Development of an *in silico* Gene Bank for Plant Abiotic Stresses: Towards Its Utilization for Molecular Analysis of Salt Tolerant and Susceptible *Casuarina equisetifolia* Clones

Mathish Nambiar-Veetil[1*], Dhanya Narayanan Nair[1], Rajendran Kamalabai Selvakesavan[1], Rathinam Saviour Caesar Jayaraj[1], Mani Roopesh[1], Samuvel Johnson Prabhu[1], Aijar Balasubramanian[1], Rathinavel Venkatachalam[1], Kottaipalayam Somasundaram Sowmiyarani[1], Shanmugan Sudha[1], Vijaykumar Waman Bachpai[1], Claudine Franche[2], Hassen Gherbi[2], Sergio Svistoonoff[2], Valérie Hocher[2], Didier Bogusz[2] and Ramanathan Vivekanandan[1]

1. Institute of Forest Genetics and Tree Breeding, Coimbatore 641002, Tamil Nadu, India
2. Equipe Rhizogenèse, UMR DIAPC, Institut de Recherche pour le Développement (IRD), 911 Avenue Agropolis, BP 64501, 34394, Montpellier Cedex 5, France

* Email: nvmathish@ yahoo. com

Abstract Worldwide, abiotic stresses represent the main cause of crop loss. Molecular marker and genetic engineering approaches are increasingly being used for breeding varieties with improved productivity in stressed sites. However, application of these techniques by Casuarina breeders requires understanding of the genetic determinants of salt tolerance. Gene isolation efforts in model systems have resulted in the identification of a large number of these polygenic determinants. Comparative genomics provide cost effective approaches for identification of gene homologues from Casuarina, a species widely planted in the coastal areas of tropics. To provide a one-point resource of gene sequences implicated in stress tolerance, a database of gene sequences implicated in abiotic stress tolerance was developed. The nucleic acid and protein sequence information for cellular signaling components, transcription factors, transporters, and those involved in protein and membrane protection were downloaded and analyzed for identification of conserved regions and deducing PCR primers that could be tested for isolation of gene homologues from salt tolerant tree species. The gene information for transcription factors and transporters were uploaded into a prototype WAMP environment. After validation, the comprehensive database will be web-enabled for access and editing by researchers. The database, together with the EST resource for nodules and non-nodulated roots of *C. glauca* developed at the Institut de Recherche pour le Développement (IRD), France, would facilitate identification of genes implicated in salt tolerance in *Casuarina equisetifolia*. Eighty-five clones of *C. equisetifolia* were assessed for their salt stress response and highly tolerant and susceptible clones were identified. While the most susceptible clones had a shoot to root ratio of sodium ranging from 1.5 to 3.6, in the tolerant clones it was

lesser than or around one indicating that the parameters could be considered as an important marker for screening of salt tolerant casuarina clones. These clones will be used for differential transcriptome analysis under salt stress.

1 Introduction

Salt tolerance is a polygenic trait. The determinants of salt stress tolerance, classified as "effector" molecules that are directly involved in stress adaptation, and "regulatory" molecules that control the amount and timing of effector molecules, and the genes implicated in these functions have been extensively reviewed (Nelson *et al.*, 1998; Hasegawa *et al.*, 2000; Grover *et al.*, 2001; Wang *et al.*, 2003; Bartels and Sunkar, 2005; Munns and Tester, 2008). The abundance of sequence information of transcripts implicated in abiotic stress responses in different plant species necessitates integration of available DNA and protein sequence information for enabling exhaustive bioinformatic analyses. Currently available websites on abiotic stresses include the *Generation Challenge Programme - Comparative Plant Stress Responsive Gene Catalogue* comprising protein sequence datasets (Wanchana *et al.*, 2008) and the *Plant Environment Stress Transcript Database* comprising transcript information from 16 crop species (Balaji *et al.*, 2006). Here we describe the development of an *in silico* gene bank specifically for nucleic acid and protein sequences implicated in abiotic stress tolerance in all plant species for which data is available. Wide variation in salt stress response has been reported in *Casuarina equisetifolia* clones. Eighty-five clones of *C. equisetifolia* were assessed, to identify highly salt tolerant and highly susceptible clones that differed in their shoot to root sodium ratio. These will be subject to differential transcriptome analysis for salt tolerance in Casuarina.

