UNIVERSITÉ DE QUÉBEC EN ABITIBI-TÉMISCAMINGUE

CONTRIBUTION DES LIENS RACINAIRES AUX COMPOSANTES GÉNÉTIQUES ET PHYSIOLOGIQUES DE LA STRUCTURE CLONALE DU PEUPLIER FAUX-TREMBLE (*POPULUS TREMULOIDES* MICHX):

THÈSE

PRÉSENTÉE

COMME EXIGENCE PARTIELLE

DU DOCTORAT EN SCIENCES DE L'ENVIRONNEMENT

PAR

HANA JELÍNKOVÁ

Mai 2013

ACKNOWLEDGEMENTS

This PhD thesis is a result of long work and could hardly be finished without the help of numerous people surrounding me at UQAT in Rouyn-Noranda and Amos, at UQAM in Montreal and at the research station in Duparquet.

Namely I would like to thank to Francine Tremblay, my director, who gave me the trust to do this work and did not lose it even over a long time and distance. I would like to greatly appreciate the way she directed my work with a lot of liberalism, independence, and understanding.

I would also like to thank to Annie DesRochers, my co-director, who guided me throughout this work. Her straight to point ideas helped greatly to improve the resulting manuscripts.

I am greatful to M. Baret who accompanied me for long hours in the study fields in the boreal forest. Thank you to him I could finish the work even when facing the frightening beauty of the wilderness far in the northwest of Quebec.

I would also like to thank to E. Whitfield, L. Blackburn, and Ch. Pack who helped me to explore the fascinating world hidden deep in the ground and C. Krause for introducing me to the realm of dendrochronological analyses.

My gratitude also goes to K. Naydenov and his wife for their not only professional but also personal presence in the laboratory of population genetics.

I would also like to express my appreciation to P. Wildová, my friend and former colleague, who helped me to make my way through the preparation of the real time expression assays.

I am also grateful to my parents for continuous support, and in particular to my mother who patiently baby-sat my little baby boy for countless hours so I could work on this text.

And finally, it is difficult to express in words my gratitude to Christopher who actually brought me to this part of the world and made me discover its colorful society, culture, language, and nature. He was always there ready to share the good and difficult moments with me.

Moreover, this work would not be possible without the support of the Centre for Forest Research, National Science and Engineering Research Council, Industrial Chair in Sustainable Forest Management, Fonds de recherche sur la nature et les technologies, and the government of Quebec.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS, SYMBOLS, AND ACRONYMS

ACT	actin
BLAST	Basic Local Alignment Search Tool
C_t	threshold cycle values
CVA	Canonical variates analyses
CVs	canonical variates
DFR	dihydroflavonol reductase
EFDs	elliptic Fourier descriptors
ESTs	expressed sequence tags
f and g	allelic frequencies of alleles f and g
h	number of heterozygote loci
ISR	induced systemic resistance
KPIs	Kunitz protease inhibitors
l	number of loci
LPI	leaf plastochron index
LRDS	living roots on dead stumps
mRNA	messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
nEFDs	normalized elliptic Fourier descriptors
PC	principal components

PCA	principal component analysis
PCR	polymerase chain reaction
p_{gen}	probability that consecutively sampled trees that actually belong to different clones would, by chance, have a similar genotype
R	raw fluorescence data
R_n	raw fluorescence normalized to ROX reference dye
TIF	translation initiation factor
λ	Wilks' lambda
$1 - \lambda_{clones}$	among clone component
$\lambda_{clones} - \lambda_{ramet}$	among ramet within clone component

