UNIVERSITI PUTRA MALAYSIA

GERMPLASM COLLECTION AND MOLECULAR DETECTION OF ENDOPHYTIC FUNGI IN IRANIAN TALL FESCUE (*FESTUCA ARUNDINACEA* SCHREB.)

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By

MOJTABA KHAYYAM NEKOUEI

Thesis Submitted in Fulfilment of the Requirement for the Degree of Doctor of Philosophy in the Faculty of Food Science and Biotechnology Universiti Putra Malaysia

January 2001

UPM

Specially Dedicated to

My Wife

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy.

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January 2001

Chairman: Dr Suhaimi Napis

Faculty: Food Science and Biotechnology

Tall fescue is a popular pasture grass grown in many countries. A systematic endophytic fungus, *Acremonium coenophialum*, lives in a symbiotic association within tall fescue and may impart superior competitiveness to the plant through increased resistance to pests, tolerance to drought and improvements in other agronomic traits. The assessment of the infection status and viability of endophytic fungi would open the possibility of identifying potentially desirable endophyte strains for improving pasture, turf and crop species. Therefore, studies of tall fescue and endophytic fungi in Iran are essential for its improvement and may provide opportunities to produce elite endophyte-infected plant population. Nineteen accessions of tall fescue were collected from various regions of Iran, identified and evaluated for the presence of endophyte based on IPGRI descriptors. The accessions were mainly distributed in the northern and western part of the country with relatively more precipitation. Seven agronomic characteristics under greenhouse and fifteen traits under field conditions were evaluated. Result obtained from cluster

analysis grouped the accessions into 3 clusters based on the parameters of the greenhouse and field experiments. Out of the 15 traits, only 10 traits under field conditions showed significant variation among the accessions. The correlation analysis showed that the yield is directly proportional to the number of inflorescence. After greenhouse and field evaluation, the accessions were evaluated for the presence of endophyte. Detection of endophytic fungi in tall fescue seeds showed that 84.2% of the accessions were infected with endophyte at infection rates of 20 to 95%. The results of the endophytic fungi detection in greenhouse-grown and field-grown tall fescue seedlings indicated that viable fungal endophyte occurred in 73.3% of total tall fescue accessions evaluated. The *in vitro* isolation and culture of endophyte confirmed the result obtained from greenhouse and field experiments. The conventional methods for detection of endophyte in tall fescue requires at least 28 days and therefore a rapid and sensitive molecular method was developed to facilitate detection and identification of endophytic fungi in tall fescue. This method could be used for the screening of large number of seed and plant samples. Diagnostic PCR was developed and optimised to evaluate and verify the infection status of collected accessions. The PCR with microsatellite (MS) and internal transcribed spacer (ITS) primers generated DNA fragments of different sizes. The infected accessions yielded amplification products with size ranging from 250 to 400 base pair for MS primers and 550 to 750 base pair for ITS primers. No amplification product was detected on the uninfected seedlings. The results indicated that ITS primers (ITS1 and ITS4) and also MS primers (MSF and MSR) appeared to be useful for the detection of endophytic infection of tall fescue accessions.

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PENGUMPULAN GERMPLASMA DAN PENGENALPASTIAN MOLEKUL FUNGI ENDOFIT DI DALAM TALL FESCUE (*FESTUCA ARUNDINACEA* SCHREB.)

Oleh

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'Tall fescue' adalah sejenis rumput ragut yang ditanam di banyak negara. Terdapat sejenis fungus endofit, *Acremonium coenophialum*, hidup melalui hubungan simbiotik di dalam rumput ini, mengakibatkan rumput ini lebih tahan kepada ancaman perosak, kemarau dan kemajuan sifat agronomik yang lain. Penilaian status jangkitan dan kemandirian fungi endofit akan membolehkan endofit yang berpotensi dikenalpasti untuk memajukan rumput ragutan, rumput turf dan tanaman. Oleh itu, kajian terhadap 'tall fescue' dan fungi endofit di Iran adalah perlu untuk kemajuan dan peluang untuk menghasilkan tanaman elit yang dijangkiti endofit. 19 'aksesi tall fescue' telah dikumpul daripada beberapa kawasan di Iran, dikenalpasti dan dinilai untuk kemandirian endofit berdasarkan garis panduan daripada IRGRI. Sebahagian besar daripada 'aksesi-aksesi' ini tersebar di sebelah utara dan barat negara Iran yang menerima tahuran hujan yong lebih secara relatif. Penilaian terhadap 7 sifat agronomi di dalam rumah hijau dan 15 sifat di ladang telah dilakukan. Keputusan yang didapati daripada analisa 'cluster' membahagikan