2 Materials and Methods

2.1 Retrieval of gene information and sequence analysis

Nucleic acid and protein sequence information for cellular signaling components, transcription factors, transporters (sodium transporters, potassium transporters, calcium transporters, proton transporters, water transporters), and those involved in protein and membrane protection (molecular chaperones, dimethyl sulfonium compounds, polyols, proteins-osmotin, ROS scavenging membrane fluidity) were downloaded into Excel spread sheets from NCBI website. ClustalW (Thompson *et al.*, 1994), the online multiple sequence alignment tool, was used for identification of conserved regions and deducing the relationship between the different species. Online software PriFi, which works with an alignment of DNA sequences from phylogenetically related species to provide a list of degenerate primer pairs that have a maximal probability of amplifying orthologous sequences in other phylogenetically related species, was used (Fredslund *et al.*, 2005).

2.2 Database development

Tables and their relationships were created for all the sequences downloaded after data normalization. Gene information was organized in Excel spread sheets for both amino acid and nucleic acid sequences with fields including gene name, locus name, sequence length, molecular type, modification date, IFGTB update, definition, accession number, version, GI, database source, taxonomic class, reference, authors, consortium, title, journal, pubmed, comment, method, IFGTB remark, features, gene, CDS, STS, miscellaneous, mRNA sequence, wet lab primers, product length, primer references, conserved regions, *in silico* primers, product length,

RNAi targets, promoter, specific conserved elements, stress conditions, information about the function of the sequence. Gene information of transcription factors and transporters were used for the development of prototype *in silico* gene bank for plant abiotic stresses in WAMP environment.

2.3 Screening of salt tolerant *C. equisetifolia* clones

To identify *C. equisetifolia* clones substantially differing in their salt stress response, 85 clones obtained from the Casuarina germplasm collection of the Institute of Forest Genetics and Tree Breeding, Coimbatore, India (Jayaraj and Savio, 1998) were screened for their salt tolerance in hydroponic condition. Nine-month-old ramets rooted in vermiculite were used for the experiment. Each clone was represented by 28 ramets, of which 20 ramets were subjected to salt stress while 8 ramets were used as control. The clones were allowed to grow in Hoagland solution for two months after which the salt concentration was gradually increased at an increment of 50 mM each week from 50 mM to 500 mM. The clones were then left in same concentration for one month after which the salt concentration was increased to 550 mM. Data on the number of survived ramets and the number of needles were taken after the clones were subjected to a NaCl concentration of 200 mM, 400 mM and 500 mM. The percent difference in the number of needles in each clone relative to the control plants when the salt concentration was increased from 200 mM (4^{th} week) to 500 mM (10^{th} week) was calculated using the formula $[(B\text{-}A)/A \times 100]_{control} - [(B\text{-}A)/A \times 100]_{treated}$, where B is the average number of needles present in the clone after 10^{th} week and A is the average number of needles present in the clone after 4^{th} week following the start of the experiment. Needles were also collected after 200 mM, 400 mM and 550 mM NaCl treatment for sodium and potassium analysis by flame photometry. Sodium and potassium analyses were also carried out in roots after 550 mM NaCl treatment.

3 Results

3.1 Retrieval of gene information and sequence analysis

A total of 2, 377 nucleotide sequences and 2, 520 amino acid sequences were downloaded from NCBI (Sayers *et al*., 2010), Swiss-prot (Boeckmann *et al*., 2003) and Plant Transcription Factor database (Perez *et al*., 2009). The sequences were analyzed for identifying conserved regions. Based on the results of multiple sequence alignments, PriFi was used to design PCR primers for the phylogenetically related species.

3.2 Development of database

The total number of fields for nucleic acid and protein sequence information for the genes were 48 and 30 respectively. These fields were subdivided into 11 nucleotide and 4 protein subtables viz, Gene-NA 1 to 11 and Gene-AA 1 to 4 respectively. The "gene name" field common to all tables was used as a primary key. The database with information for transcription factors and transporters in WAMP environment using phpMyAdmin, dreamweaver software was used to develop the web pages. PHP scripts were included in the site to facilitate retrieval of information for sodium antiporter genes.

