UNIVERSITI PUTRA MALAYSIA

GENETIC DIVERSITY AND CHARACTERIZATION OF INDIGENOUS RHIZOBIUM LEGUMINOSARUM BIOVAR VICIAE ISOLATES OF COOL-SEASON FOOD LEGUMES GROWN IN THE HIGHLANDS OF ETHIOPIA

NEGASH DEMISSIE TEGEGN.

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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GENETIC DIVERSITY AND CHARACTERIZATION OF INDIGENOUS *Rhizobium leguminosarum* BIOVAR *viciae* ISOLATES OF COOL-SEASON FOOD LEGUMES GROWN IN THE HIGHLANDS OF ETHIOPIA

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NEGASH DEMISSIE TEGEGN

February 2006

Chairman: **Associate Professor Halimi Mohd Saud, PhD**

Faculty: **Agriculture**

Cool-season food legumes (CSFLs) are legumes of the temperate cool subtropical origin. In Ethiopian context these legumes encompass 5 legumes such as faba bean (*Vicia faba)*, field pea (*Pisum sativum*), lentil (*Lens culinaris*), Chickpea (*Cicer arietinum*) and Grasspea (*Lathyrus sativus*) and are cultivated on the highlands. These legumes have high economic values and provide rich protein sources for human and animal consumption. Although it was reported that, a few species taxonomically related to cultivated CSFL exist in Ethiopian. Thus, there is reason to believe that Ethiopian soils harbour diverse rhizobial isolates, which form symbiotic relationships with CSFLs. In general, there is little or no information is available on the diversity CSFL rhizobia across the country. This indicates that the extent and divergent of the local rhizobial populations belonging to the long cultivated lands are yet to discover. Thus, the importance of characterizing indigenous rhizobia cannot be overemphasized. It is important to establish which rhizobia nodulate which host(s)

and how effectively and which rhizobia predominate which region in order to develop broad host range inoculants in the country. Hence, the current study was conducted with the objectives to isolate, characterise, and determine the morphological, biochemical, and genetic diversity of rhizobial symbionts to CSFLs grown of Ethiopia, and to determine inoculation effects of selected elite strains against introduced/exotic strains on the symbiotic growth and development of lentil (*Lens culinaris*).

Over 150 indigenous *Rhizobium* species, symbionts to CSFLs, were collected from farmers' fields in the highlands of Ethiopia and categorized based on their rhizosphere pH and their agro-ecological zones (AEZ) origin. These isolates were characterised for their colony morphology, host specifity, cell growth rate and mean generation time, acid producing and intrinsic antibiotic resistance (IAR) characteristics. However, a more comprehensive and detail physiological (ATR, STL, IAA, C SUP), and genetic (RAPD, RE RFLP) characterization studies were made apparently for 90 representative isolates of the 150 isolates. Finally, field inoculation experiment was conducted on lentil plant using two elite and 2 exotic inoculant strains. The collection, isolation, colony morphological characterization, and field experiment studies were carried on in Ethiopia, while the physiological and genetic characterization studies were at the Universiti Putra Malaysia (UPM) laboratories in Malaysia.

The study recognized the different rhizosphere pH for the host and existing agro-ecological zones (AEZs) for the initial isolates sampling points and used as a tool to categorize the bulk rhizobial isolates. Thus, isolates constituted 3 and 4 categories for

rhizosphere pH and AEZs, respectively. All isolates were Gram negative *Rhizobium leguminosarum* bv. *viciae* species. Results of the host range specifity study showed that of the 3 rhizobial biovars *Vicia faba* rhizobia were the most host discriminative rhizobia that formed less number of nodules on other host plants. Thus, *Lens culinaris* and *Pisum sativum* rhizobia showed almost similar host discriminative capacity. Isolates differentiated by morphological, acid/alkali production capacity and growth characteristics into 4, 2 and 4 categories, respectively. Approximately 40 % of the total isolates similarly exhibited a colony category of mucoid moisture, with circular shape, white opaque color, and raised structure. However, *Lens culinaris* rhizobia were distinguished apparently by two morphological characteristics. In general, 92% of the indigenous rhizobia isolates examined in this study were fast to very fast growing types acid producing types with overall MGT of 5.88 to 5.9 h.