RÉSUMÉ

Dans cette thèse, nous avons examiné une nouvelle approche pour l'identification des clones de tremble. Nous avons utilisé les liens racinaires clonaux pour l'exploration de leurs fonctions potentielles dans le maintien de la diversité clonale et de la communication entre les tiges interconnectées. En particulier, nous avons examiné les hypothèses suivantes: (1) les clones de tremble peuvent être identifiés sur la base de la morphométrie digitale des feuilles; (2) les liens racinaires clonaux facilitent le maintien de la diversité clonale en intégrant différents génotypes par les greffes racinaires; et (3) les liens racinaires permettent la propagation et déclenchement des mécanismes de défense contre une attaque d'herbivore entre les individus interconnectés avant l'arrivée des insectes. Pour vérifier ces hypothèses, nous avons excavés les systèmes racinaires de trois stations de tremble purs où les clones ont été identifiés à l'aide de marqueurs moléculaires (microsatellites), morphologiques (forme de feuille, couleur et type de l'écorce) et phénologiques (apparition des feuilles). Les feuilles ont été récoltées et scannées. Les résultats ont été analysés à l'aide des descripteurs Fourier elliptiques normalisés (nEFDs). Les analyses dendrochronologiques ont été utilisées pour reconstruire le développement du système racinaire et le greffage racinaire. L'expression temporelle des mécanismes de défense induits (inhibiteurs de la trypsine de type Kunitz et dihydroflavonol réductase) a été suivie par une PCR quantitative en temps réel. Nous avons montré que l'évaluation quantitative de la forme des feuilles est difficilement applicable à l'identification des clones dans des peuplements naturels de peupliers caractérisés par la présence d'un grand nombre de clones et de génotypes uniques. Les systèmes racinaires excavés ne contenaient pas de génotypes uniques et qui n'étaient pas présent dans les tiges des arbres vivants. Néanmoins, l'interconnectivité substantielle à l'intérieur des clones ainsi qu'entre les clones, due à la présence de nombreuses greffes, conduit à la formation de vastes réseaux racinaires génétiquement diversifiés. Nous avons constaté que ces systèmes racinaires n'entraînent pas automatiquement l'induction des mécanismes de défenses, tels qu'ils sont exprimés dans les plantes directement exposées aux attaquesd'insectes phytophages. Il semble plutôt que d'autres moyens de communication, comme les composés volatils, puissent servir de voies de transmission des signaux entre les individus interconnectés.

Mots clés: *Populus tremuloides,* identification clonale, effet de l'environnement, morphométrie, ramet, diversité foliaire, phénologie printanière, indicateurs microsatellites, intégration clonale, dendrochronologie, diversité, greffe racinaire, système racinaire, attaque herbivore, expression génétique, communication entre plantes, défenses induites.

ABSTRACT

In this thesis, we investigated a novel approach of aspen clone recognition and used the delineated clonal root networks for exploration of their possible functions in diversity maintenance and communication among network members. In particular, we hypothesized that: (1) aspen clones could be delineated based on digital morphometrics of leaf shape; (2) clonal root networks could facilitate diversity maintenance by integration of different genotypes through natural root grafts; and (3) clonal root networks could serve as an early-warning system triggering antiherbivore defenses in network members even before arrival of pests. In order to verify these hypotheses, we uncovered root systems of three pure aspen stands where clones had been identified with the help of molecular (microsatellite loci), morphological (leaf shape, bark colour and type) and phenological (timing of leaf flush) characteristics. Scanned leaves were analyzed using normalized elliptic Fourier descriptors (nEFDs). Dendrochronological analyses were employed to reconstruct the root system development with respect to natural root grafting. Temporal expression of induced defensive traits (Kunitz trypsin inhibitors and dihydroflavonol reductase) was followed by quantitative real-time PCR. We showed that despite quantitative evaluation of the complex leaf shape, this approach to clone identification is hardly feasible in natural aspen stands with high number of clones and many singletons. The uncovered root networks did not include unique genotypes that would not occur among stems. Nevertheless, substantial interconnectivity both within as well as between clones due to frequent grafting resulted in formation of large genetically diverse root networks. We found that these root systems did not automatically lead to induction of defensive traits that are expressed in plants directly under simulated herbivory. Rather, it seemed that other communication means such as air borne volatiles could serve as signal transmission pathways among network members.

Key words: *Populus tremuloides*, clone identification, leaf flush, environmental effect, morphometrics, ramet, foliar diversity, spring phenology, microsatellite markers, clonal integration, dendrochronology, diversity, natural root grafting, root system, herbivory, gene expression, interplant communication, induced defenses

INTRODUCTION

Trembling aspen is an important tree species, both economically and ecologically (Bradshaw *et al.*, 2000). As being a major component of many ecosystems, numerous aspects of its biology have been thoroughly explored. Considerably less attention has been paid to aspen root system. In this thesis, we studied aspen clones and their underlying root networks. A few studies showed that natural root grafting occurs in aspen (Barnes, 1966; Shepperd, 1993; Desrochers and Lieffers, 2001). But can grafts be found even among roots of different clones? Could roots of dead stumps surviving due to grafting to living trees have unique genotypes that are not present among the living trees? And could, thus, aspen root systems represent a "genotype bank" that helps maintain high levels of clonal diversity despite the prevailing vegetative reproduction? Does aspen trees or even clones function as discrete physiologic units or rather as a part of a larger physiological entity? And in particular, can aspen root systems be use as networks for sharing information among network members? Moreover, we tried to facilitate clone recognition by exploring a new method for clone identification and addressed the following questions: Is it possible to identify aspen clones solely based on the shape of leaves provided we evaluate the complex shape? Is there enough foliar variation among clones growing in close proximity? Is the leaf shape invariant enough within a single tree? Are other traditionally used traits such as timing of spring leaf flush reliable for clone identification?