3.3 Screening for salt tolerant and susceptible clones of *C. equisetifolia*

Eighty five clones were screened for their salt stress response in hydroponic condition. The survival percentage (Fig. 1) and visual observation on the overall health of the plants were used to identify 17 highly salt tolerant and 14 susceptible clones. These 31 identified clones were analyzed for percent

difference in the number of needles during salt stress (data not shown), and sodium and potassium content in needles and roots (Fig. 2 and Fig. 3). These data along with visual observation on the overall health of the plants were used to identify 5 most salt tolerant (TNIPT-4, TNKBM-407, APKKD-10, APVSP-14 and TNMT-2) and 6 most susceptible clones (PYN, JKCE-8, APVJM-33, TNPP-4, TNVM-3 and TNPV-2).

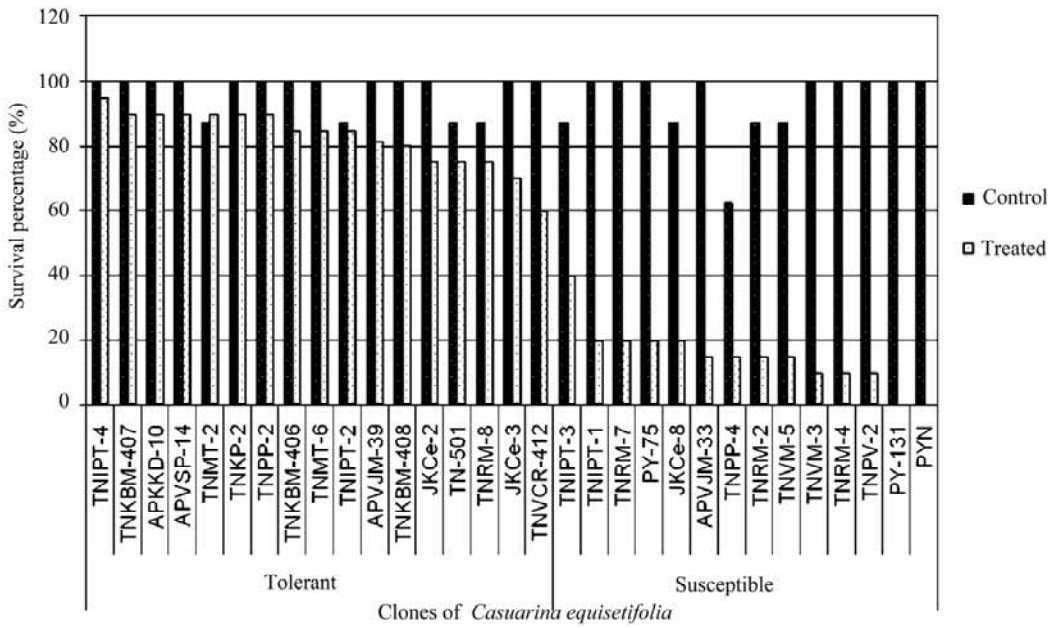

Fig. 1 Survival percentage of *C. equisetifolia* clones after 500 mM NaCl treatment

3.4 Effect of NaCl treatment on sodium concentration in shoots and roots

Analysis of sodium and potassium content in needles and roots collected after 550 mM NaCl treatment in the 31 clones (Fig. 2) revealed that the susceptible clones tended to accumulate more sodium in the shoots than in the roots in contrast to tolerant clones where in the shoot to root sodium was lesser than or around one.

4 Discussion

4.1 Database development

The database being developed is intended to be a one-point resource for molecular breeders for accessing information on protein and nucleotide sequences of different abiotic stress tolerance conferring genes. This database of abiotic stress tolerance would complement the EST database developed for nodules and non-nodulated roots of *C. glauca* at the Institut de Recherche pour le Développement (IRD), France (Hocher *et al*., 2006), for facilitating identification of gene homologues related to salt tolerance in *C. equisetifolia*. Differential transcript and functional analysis of these genes using the composite transgenic approaches (Gherbi *et al*., 2008) would provide information on the major genes contributing to salt tolerance in *C. equisetifolia*. The information would, therefore, provide inputs for development of candidate gene based markers in *C. equisetifolia*.