Eighty-three representative indigenous isolates and 4 reference strains were examined for their physiological characteristics such as ATR, STL, IAA and C SUP and isolates showed variable response. Few rhizobial isolates were able to grow on acid media of pH 4.75 - 6.00. Two *Vicia faba* and one *Pisum sativum* rhizobia were the most acid tolerant isolates that grew at pH 4.75. A total of four *Vicia faba* and two *Pisum sativum* rhizobia were identified to be acid tolerant isolates. In contrary, some isolates of central and northwest highland origins were very sensitive to slightly low pH media of 6. TAL1399 grew apparently on pH 5.5 the reset ph>5.5. Some isolates of the same agro-ecology found to have uniform ATR. The response of representative isolates to growth-inhibiting salt (NaCl) concentrations showed relatively law variations among isolates. Most (92%) isolates were able to grow well

at salt concentration of 0.1% and less. The rest 54 % isolates remained unchanged. Almost 78% isolates were tolerated to NaCl upto 0.2%. However, apparently, 3 isolates survived at the highest NaCl concentration of 0.3%. Isolates were homologues with the increased similarity level (>0.60) and 17 isolates had shown identical response with that of reference strains. The IAA concentration for the investigated isolates showed that indigenous isolates varied greatly in their IAA production capacity and formed 17 clusters. The IAA concentration reached up to 25.92 mg L^{-1} with a mean of 9.9 mg L^{-1}. In general, IAA producing capacity of isolates was remained the best indicator to distinguish and group rhizobial isolates. Isolates cluttered into 5 clusters at increased similarity level of >0.60. IAA was the best indicator among the tested physiological parameters for the divergence of isolates among each other. With respect to C SUP, most isolates preferred polyols, monosaccharide, and disaccharides as their first, second, and third choice carbon sources, respectively. Lentil rhizobia were indifferent for about 6 of the 8 C sources.

Also, results from the current study showed great diversity among isolates with respect to their IAR capacity, making this test useful for distinguishing among isolates. Thirty-three isolates have shown multi-resistant characteristics and formed 18 identical antibiotic resistance profiles. However, number of similar clusters varied with an increase in similarity level of 0.60. Thirteen isolates were found significantly divergent from the bulk of isolates examined. Faba bean and field pea rhizobia had shown more or less uniform IAR capacity, whilst lentil rhizobia showed different IAR capacity for the examined antibiotics.

DNA fragment analysis carried out for 95 representative isolates have shown a total of 83, 79, and 75 fragment patterns for RAPD-PCR, *Hae*III and *Msp*I RE-RFLPs, respectively. These two enzymes per se showed highly polymorphic and distinct DNA profiles indicating the divergence of *Rhizobium* isolates. RE digestion of *Lens culinaris* rhizobia with both enzymes yielded single PCR products with approximately 750 base pairs while the single band for *Vicia faba* and *Pisum sativum* yielded larger fragment of up to 1800 base pairs. Majority (76.17 %) showed significant genetic similarity, while the rest (23.83 %) isolates were divergent among each other. The RAPD-PCR and RE digestions methods formed 18, 13 and 20 clusters respectively. Hence, the DNA profile analysis showed that isolates were distinctly divergent among each other at higher similarity level of >0.60.

Field inoculation experiment on lentil showed that elite inoculant strain EAL400 and imported/ commercial strain TAL1402 in both single as well as mixed inoculant form(s) showed significant ($P<0.05$) increase in seed yield, yield components, nodulation and symbiotic growth of lentil. The study demonstrated that inoculant type of rhizobial strain sounds better than apparent use of different form(s) (single or mixed inoculants) of inoculants under lentil. Moreover, divergent of rhizobia within *Rhizobium leguminosarum* bv. *viciae* nodulating CSFLs, the field performance of the 2 strains (EAL400 and TAL1402) and the presence indigenous rhizobia that have similar characteristics with strain EAL400 and TAL1402 indicates the future potential for identification of new competitive and efficient *Rhizobium leguminosarum* strains for the country.