STATE OF THE ARTS

Trembling aspen (*Populus tremuloides* Michx.)

Trembling aspen (*Populus tremuloides* Michx.), a member of the Salicaceae family, is a deciduous tree species widely distributed over the Northern Hemisphere

(Bradshaw *et al.*, 2000). Aspen stands play an important role in maintaining ecosystem biodiversity by providing nutrition and habitat for a number of species (Griffis-Kyle and Beier, 2003).

Aspens are dioecious, wind-pollinated, and produce large numbers of wind-dispersed seeds. Successful seed germination requires favorable moisture conditions, absence of competition, and availability of exposed mineral soil. However, these conditions allowing new seedling recruitment rarely occur at the same time (Romme *et al.*, 2005). Some even suggested that massive colonization of vast areas through seedling establishment has not occurred in North America since the end of the last glacial period 10 000 years ago (Kemperman, 1976). Vegetative propagation from root suckering, thus, remains the prevailing mode of reproduction making aspen a clonal tree species.

Rapid aspen regeneration occurs after either man-made or natural disturbances such as fires, outbreaks of insects, or gaps created by fallen trees (Bergeron, 2000). Release of apical dominance following the tree removal allows sucker initiation from the shallow lateral roots that were left in the ground after the disturbance (Frey *et al.*, 2003). Vegetative reproduction of aspen leads to the formation of a clone or genet that can be composed of several trees termed ramets (Scrosati, 2002).

Clone identification

Identification of clones can be done based on similarities in phenotype or genotype among individuals. Ideal phenotypic or genotypic criteria used for clone identification should exhibit a large variation among clones and be highly consistent within clones. As not a single phenotypic feature was sufficient to provide enough variation, a combination of a few was traditionally used (Barnes, 1966; Barnes, 1969; Kemperman, 1976; Barnes and Han, 1993). Ideal criteria should be also insensitive to

environmental factors. Some morphological characteristics were traditionally assessed only categorically and such characterization has nowadays been abandoned as being too subjective. Value of other morphological traits was questioned for being influenced by environmental factors (Persson and Gustavsson, 2001; Rumpunen and Bartish, 2002; Lopez-de-Heredia *et al.*, 2004). Recently, molecular markers have become the method of choice in most population studies (Wyman *et al.*, 2003; Namroud *et al.*, 2005; Suvanto and Latva-Karjanmaa, 2005; Mock *et al.*, 2008; De Woody *et al.*, 2009; Jelinkova *et al.*, 2009; Liesebach *et al.*, 2010).

Nonetheless, morphological trait assessment had several advantages over the use of molecular markers. It was mainly the rapidity and ease of use directly in the field without the need of any laboratory analyses. Development of new methods for evaluation of various biological shapes in animals and plants brought also new possibilities for clone delineation (McLellan and Endler, 1998; Jensen *et al.*, 2002; Neto *et al.*, 2006; Viscosi and Fortini, 2011; Cope *et al.*, 2012). A new method of leaf shape analysis was explored in the first chapter.

Genotype conservation and aspen root system

Clonal diversity in species with limited sexual reproduction tends to diminish over time (Balloux *et al.*, 2003). Despite the limited recruitment of seedlings, aspen remains one of most diverse tree species letting stranded those who tried to find a decline of clonal richness during succession (Erickson and Hamrick, 2003; Pluess and Stocklin, 2004; Namroud *et al.*, 2005). In the second chapter, we sought an additional mechanism of diversity maintenance in aspen clonal root network.