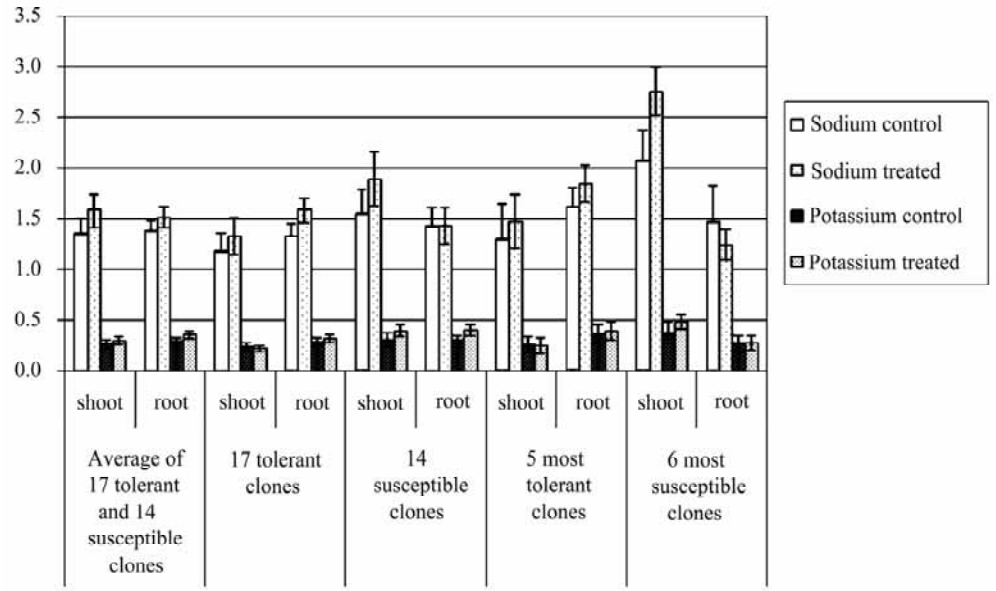

Fig. 2 The average sodium and potassium percentage in the shoots and roots of identified tolerant and susceptible *C. equisetifolia* clones at 550 mM NaCl treatment

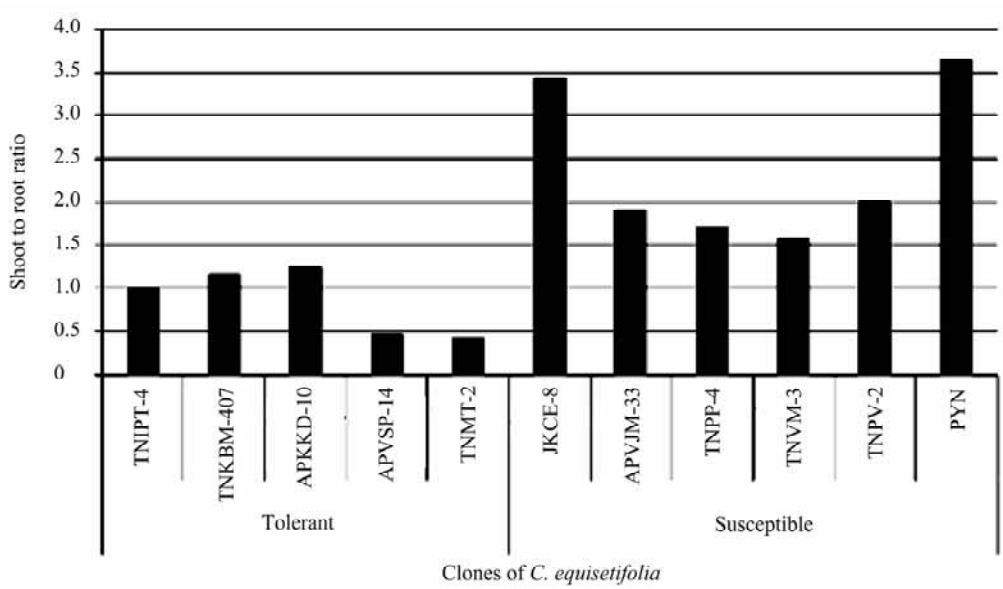

Fig. 3 The shoot to root ratio of sodium content of selected tolerant and susceptible *C. equisetifolia* clones