'accession' kepada 3 'cluster' berdasarkan parameter di dalam rumah hijau dan di ladang. Daripada 15 sifat, hanya 10 sahaja sifat agronomi di ladang yang menunjukkan variasi yang signifikan di antara 'aksesi'. Analisa korelasi menunjukkan bahawa hasil adalah berkadar terus dengan bilangan infloresen. Selepas itu, 'aksesi' dinilai untuk kehadiran endofit. Didapati 84.2% daripada 'aksesi' dijangkiti dengan kadar jankitan daripada 20 hingga 95%. 73.3% daripada anak benih yang ditanam di dalam rumah hijau dan di ladang didapati dijangkiti dengan endofit yang hidup. Pemencilan dan pengulturan endofit secara *in vitro* mengesahkan keputusan yang didapati daripada rumah hijau dan ladang. Biasanya, untuk mengesan endofit daripada 'tall fescue' memerlukan sekurang-kurangnya 28 hari. Oleh itu, kaedah molekul biologi yang cepat dan sensitif telah dibangunkan untuk membantu pengesanan dan pengenalpastian fungi endofit di dalam 'tall fescue'. Kaedah ini boleh digunakan untuk penyaringan jumlah benih dan anak benih yang banyak. Diagnosa PCR telah dibangunkan dan dioptimakan untuk menilai dan mengesah tahap jangkitan 'aksesi' yang telah dikumpul. PCR dengan pencetus mikrosatelit (MS) dan 'internal transcribed spacer' (ITS) menghasilkan serpihan DNA dengan saiz yang berbeza. 'Aksesi yang dijangkiti menghasilkan serpihan DNA dengan julat saiz di antara 250 hingga 400 basa untuk pensetus MS dan di antara 550 hingga 750 bes untuk pensetus ITS. Tiada serpihan DNA dikesan pada anak benih yang tidak dijangkiti. Keputusan ini menunjukkan pensetus ITS (ITS1 dan ITS 4) dan juga pensetus MS (MSF dan MSR) berguna untuk pengesanan jangkitan endofit pada 'aksesi-aksesi tall fescue'.

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LIST OF ABBREVIATIONS

α	alpha
β	beta
λ	lambda
%	Percentage
bp	base pair
CMM	Corn Meal Malt extract agar
CTAB	Cetyl trimethyl-ammonium bromide
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotides
dATP	2'-deoxy-adenosin-5'-triphosphate
dCTP	2'-deoxy-cytidin-5'-triphosphate
dGTP	2'-deoxy-guanosine-5'-triphosphate
dTTP	thymidine-5'-triphosphate
dH_2O	distilled water
E+	endophyte infected
E -	endophyte free
EDTA	ethylene glycol bis-(β- aminoethyl ether)
g	gram
HCl	hydrochloric acid
hr	hours
ITS	Internal transcribed spacer
LB	Luria-Bertani
k	Kilo

kb	Kilobase
KCl	potassium chloride
L	litter
M	Molar
mg	milligram
min	minute(s)
ml	millilitre
mM	Millimolar
$MgCl_2$	Magnesium chloride
MS	Microsatellite
NaCl	sodium chloride
NaOAc	Sodium Acetate
OD	Optical density
PCI	phenol: chloroform: isoamylalcohol
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
RNA	Ribonucleic acid
rRNA	ribosomal RNA
RNase	Ribonuclease
rpm	revolution per minute
SDS	sodium dodecyl sulphate
SSRs	simple sequence repeat
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
U	Unit

µg	microgram
µl	microlitter
UV	Ultraviolet
v/v	volume per volume
w/v	weight per volume

Glossary

***Acremonium coenophialum*:** A symbiotic endophytic organism that is an obligate inhabitant of tall fescue.

Alkaloid: A general term to describe a class of basic organic compounds containing nitrogen in their structure.

Endophyte: An organism that lives its life cycle within a host plant without causing disease; not specific enough to comply *A. coenophialum* unless defined earlier.

Endophyte free seed: Seed that has been determined to contain no viable endophyte; applies to *A. coenophialum.*

Endophyte-infected seed: Seed that has been determined to contain viable endophyte; applies to *A. coenophialum.*

Endophytic fungus: A fungus that lives its life cycle within a host plant without causing disease.

Ergopeptide (ergopeptine) alkaloids: Any of the lysergic acid derivatives formed with a peptide bond between the acid group of lysergic acid and the reacting amine group.

Ergot alkaloids: The alkaloids described as produced by the fungi *Claviceps purpurea, C. paspali,* and *C.fusiformis;* may be produced by other organisms; these alkaloids are derived from ergoline and include the clavine alkaloids, lysergic acid, lysergic acid amides, and ergopeptide alkaloids.

Fescue: A grass classified in the *Festuca* genus; a vernacular but incomplete description for tall fescue, unless defined earlier in the publication.