Shallow root systems of aspens comprise the principal and thin lateral roots. They are mainly the lateral roots that initiate new suckers. Aspen trees that have regenerated from the root suckers remain connected to the original parental roots

even after reaching maturity (DesRochers and Lieffers, 2001). Besides, aspen root systems often include surviving roots of dead stumps grafted to roots of living stems (DesRochers and Lieffers, 2001). It is not known whether these grafts occur only between roots of the same genotype or whether they could be also established between trees of different clones. Montalvo and his colleagues (Montalvo *et al.*, 1997) found that some interconnected trees of another clonal tree species, *Quercus chrysolepis,* differed in genotypes. Moreover, many aspen clones are often intermingled at the same site (Wyman *et al.*, 2003; Namroud *et al.*, 2005; Suvanto and Latva-Karjanmaa, 2005). These findings suggest that different genets of an aspen stand might be linked by their root systems and could include roots of unique genotypes that are not present among the stems. We explored this "genotype bank" hypothesis in the second chapter.

Physiological integration

The above described formation of physical root connections which allow exchange of substances among interconnected trees is known as physiological integration. The root connections are not only physical but also physiological allowing for the exchange of water, nutrients, and carbohydrates. Nonetheless, little is known about sharing of hormones, pathogens and non-nutritional molecules. It has been suggested that these connections may represent highways for long-distance transport of signals (Stuefer *et al.*, 2004). Viewed from this angle, the physiological integration might have far-reaching physiological and ecological implications. Clonal root networks could be, for instance, used as a mean of communication of defense signals among the inter-connected individuals.

Plant defenses against herbivores and herbivore-simulated wounding

Trees have efficient defense mechanisms (Philippe and Bohlmann, 2007; Duplessis *et al.*, 2009; Ralph, 2009; Barton and Koricheva, 2010) that can be divided into two types; constitutive defense mechanisms are permanently at work even in absence of potential threats (Robison and Raffa, 1997; Philippe and Bohlmann, 2007; Brilli *et al.*, 2009), whereas, inducible defenses occur only after pathogen or herbivore attack triggering off the defense responses (Metraux *et al.*, 2002; Philippe and Bohlmann, 2007; Germain and Seguin, 2011). Some defense mechanisms effect herbivores directly by producing toxins or other substances that change the tissue quality (Lindroth, 2001; Osier and Lindroth, 2001; Bailey *et al.*, 2007; Donaldson and Lindroth, 2008). Other defense mechanisms may tackle the herbivores in an indirect way by releasing gaseous chemicals called volatiles that attract the natural enemies of the herbivores (Parry *et al.*, 2003; Arimura *et al.*, 2004; Brilli *et al.*, 2009).

The induced defense mechanisms can be either confined to the damaged site or spread to remote unaffected parts of the plant. This phenomenon is referred to as induced systemic resistance (ISR; Metraux *et al.*, 2002). After recognition of an initial stimulus (herbivory) by the plant, a signal transduction pathway is switched on resulting in activation of defense mechanisms. These defense mechanisms principally involve expression of new genes (Haruta *et al.*, 2001a; Haruta *et al.*, 2001b; Peters and Constabel, 2002; Arimura *et al.*, 2004; Christopher *et al.*, 2004; Wang and Constabel, 2004; Ralph *et al.*, 2006; Tsai *et al.*, 2006; Philippe *et al.*, 2009; Philippe *et al.*, 2010).

Among hundreds of systemically wound-induced genes, there are chitinases, peroxidase, lipoxygenase, and cysteine proteinase, which have direct roles in herbivore defenses, and numerous others, which have a variety of other functions in metabolism, signal transduction, and other cellular processes (Metraux *et al.*, 2002).

Mechanical wounding has been used in many studies as a simple imitation of the real herbivory (Constabel *et al.*, 2000; Haruta *et al.*, 2001a; Christopher *et al.*, 2004; Dervinis *et al.*, 2010). At the level of gene expression, both the artificial and real herbivory led to the production of qualitatively similar response, nonetheless these two responses differ quantitatively (Major and Constabel, 2006). Ultimate proofs of defense mechanism activation were brought by studies using real insect feeding that demonstrated that defense induction affected insect behavior (food choice tests) or development and survival of insect larvae (Osier, 2000; Osier and Lindroth, 2001; Dervinis *et al.*, 2010).