4.2 Effect of salt on survival of *C. equisetifolia*

Wide variation in the response of *C. equisetifolia* clones to salt stress has been observed (Balasubramanian, 2001). Further screening of the vegetative propagules from these clones at 340 mM NaCl stress (under hydroponic condition) identified a Casuarina clone able to survive for 30 days, while other clones failed to survive beyond 8 days. Altered protein profiles under salt stress

were observed (Tripathi *et al.*, 2001). In the present study, 85 clones were assessed for their salt stress response and 17 highly tolerant and 14 highly susceptible clones were identified based on the percentage of survival and visual observations. The percent difference in the number of needles in each clone when the salt concentration was increased from 200 mM to 500 mM, the percent decrease in the needles calculated by comparing the difference in the number of needles in the treated and control plants (data not shown), the overall health of the plants and the sodium content in needles and roots were used to identify 5 most salt tolerant and 6 most susceptible clones (Fig. 3). The most susceptible clone PYN wilted at a salt concentration of 400 mM, while the most tolerant clone TNIPT-4 survived for 45 days post 550 mM NaCl stress. The tolerant clones could be good candidate clones for further testing in flooded coastal saline areas.

4.3 Effect of salt on sodium concentration in shoots and roots

In this study swelling of older needles was observed in few of the identified resistant as well as susceptible clones. No red phylloclades were observed. However, Dutt *et al.* (1991) reported that salinity stress caused higher levels of Na^+ accumulation in the older red phylloclades. Salt tolerance in Casuarina species have been attributed to a more prolonged intake of ions by older needles via the transpiration stream, reduced mobility into younger tissues (Greenway, 1962), greater compartmentation in the older tissues, or efficient retranslocation (Aswathappa and Bachelard, 1986). The sodium and potassium contents in shoots and roots were estimated (Fig. 2). When compared to the susceptible clones, the resistant clones accumulated lesser sodium in shoots and higher sodium in the roots. While the shoot to root sodium content was less than or close to one in resistant clones, in the case of susceptible clones, shoots accumulated 0.5 to 2.6 times more sodium than in the roots (equivalent to shoot to root ratio of 1.5 to 3.6) (Fig. 3). Studies by Clemens *et al.* (1983) and Aswathappa and Bachelard (1986) have also shown that highly salt tolerant species like *C. equisetifolia* and *C. glauca* exclude both Na^+ and Cl^- from their shoots. The species (*C. inophloia*, *C. stricta*, *C. instata* and *C. decaimeana*) that accumulated the highest concentrations of Na^+ and Cl^- in the shoot tip were those that suffered the greatest reductions in growth, shoot tip chlorosis and/or death, while more tolerant species like *C. equisetifolia* and *C. glauca* showed higher levels of Na^+ and Cl^- in the roots (Clemens *et al.*, 1983). Thus, sodium transporter homologues involved in xylem unloading, vacuolar compartmentation, Na^+ efflux and Na^+ influx may have significant roles in imparting salt tolerance in Casuarina. However, in this study, shoot to root ratio greater than 1.4 was observed in 4 of the 17 tolerant clones viz, TNKP-2 (1.4), TNPP-2 (1.6), TNIPT-2 (1.4) and TNKBM-408 (1.4). Similarly, shoot to root ratio lesser than 1 was observed in 6 of the 14 susceptible clones TNIPT-1 (0.9), PY-75 (0.4), TNRM-2 (0.6), TNVM-5 (0.9), TNRM-4 (0.9) and PY-131 (0.3). The tolerance or susceptibility in these clones may be attributed to other genetic determinants. The most tolerant and most susceptible clones identified in this study will be used for identification of sodium transporter gene homologues and the other genetic determinants contributing to salt tolerance.

5 Conclusions

The database on plant abiotic stresses would provide a ready reference of gene sequences implicated in stress tolerance, their conserved motifs, and consensus primer sequences for use in gene isolation and marker assisted selection in Casuarinas. The database after validation and testing would be hosted on the web and would complement the EST resource of *C. glauca* developed at the IRD. The study identified 5 most salt tolerant and 6 most susceptible clones that would serve as a base material for

differential expression studies for identification of gene homologues, and development of molecular marker approaches. The identified tolerant clones could be good candidates for further testing in flooded coastal saline tracts. The higher root to shoot ratio of sodium could be considered as one of the markers for screening of salt tolerance in *C. equisetifolia*.