Abstrak tesis yang Dikemukakan kepada Senat Universiti Putra Malaysia sebagai Memenuhi Keperluan untuk Ijazah Doktor Falsafah

KEPELBAGAIAN GENETIK DAN PENCIRIAN ISOLAT TEMPATAN *Rhizobium leguminosarum* BIOVAR *viciae* YANG BERSIMBIOSIS DENGAN DALAM TANAMAN MAKANAN MUSIM SEJUK KEKACANG DITANAM DI KAWASAN TANAH TINGGI ETHIOPIA

Oleh

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Tanaman kekacang musim sejuk (CSFLs) adalah tanaman kekacang yang berasal dari subtropika bercuaca sejuk. Di dalam konteks Ethiopia kekacang ini terdiri daripada 5 jenis kekacang seperti faba bean (*Viciae faba*), field pea (*Pisum sativum*), lentil (dal) (*Lens culinaris*), chickpea (*Cicer arietinum*) dan Graspea (*Lathyrus sativus*) dan ia ditanam di atas tanah tinggi. Kekacang ini mempunyai nilai ekonomi yang tinggi dan membekalkan sumber protein yang kaya untuk kegunaan manusia dan haiwan. Walaupun ia telah dilaporkan bahawa, beberapa spesis secara taksonomik berkait dengan penanaman CSFL yang wujud di Ethiopia. Oleh itu, ia adalah sebab untuk dipercayai bahawa tanah perlindungan Ethiopia mempunyai pelbagai pencilan rhizobia, di mana ia membentuk hubungan simbiotik dengan CSFLs. Secara umum, terdapat sedikit atau tiada maklumat yang sedia ada ke atas kepelbagaian rhizobia CSFL merentasi negara. Ini menunjukkan bahawa peluasan dan penyebaran populasi asal rhizobia adalah kepunyaan tanah yang telah ditanam

sekian lama belum lagi ditemui. Oleh itu kepentingan bagi mencirikan rhizobia tempatan tidak boleh terlalu dititikberatkan. Ia adalah penting untuk membuktikan rhizobia yang mana menodulkan perumah dengan berkesan dan rhizobia yang mana mempengaruhi kawasan mana dalam membentuk jumlah inokulan perumah paling banyak dalam negara. Oleh sebab itu, kajian yang sedia ada dijalankan dengan tujuan untuk pencilan, pencirian, dan penentuan morfologi, biokimia, dan kepelbagaian genetik bagi simbion rhizobia terhadap CSFLs yang ditanam di Ethiopia, dan untuk menentukan kesan inokulasi bagi strain elit terpilih terhadap strain eksotik ke atas pertumbuhan simbiotik dan pembentukan lentil (*Lens culinaris*).

Lebih 150 *Rhizobium* asli, adalah simbion kepada CFLS, telah dikumpulkan daripada ladang petani di tanah tinggi Ethopia dan dikategorikan berdasarkan pH rhizosfera dan zon agro-ekologi asal. Pencilan ini telah dicirikan terhadap morfologi koloni, perumah specifik. Kadar pertumbuhan sel dan min generasi masa, penghasilan asid dan ciri rintang terhadap asid. Walaubagaimanapun, kajian yang lebih komprehensif dan fisiologikal lengkap (ATR, STL, IAA, C SUP) dan pencirian genetik (RAPD, RE RFLP) telah dibuat dengan nyata untuk 90 pencilan terpilih daripada 150 pencilan. Akhirnya, eksperimen inokulasi di ladang telah dijalankan terhadap pokok lentil dengan menggunakan 2 strain inokulasi elit dan eksotik. Pengumpulan, pencilan, pencirian morfologi koloni dan kajian eksperimen di ladang telah dilakukan di Ethiopia, manakala kajian fisiologi dan genetik dijalankan di makmal Universiti Putra Malaysia (UPM) di Malaysia. Kajian ini telah mengenalpasti perbezaan rhizosfera bagi perumah dan zon agroekologi (AEZs) yang sedia ada untuk titik permulaan dan digunakan sebagai alat untuk mengkategorikan jumlah keseluruhan pencilan rhizobia. Demikian, secara respektif pencilan mengandungi 3 dan 4 kategori untuk pH rhizosfera dan AEZs. Kesemua pencilan adalah spesis Gram negative