In trembling aspen, three trypsin inhibitors were found to be involved in induced protein-base defenses against insect herbivores (Haruta *et al.*, 2001*a*). Trypsin inhibitors are one of protease inhibitors which intervene with the proteolytic activity of enzymes in herbivore guts leading to loss of essential amino acids, and consequently, to an inhibition of insect growth (Dicke and Hilker, 2003). Other well-characterized, both constitutive and inducible, anti-herbivore defense systems of trembling aspens are based on production of phytochemicals such as condensed tannins and phenolic glycosides (Haruta *et al.*, 2001b; Peters and Constabel, 2002; Peters and Constabel, 2003). Condensed tannins are polyphenolic compounds, typically found in woody plants, which reduce efficiency in nutrient absorption in insect midgets.

Defense signaling in a clonal plant species

Herbivory feeding (or herbivory simulation by wounding) initiates production of defense induction signals at the site of the damage. These signals then spread throughout the plant body and trigger off the systemic defense mechanisms (Metraux *et al.*, 2002). The nature of these signals is not well understood, nonetheless some

factors influencing their movement are known. For instance, it is clear that signal transmission is limited by plant vascular architecture (Jones *et al.*, 1993). The vascular architecture of trembling aspen is ortosticious, which means that a selected leaf alongside the stem is directly connected to the eighth leaf growing upward or downward from the selected one. These leaves share direct vascular connection, and, thus, signal transmission among these leaves is not limited by vascular connectivity. Moreover, it has been demonstrated that the movement of the defense induction signals is influenced by the source and sink relationship within the plant body (Arnold *et al.*, 2004; Arnold and Schultz, 2002). As fast growing young leaves at the top of stems represent the strongest sinks for assimilates, strong systemic defense induction was detected particularly in these leaves. However, herbivory feeding modifies the flow of assimilates through the plant body partially diverting carbohydrates to roots (Babst *et al.*, 2005; Babst *et al.*, 2008).

Provided that some herbivore damage occurred in a stand of a clonal plant species like aspens, the defense induction signal might be transmitted from the tree under attack to intact trees through their integrated root system (Stuefer *et al.*, 2004). Thus, the trees that have not been damaged so far might activate their defense mechanisms before the arrival of the herbivores. Such defense signaling was experimentally tested in the third chapter.

METHODS

With the aid of forestry maps established for the region in the archive of Nordbord Company, we found three pure aspen stands of sucker origin aged 20 - 45 years and having a high density of stems. One study plot of approximately 30 m^2 encompassing the maximum number of trees was established in every site. All aspen

trees within these plots were genotyped using a set of microsatellite markers (Dayanandan *et al.*, 1998; Rahman *et al.*, 2000; Wyman *et al.*, 2003; Namroud *et al,* 2005*a*, 2005*b*). In the first chapter, aspen trees within these plots were characterized with respect to spring phenology (appearance of the first fully expanded leaves) and bark features. Eight intact leaves from every tree were collected for an analysis of leaf shape. The leaf shape was assessed quantitatively using elliptic Fourier descriptors. In the second chapter, the three plots were excavated and the uncovered root systems were mapped. The roots forming grafts and the living roots on dead stumps were genotyped using the set of microsatellites. Cross-sectional disks of all principal roots, root grafts, and stems were taken. Dendrochronological analysis was used to distinguish the root types (new and mother roots) and date the formation of root grafts. In the third chapter, we conducted a green house experiment. We cultivated root cuttings of a single genotype originating from the mixed boreal forest of northwestern Quebec under the conditions promoting root suckering. Root suckers were used to follow the expression of two induced antiherbivore defense traits, Kunitz trypsin inhibitor and dihydroflavonol reductase. Their expression was followed after a wound treatment simulating insect herbivory in systemic (unwounded) leaves of wounded and untreated suckers and in controls.

OBJECTIVES AND EXPERIMENTAL APPROACH

The general aim of this thesis was to investigate a novel approach of aspen clonal identification and to explore possible functions of aspen clonal networks in diversity maintenance and inter-ramet communication. To meet the objectives, we employed an automated image processing of leaf shape, molecular and dendrochronological approach for studying aspen root systems, and quantitative real-time PCR to follow gene expression in interconnected aspen ramets. The second and

third chapters of this thesis have been published as research papers in peer-reviewed journals. The first chapter is now being evaluated by anonymous reviewers.

Chapter 1: H Jelínková, F Tremblay, A DesRochers. The use of digitalmorphometrics and spring phenology for clone recognition in trembling aspen (*Populus tremuloides* Michx.) and its comparison to microsatellite markers. Submitted.