Acknowledgments

The funding support from the Indian Council of Forestry Research and Education and the Department of Biotechnology, Government of India, are gratefully acknowledged.

References

Aswathappa, N. and Bachelard, E. P. 1986. Ion regulation in the organs of *Casuarina* species differing in salt tolerance. Australian Journal of Plant Physiology, 13: 533-545.

Balaji, J., Crouch, J. H., Petite, P. V. and Hoisington, D. A. 2006. A database of annotated tentative orthologs from crop abiotic stress transcripts. Bioinformation, 1(6): 225-227.

Balasubramanian, A. 2001. Screening for salinity resistance in clones of *Casuarina equisetifolia* Forst. PhD thesis submitted to the Forest Research Institute (Deemed University), India.

Bartels, D. and Sunkar, R. 2005. Drought and salt tolerance in plants. Critical Reviews in Plant Sciences, 24: 23-58.

Boeckmann, B., Bairoch, A., Apweiler, R., Blatter, M., Estreicher, A., Gasteiger, E., Martin, M. J., Michoud, K., O'Donovan, C., Phan, I., Pilbout, S. and Schneider, M. 2003. The Swiss-Prot protein knowledge base and its supplement TrEMBL in 2003. Nucleic Acids Research, 31: 365-370.

Clemens, J., Campbell, L. C. and Nurisjah, S. 1983. Germination, growth and mineral ion concentrations of *Casuarina* species under saline conditions. Australian Journal of Botany, 31: 1-9.

Dutt, S. K., Bal, A. R. and Bandyopadhayay A. K. 1991. Salinity induced changes in *Casuarina equisetifolia* Forst. Egyptian Journal of Soil Science, 31: 57-63.

Fredslund, J., Schauser, L., Madsen, L. H., Sandal, N. and Stougaard, J. 2005. PriFi: using a multiple alignment of related sequences to find primers for amplification of homologs. Nucleic Acids Research, 33: 516-520.

Gherbi, H., Nambiar-Veetil, M., Zhong,C., Félix, J., Autran, D., Girardin, R., Vaissayre, V., Auguy, F., Bogusz, D. and Franche, C. 2008. Post- transcriptional gene silencing in the root system of the actinorhizal tree *Allocasuarina verticillata*. Molecular Plant-Microbe Interactions, 21: 518-524.

Greenway, H. 1962. Plant responses to saline substrates. II. Chloride, sodium and potassium uptake and translocation in young plants of *Hordeum vulgare* and during and after short sodium chloride treatment. Australian Journal of Biological Sciences, 15: 39-57.

Grover, A., Kapoor, A., Lakshmi, O. S., Agarwal, S., Sahi, C., Katiyar-Agarwal, S., Agarwal, M. and Dubey, H. 2001. Understanding molecular alphabets of the plant abiotic stress responses. Current Science, 80: 206-216.

Hasegawa, P. M., Bressan, R. A., Ahu, J. K. and Bohnert, J. 2000. Plant cellular and molecular responses to high salinity. Annual Review of Plant Physiology and Plant Molecular Biology, 51: 463-499.

Hocher, V.,Florence, A., Argout, X., Laplaze, L., Franche, C. and Bogusz, D. 2006. Expressed sequence-tag analysis in *Casuarina glauca* actinorhizal nodule and root. New Phytologist, 169: 681-688.

Jayaraj, R. S. C. and Savio M. M. D. 1998. Germplasm collections in forestry. Sylva Plus, 6(2): 14-22.

Munns, R. and Tester, M. 2008. Mechanisms of salinity tolerance. Annual Review of Plant Biology, 5: 651-681.

Nelson, D. E., Shen, B. and Bohnert, H. J. 1998. Salinity tolerance-mechanisms, models and the metabolic engineering of complex traits. Genetic Engineering, 20: 153-176.

Perez-Rodriguez, P., Riano-Pachon, D. M., Guedes Correa, L. G., Rensing, S. A., Kersten, B. and Mueller-Roeber, B. 2009. PlnTFDB: updated content and new features of the plant transcription factor database. Nucleic Acids Research, 38: 822-827.