Rhizobium leguminoserum bv *viciae*. Keputusan kajian bagi kadar spesifikasi perumah menunjukkan bahawa 3 biovar rhizobia *Vicia faba* adalah rhizobia perumah paling diskriminasi yang membentuk bilangan nodul paling sedikit ke atas pokok perumah yang lain. Demikian, *Lens culinaris* dan *Pisum sativum* menunjukkan kapasiti diskriminasi perumah yang hampir serupa. Pencilan dibezakan melalui morfologi, kapasiti penghasilan asid/alkali dan ciri-ciri pertumbuhan kepada 4, 2 dan 4 kategori secara respektif. Lebih kurang 40% daripada jumlah pencilan menunjukkan persamaan kategori koloni yang berlendir dan lembap, berbentuk bulat, berwarna putih legap, dan struktur tertimbul. Bagaimanapun, rhizobia *Lens culinaris* telah dibezakan melalui dua ciri morfologi. Secara umum, 92% pencilan rhizobia tempatan yang diselidik dalam kajian ini adalah jenis pertumbuhan cepat kepada paling cepat, jenis penghasilan asid dengan jumlah MGT 5.88 ke 5.9 j.

83 wakil pencilan tempatan dan 4 strain rujukan diuji untuk ciri-ciri fizikal seperti ATR, STL, IAA dan C SUP dan pencilan menunjukkan tindak balas yang berubah-ubah Sesetengah pencilan rhyzobia berkebolehan untuk cepat tumbuh di atas media berasid pH 4.75 – 6.00. Dua *Vicia faba* dan satu *Pisum sativum* rhizobia adalah paling rintang terhadap asid dan tumbuh pada pH 4.75. Sejumlah empat *Vicia faba* dan dua *Pisum sativum* rhizobia telah dikenalpastikan pencilan yang rintang terhadap asid. Berlawanan pula dengan sesetengah pencilan asal di tengah dan barat laut kawasan pergunungan yang sangat sensitif kepada media pH serendah 6. Nampaknya TALI399 tumbuh pada pH 5.5 selebihnya pH > 5.5. Sesetengah pencilan yang sama Agro-Ekologi mempunyai ATR yang seragam. Tindak balas wakil pencilan terhadap tahap kepekatan garam yang boleh merencatkan pertumbuhan meunujukkan kepelbagaian di antara pencilan. Kebanyakan (92%) pencilan dapat tumbuh dengan

baik pada kepekatan garam 0.1% dan kurang daripadanya. Selebihnya 54% baki pencilan tidak berubah. Hampir 78% pencilan dapat menahan [NaCl] sehingga 0.2%. Akan tetapi, hanya 3 pencilan yang hidup pada kepekatan NaCl yang tinggi iaitu 0.3%. Pencilan adalah homolog dengan pertambahan persamaan paras (>0.60) dan 17 pencilan menunjukkan tindak balas serupa dengan strain rujukan. Kepekatan IAA untuk pencilan yang dikaji menunjukkan variasi tinggi dalam kadar IAA yang dihasilkan dan dapat dikumpulkan dalam 17 kelompok. Kepekatan IAA mencapai lingkungan 25.92 mgL^{-1} dengan purata 9.9 mgL^{-1}. Secara umumnya, baki pencilan kadar penghasilan IAA merupakan penunjuk yang terbaik dalam membezakan dan mengumpulkan pencilan rhizobia. Taburan pencilan dalam lima kelompok pada tambahan persamaan paras >0.60. IAA adalah penunjuk terbaik di antara satu sama lain. Dengan pemerhatian kepada C SUP, kebanyakan pencilan memilih polyols, monosakarida dan disakarida pada pilihan pertama, kedua dan ketiga sumber karbon masing-masing. Lentil rhizobia adalah berbeza di antara 6 hingga 8 C sumber.