In this chapter, we investigated a novel method for clone identification using complex evaluation of leaf shape, bark characteristics, and spring phenology. H. Jelínková did sampling in the study sites, genotyping using microsatellites in the laboratory, analysis of leaf shape from data acquisition to statistical analysis, and wrote the manuscript. Dr. Tremblay and Dr. DesRochers set the framework of the research and experimental approach and edited the manuscript.

Chapter 2: H Jelínková, F Tremblay, A DesRochers (2009). Molecular and dendrochronological analysis of natural root grafting in *Populus tremuloides* (Salicaceae). American Journal of Botany 96: 1500-1505.

In this chapter, we explored a possible mechanism of clonal diversity maintenance through genotype conservation in aspen root system. H Jelínková participated in site excavation, mapping and sampling, conducted the molecular and dendrochronological analyses, and wrote the manuscript. Dr. Tremblay and Dr. DesRochers set the framework of the research and experimental approach, contributed to the hypothesis definition, and edited the manuscript.

Chapter 3: H Jelínková, F Tremblay, A DesRochers (2012). Herbivore-simulated induction of defenses in clonal networks of trembling aspen (*Populus tremuloides*). Tree Physiology 32: 1348-1356.

In this chapter, we explored defense responses to herbivory simulation in interconnected aspen ramet. H Jelínková conducted the stress treatment, sampling,

real-time PCR assays in the laboratory, analyzed the data and wrote the manuscript. Dr. Tremblay and Dr. DesRochers set the framework of the research and experimental approach and edited the manuscript.

CHAPTER I

THE USE OF DIGITAL MORPHOMETRICS AND SPRING PHENOLOGY FOR CLONE RECOGNITION IN TREMBLING ASPEN (*POPULUS TREMULOIDES* MICHX.) AND ITS COMPARISON TO MICROSATELLITE MARKERS

1.1 RÉSUMÉ

Les clones de tremble ont traditionnellement été identifiés sur la base des similarités des caractères phénotypiques, y compris la forme des feuilles. Cela nécessitait plusieurs visites des stations, de nombreuses mesures et évaluations visuelles subjectives. Dans cette étude, nous avons examiné une nouvelle approche pour l'identification des clones en utilisant respectivement la morphométrie digitale de la forme des feuilles, les caractéristiques de l'écorce et la phénologie printanière. Les clones de tremble ont été délimités selon leur caractéristiques moléculaires (marqueurs microsatellites), morphologiques (forme des feuilles, couleur et type d'écorce) et phénologiques (temps de débourrement). Les feuilles ont été scannées et les images analysées en utilisant les descripteurs Fourier normalisés (nEFDs) et l'analyse en composantes principales. Le pouvoir discriminant des nEFDs a été testé par l'analyse canonique des variables (CVA). Nous avons identifié 18 clones parmi les 60 tiges analysées sur les trois stations. L'analyse de regroupement des variables de la forme des feuilles a permis d'unifier des types foliaires similaires dans deux sites qui correspondaient aux clones définis par les marqueurs microsatellites. Toutefois plusieurs ramets de la troisième station n'ont pas été identifiés correctement. Le test de reclassement indique que la forme des feuilles contient des fonctions suivant lesquelles les clones très similaires peuvent être différenciés avec des faibles taux d'erreur. Cependant, comme il n'était pas possible de fixer un seuil pour les distances maximales à l'intérieur des clones, l'application de cette approche d'identification dans les peuplements naturels de peupliers avec un nombre élevé de clones multi-ramets et beaucoup de génotypes uniques est irréalisable. Parmi les critères examinés, la phénologie printanière semble le caractère le moins fiable pour la reconnaissance des clones. Les causes possibles du débourrement hétérogène parmi les ramets du même génotype sont discutées

1.2 ABSTRACT

Aspen clones were traditionally identified based on similarities in several phenotypic traits including leaf shape. This required several visits of the stands, laborious measurements and subjective visual assessments. In this study, we investigated a novel approach to clone identification using digital morphometrics of leaf shape complemented with bark characteristics and spring phenology. Aspen clones were delineated based on molecular (microsatellite loci), morphological (leaf shape, bark colour and type) and phenological (timing of leaf out) characteristics. Leaves were scanned and images were analyzed using normalized elliptic Fourier descriptors (nEFDs) and principal component analysis. Discriminatory power of nEFDs was tested by canonical variates analysis (CVA).We identified 18 clones among 60 aspen trees in the three sites. Cluster analysis of the leaf shape variables grouped together similar foliar types that in two out of the three sites reflected the clones defined by a set of microsatellite markers. Many ramets from the third site were clustered erroneously into incorrect clones. The reclassification test indicated that leaf shape contains features according to which very similar clones can be differentiated with low error rates. However, because it was not possible to set a threshold for maximum distances within clones, application of this approach of clone identification in natural aspen stands with a high number of multi-ramet clones and many singletons is unfeasible. We judged spring phenology as the least reliable trait for clone recognition and suggested possible causes of heterogeneous leaf flushing among ramets of the same genotype.