Sayers *et al*. 2010. Database resources of the National Centreg for Biotechnology Information. Nucleic Acids Research, 38: 5-16.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22: 4673-4680.

Tripathi, S. B., Nambiar-Veetil, M. and Gurumurthi, K. 2001. Changes in protein profiles in *Casuarina equisetifolia* during salt stress. In: Proceedings of the 5th Annual Workshop on Casuarina (compiled by K. Gurumurthi). October 8-9, 2001, Regional Forest Research Centreg, Rajahmundry, pp126-134.

Wanchana, S., Thongjuea, S., Ulat, V. J., Anacleto, M., Mauleon, R., Conte, M., Rouard, M., Ruiz, M., Krishnamurthy, N., Sjolander, K., van Hintum, T. and Bruskiewich, R. M. 2008. The generation challenge programme comparative plant stress-responsive gene catalogue. Nucleic Acids Research, 36: 943-946.

Wang, W., Vinocur, E. B. and Altman, E. A. 2003. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta, 218: 1-14.

Improving Smallholder Livelihoods through Improved Casuarina Productivity



Proceedings of the 4th International Casuarina Workshop
Haikou, China
21-25 March 2010

Edited by

Chonglu Zhong
Khongsak Pinyopusarerk
Antoine Kalinganire
Claudine Franche

CHINA FORESTRY PUBLISHING HOUSE

Improving Smallholder Livelihoods through Improved Casuarina Productivity

Proceedings of the 4th International Casuarina Workshop
Haikou, China
21-25 March 2010

Edited by

Chonglu Zhong
Khongsak Pinyopusarerk
Antoine Kalinganire
Claudine Franche

China Forestry Publishing House

Organizing Committee

International

Daping Xu, Research Institute of Tropical Forestry, Chinese Academy of Forestry, China
Khongsak Pinyopusarerk, CSIRO Plant Industry, Australia
Antoine Kalinganire, World Agroforestry Centre (ICRAF-WCA/Sahel), Bamako, Mali
Didier Bogusz, Institut de Recherche pour le Développement (IRD), France
Claudine Franche, Institut de Recherche pour le Développement (IRD), France
Chonglu Zhong, Research Institute of Tropical Forestry, Chinese Academy of Forestry, China

Local

Shirong Liu, Chinese Academy of Forestry
Wenming Lu, Chinese Academy of Forestry
Zhongmin Wu, Research Institute of Tropical Forestry, Chinese Academy of Forestry
Mei Li, Research Institute of Tropical Forestry, Chinese Academy of Forestry
Yong Zhang, Research Institute of Tropical Forestry, Chinese Academy of Forestry

Front cover photos by: Chonglu Zhong, Yong Zhang and Yu Chen
Back cover photos by: Chonglu Zhong, Khongsak Pinyopusarerk and Qingbin Jiang

图书在版编目(CIP)数据

提高木麻黄林木生产力 改善林农生计: 2010 年 3 月 21-25 日中国海口第四届木麻黄国际会议论文集 = Improving Smallholder Livelihoods through Improved Casuarina Productivity: Proceedings of the 4th International Casuarina Workshop, Haikou, China 21-25 March 2010: 英文 / 仲崇禄等编. -北京: 中国林业出版社, 2011.11

ISBN 978-7-5038-6365-3

Ⅰ. ①提… Ⅱ. ①仲… Ⅲ. ①木麻黄-国际学术会议-文集-英文 Ⅳ. ①S792.93-53

中国版本图书馆 CIP 数据核字(2011)第 221766 号

Chinese Publications Number of Archives Library: 2011-221766
ISBN 978-7-5038-6365-3
Improving Smallholder Livelihoods through Improved Casuarina Productivity: Proceedings of the 4th International Casuarina Workshop, Haikou, China 21-25 March 2010. Chonglu Zhong, Khongsak Pinyopusarerk, Antoine Kalinganire and Claudine Franche (eds) 2011.11:272pp.
1. Improving… 2. Zhong… 3. Casuarina-International Meeting-Proceedings-English 4. S792.93-53

First Published in the P. R. China in 2011 by China Forestry Publishing House
No.7, Liuhaihutong, Xicheng District, Beijing 100009
Http://lycb.forestry.gov.cn
Email:wildlife_cfph@163.com

Price: RMB 60.00