Fescue endophyte: The fungus *Acremonium coenophialum* that lives symbolically within the tall fescue plant; the term should be defined in each publication, since a number of different endophytic fungi may exist in tall fescue and become widely adapted.

Fescue toxicosis: The generic term used to describe collectively the animal syndromes associated with ingestion by animals of *A. coenophialum*-infected tall fescue, such as fescue foot, fat necrosis, agalactia, and other disorder; the disease state in the animal is implied by this generic term and may result in reduced growth. rough haricoat, excessive salivation, elevated body temperature, and impaired reproductive performance; since many of these signs can be exacerbated by elevated ambient temperatures, the term summer syndrome has been used colloquially to describe fescue toxicosis observed in summer.

Fungus: Use of the word "fungus" alone should be avoided unless it has been clearly defined earlier in the publication.

Fungus-free: The state of being free of any fungus; the term applies to a plant that is totally free of the fescue endophyte.

Fungus-infected: A plant that has been invaded by a fungus; the fungus should be identified earlier.

Fungus-infested: A field or a population of plants in which a number of individuals are infected by a fungus; the fungus should be identified earlier.

Incidence: The proportion or percentage of individuals within a defined population that possess a measured characteristic; it does not refer to the number of infected plants.

Infected: A plant that has been invaded by a symbiont, a parasite, or a pathogen.

Infection: The state produced by the establishment of an infective agent in or a suitable host; at this time, no research supports the idea of a pathogenic relationship between *A. coenophialum* and tall fescue. However, infection often is used as a generic term to denote the presence of a symbiont, such as mycorrhyzal fungi or *Rhizobia.*

Infestation level: The proportion or percentage of individuals examined that are infected; the term needs very careful definition in each publication.

Infested: A plant cannot be infested with an endophytic fungus, it is infected. The usage has developed to describe a population as being infested a field, a pasture, or a seed lot. A population is never infected; its component individuals are.

Low-endophyte seed: A seed lot of all fescue in which the percentage of *A. coenophialum*-infected seed is small, generally less than 5%; the endophyte should have been defined earlier in the publication.

Non-infected: The specific infecting organism, which should be specified, is not present.

Summer syndrome: An unsatisfactory term for tall fescue toxicosis, since it implies the problem is restricted to summer.

Tall fescue: *Festuca arundinacea* Schreb.

Toxic fescue: An unsatisfactory term for referring to tall fescue infected by *A .coenophialum.*

CHAPTER I

INTRODUCTION

Among the most important and widely grown pasture grasses for cattle are tall fescue (*Festuca arundinacea* Schreb.) and perennial ryegrass (*Lolium perennel*). Tall fescue is a popular pasture grass grown in many countries.

A systematic endophytic fungus, *Acremonium coenophialum*, lives in a symbiotic association within tall fescue. Comparisons of endophyte infected and endophyte-free tall fescue genotypes have shown that the endophyte imparts superior competitiveness to the plant through increased resistance to pests, tolerance to drought and improvement in other agronomic traits. Unfortunately, animals grazing on these grasses often show symptoms of fescue toxicosis. It has been established that grasses infested with fungal endophytes are responsible for the observed toxicosis. Although the problem may exist in several countries in Europe, its economic impact has not been as apparent as in the United States, which has far the most tall fescue acreage together with the more extreme climatic conditions.

Fungal endophytes have been reported to occur in several species of fescue grass (*Festuca spp*) and ryegrass (*Lolium spp*) and the tall fescue endophyte *A. coenophialum*, was previously referred to as *Epichloe typhina*. The fungus is a true endophyte in that it completes its entire life cycle within the host plant. Spores of this fungus have not been reported to occur on or in plants but conidia are produced on several complex media.

Ergopeptine alkaloids produced by *A. coenophialum* is thought to be responsible for the livestock disorder known as fescue toxicosis. Also it is found that ergovaline content is partially depended upon plant genotype and does not appear to influence superior plant performance. Furthermore the genetic diversity of fungal endophytes among different species of grasses could be of great importance. Therefore, it may be possible to produce an endophyte-infected plant population with little or no ergopeptine alkaloids. This would be significant because the beneficial effects of the endophyte on the plant could be maintained but the component toxic to livestock reduced. An understanding on the important role of endophytes has led to the development of more efficient breeding and evaluation programs, including the ability to select for and utilise genetic mechanisms of most resistant and stress tolerant.

This requires collection and evaluation of germplasm indigenous to different regions of the world including countries that are considered centre of origin and diversity to many grasses species such as Iran. The demand for germplasm (ranging from individual genes to co-adapted genes complexes to entire genotypes or even populations) is unpredictable and dynamic. There is no way of telling what tomorrow's needs may be, and what plants may be able to fulfil them. The more diversity is conserved and made available for future use, the better the chances of fulfilling future demand. In practice, however, some prioritisation is necessary, as to both species and geographic regions.