Sepertimana juga, keputusan daripada kajian semasa menunjukkan kepelbagaian yang besar di antara pencilan dengan hubungan kadar IAR, menyebabkan ujikaji berguna untuk membezakan di antara pencilan. 33 pencilan menunjukkan sifat rintangan-pelbagaian dan menghasilkan 18 profail rintangan antibiotik yang sama. Akan tetapi, bilangan pelbagai kelompok yang sama dengan tambahan di dalam persamaan paras 0.60. Nyata sekali 13 pencilan yang ditemui berbeza di antara sejumlah pencilan yang diuji. Kacang Faba dan rhizobia kacang menunjukkan lebih atau kurang persamaan kadar IAR, sementara lentil rhizobia menunjukkan perbezaan kadar IAR terhadap antibiotic yang diuji.

Analisis fragmen DNA memberikan 95 contoh pencilan yang menunjukkan jumlah 83, 79 dan 75 corak fragmen untuk RAPD-PCR, *Hae*III dan *Msp*I RE-RFLPs, masing-masing. Dua enzim sangat menunjukkan polimorfik dan jelas sekali profail DNA menunjukkan pelbagai pencilan *Rhizobium*. Penguraian RE oleh *Lens culinaris* rhizobia dengan kedua-dua enzim menghasilkan satu produk PCR sekurang-kurangnya 750 bes berpasangan sementara satu jalur untuk *Vicia faba* dan *Pisum sativum* menghasilkan fragmen besar sehingga 1800 bes berpasangan. Majoriti (76.17%) nyata sekali menunjukkan persamaan genetic, sementara selebihnya (28.83%) pencilan berbeza di antara satu sama lain. RAPD-PCR dan kaedah penguraian RE membentuk 18, 13 dan 20 kelompok masing-masing. Dengan itu, analisis profail DNA menunjukkan pencilan tersebut jelas sekali berbeza di antara satu sama lain pada ketinggian persamaan paras 0.60.

Eksperimen penginokulatan ladang ke atas lentil menunjukkan inokulan pencilan elit EAL400 dan strain komersail TAL1402 di dalam keadaan tunggal serta campuran inokulan nyata sekali menunjukkan ($P<0.05$) tambahan dalam hasil biji benih, hasil-hasil bahan, bintil dan pertumbuhan simbiotik lentil. Kajian membuktikan jenis inokulan tersebut strain rhizobia adalah lebih baik berbanding yang biasa digunakan bagi membezakan bentuk (satu atau campuran inokulan) inokulan di bawah lentil. Lagipun, pelbagai rhizobia dalam lingkungan *Rhizobium leguminosarum* bv. *viciae* bernodulat CSFLs, menunjukkan dua strain (EAL400 dan TAL1402) dan kehadiran rhizobia tempatan mempunyai ciri-ciri persamaan dengan strain EAL400 dan TAL1402 menunjukkan potensi pada masa akan datang untuk mengenalpasti persaingan baru dan keberkesanan strain *Rhizobium leguninosarum* strains kepada negara.

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I certify that an Examination Committee has met on 17th February, 2006 to conduct the final examination of Negash Demissie Tegegn on his Doctor of Philosophy thesis entitled "Genetic Diversity and Characterization of Indigenous *Rhizobium leguminosarum* Biovar *viciae* Isolates of Cool-season Food Legumes Grown in the Highlands of Ethiopia". In accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Member of Examination committee are as follows

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

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Date: 11 MAY 2006

DECLARATION

I hereby declare that the thesis is based on my original work except for equations and citations, which have bean duly acknowledged. I also declare that it has not been previously or currently submitted for any other degree at UPM or any other institutions.

NEGASH DEMISSIE TEGEGN

Date 18 APR 2006

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