1.3 INTRODUCTION

Natural populations of aspen exhibit a clonal growth as an admixture of clones of several ramets and singletons (Scrosati, 2002). Identification of clones was traditionally based on the morphological characteristics such as floral, foliar, stem, and bark traits, growth form, and susceptibility to diseases or injuries (Barnes, 1966; Barnes, 1969; Barnes andand Han , 1993). A putative large aspen clone consisting of 47 000 ramets was identified in this way by Kemperman and Barnes (1976). However, when molecular markers were used, many distinct genotypes were found in and around the "pando" clone (Mock *et al.*, 2008). Morphological characterization for clonal identification was quickly abandoned mainly for two reasons: Firstly, evaluation of some phenotypic traits such as leaf shape appeared too subjective to be reliable. Secondly, many phenotypic traits are likely to be influenced by environmental factors (Persson and Gustavsson, 2001; Rumpunen and Bartish, 2002; Lopez-de-Heredia *et al.*, 2004). Hence, after the discovery of molecular markers, morphological identification of intermingling clones in aspen stands was quickly replaced by an almost exclusive use of a set of microsatellites (Wyman *et al.*, 2003; Namroud *et al.*, 2005; Suvanto and Latva-Karjanmaa, 2005; Mock *et al.*, 2008; De Woody *et al.*, 2009; Jelinkova *et al.*, 2009; Liesebach *et al.*, 2010).

Nonetheless, recent advances in morphometrics, in particular automated image processing, brought new possibilities for genotype recognition that could help overcome the above mentioned disadvantages of morphological traits assessment. For instance, there is an array of new methods for analyses of biological shapes (McLellan and Endler, 1998; Jensen *et al.*, 2002a; Neto *et al.*, 2006; Viscosi and Fortini, 2011; Cope *et al.*, 2012). They have been primarily developed for the needs of taxonomists to give an objective, quantitative shape evaluation. Among a number of descriptor suits that have been proposed, elliptic Fourier descriptors (EFDs) have proved especially useful in a variety of contexts in several plant species (Mancuso,

1999; Iwata *et al.*, 2002a; Rumpunen and Bartish, 2002; Yoshioka *et al.*, 2004; Neto *et al.*, 2006; Menesatti *et al.*, 2008; Torres *et al.*, 2008; Viscosi and Fortini, 2011).

Elliptic Fourier descriptors (EFDs) can delineate any type of shape with a closed two-dimensional contour and are sensitive to both subtle and complex changes in a specimen's outline (Kuhl, 1982; McLellan and Endler, 1998). They have been shown to be more efficient in assigning plant material to correct clones (Persson and Gustavsson, 2001; Rumpunen and Bartish, 2002; Cope *et al.*, 2012). Availability of software packages such as LAMINA or SHAPE makes the leaf shape assessment easy, fast, and inexpensive (Iwata and Ukai, 2002; Bylesjo *et al.*, 2008). Hence, unlike molecular markers, leaf shape could be used as an inexpensive marker for early clone identification in situ during a single field visit.

Besides the need for an objective evaluation, clone identification also requires a selection of phenotypic characteristics that exhibit low sensitivity to environmental effects. Development of leaf shape and size is a highly complex process under the control of many genes which is further modulated by hormonal and environmental factors (Wu *et al.*, 1997; Wu, 2000). Although leaf size can be conditioned by environmental factors (such as light exposure or water availability) leaf shape is usually less affected; Iwata *et al.* (2002b) tested genotype x environment interactions in a field trial of citrus and showed that the genotype was the main source of variation in leaf shape, but not in size. A study of European aspen (*Populus tremula*) also indicated that the genotype influence was smaller for size than for shape related traits (Lopez-de-Heredia *et al.*, 2004). Moreover, leaf shape can be described by symmetrical and asymmetrical features. It has been shown that symmetrical features are highly heritable while the asymmetrical ones are consequences of environmental effects. The two types can be devided and analyzed separately (Iwata *et al.*, 2002; Iwata and Ukai, 2002).

