. sylvatica* (Mueller-Strack, 1985, 1989; Bergmann and Scholz, 1987; Gebruek et al., 1987) and *Picea rubens* (DeHayes and Hawley, 1992). In these studies, groups tolerant to varied environmental stress tend to be higher in heterozygosity than sensitive groups.

In another study on the dynamics of genetic diversity during early stages of *P. elegans* using nuclear SSR markers, Hufford and Hamrick (2003) observed that there was selection against homozygotes during the early stages of *P. elegans* thereby leaving essentially an out-crossed adult population. This support the proposition that increased heterozygosity is one of the factors that ensure greater adaptability of trees (Mueller-Starck, 1985), which might have enabled some *R. pseudoacacia* seedlings population to survive the salt stress. The phenomenon whereby abiotic stress resulted in increase in heterozygosity has been attributed to homozygosity of deleterious alleles, to differing degrees of heterozygote advantage, or combination of both (Geburek, 2000). Therefore, increase in heterozygosity as soil salinity increased might be due to the selective disadvantage of homozygotes, and not a direct consequence of increased salinity. Furthermore, as regard the locus by locus assessment in this study, the result in most of the loci was similar to the pooled data except at Rops15 where H_o was marginally stable as salt concentration increased from 0.06 to 6 g/kg, and F which was positively increased gently with increase in salt concentration. To interpret the increment in F , it is important to recall that positive F means heterozygote deficit, which at Rops15 increased as salt concentration increased. In addition, Rops15 contained a rare allele which was only observed at the lowest salt concentration (0.06 g/kg) but was not present in other salt concentrations. This probably supports the

hypothesis that some alleles at certain loci which are deleterious could be lost due to stress.

In the cluster analysis, the treatments appeared to clump together based on the level of salt concentration. Since seeds used in the study were thoroughly mixed together to allow homogeneity, the cluster analysis suggests that apart from geographic distance, other factors like salinity gradient could also lead to genetic differentiation. This is in agreement with the study of Eleuterius (1989) on *Juncus roemerianus* (needlegrass rush), which indicated that soil water salinity is the selective force causing genetic differentiation in *J. roemerianus*.

Conclusion

The result presented in this study represents responses of genetic diversity of *R. pseudoacacia* seedlings to salt stress, and not the entire life stage of the tree species. Our samples were restricted to seedlings since adult population was difficult to study. It is probable that responses of genetic diversity of adult population to salt stress could be different. In any case, our results further add to the body of information that proves that heterozygosity is a key factor in the adaptability of trees. Therefore, management of forest tree populations should be geared towards managing genetic diversity in order to engender survival under abiotic stress.

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