Studies employing both molecular and morphological markers are rare in forest tree species (Cannon and Manos, 2001; Jensen *et al.*, 2002b; Penaloza-Ramirez *et al.*, 2010) and in genus *Populus* in particular (Lopez-de-Heredia *et al.*, 2004; Suvanto and Latva-Karjanmaa, 2005; Lexer *et al.*, 2009). In the present study we investigated a method for clone recognition that could be used in a variety of contexts of ecological research, in studies of genotype-phenotype correlation, or in selection and identification of breeding material. The tested method employed image analysis of leaf shape complemented with bark characteristics and spring phenology. We compare our results with clones identified with a commonly used set of microsatellite markers. To our knowledge, there is neither previous study applying digital morphometric to aspen nor study comparing morphological and microsatellite markers.

1.4 MATERIALS AND METHODS

1.4.1 Study Sites

Aspen clones were delineated based on molecular (microsatellite loci), morphological (leaf shape, bark color and type) and phenological (timing of leaf out) characteristics in three natural stands in the southern boreal forest in northwest Quebec, Canada. The three sites (K, M, W) consisted of pure even-aged trembling aspen forest that regenerated after clear cuts in 1983, 1968, and 1964, respectively. The area stretches from 48°11' N to 48°30' N of latitudes and from 78°45' W to 79°23' W of longitudes. A plot of 30 m^2 encompassing 33, 17, and 18 aspen trees, respectively was established at each site.

1.4.2 Microsatellite Characteristics

Bark sample was taken from every stem within the plots for DNA extraction. DNA was extracted from cambial tissue with the help of the GenElute Plant Genomic

DNA Miniprep Kit (Sigma-Aldrich Canada Ltd, Oakville, Canada) according to the manufacturer's protocol. Amplification of seven microsatellite loci (Dayanandan *et al.*, 1998; Rahman *et al.*, 2000) was done using dye-labeled oligonucleotide primers and Taq polymerase (Gibco, Invitrogen™ Life Technologies, Burlington, Canada). For more details on the protocol see Jelínková *et al.* (Jelinkova *et al.*, 2009). In order to determine the resolution power of the set of microsatellites used, the round robin method was employed (for more details, see chapter 2.2.4 page 60).

1.4.3 Leaf Shape Description

Leaves were collected from the mid-position in the crowns and from the same position on the branch (the oldest fully expanded leaf). Eight healthy leaves with perfectly preserved contours were taken from every tree. Petioles were removed and the blades were pressed. The image analysis was conducted with the software package SHAPE v1.2 (Iwata and Ukai, 2002) using elliptic Fourier coefficients (Kuhl, 1982). Precision of contour description increases with increasing number of harmonics (trigonometric functions describing the shape). As clonal differences in leaf shape can be minor, in particular when attempting to differentiate clones growing in close proximity, a series of elliptic Fourier transformations employing 20, 40, and 80 harmonics were used in this study. The elliptic Fourier descriptors (EFDs) were manually normalized to be invariant in size, thus the size component of the variation was excluded from the analysis (Kuhl and Giardina, 1982). Moreover, only components describing the symmetrical features of the leaf shape were analyzed.

1.4.4 Leaf Shape Analysis

In order to summarize the information contained in normalized EFDs (nEFDs) and to reduce the number of variables describing every leaf, principal component analysis (PCA) was performed based on a variance-covariance matrix of nEFDs in SHAPE v1.2 (Iwata and Ukai, 2002). The variation in leaf shape accounted for by

every principal component score (PC) was visualized by letting the score be equal to the mean plus and minus two times the standard deviation and the remaining components be zero. A following inverse Fourier transformation allowed reconstruction of the mean shape and its variation described by every PC (FURUTA *et al.*, 1995). Separate PCAs and subsequent inverse Fourier transformations also allowed for reconstruction of mean leaf shapes of clones defined by microsatellite markers.

Principal components were used as input variables for a cluster analysis using the unweighted pair-group method of averages (UPGMA) to generate dendrograms. Averages of the principal component scores were calculated from 8 leaves of every tree prior to the clustering (clustering was, hence, based on an average leaf shape of every ramet).

Canonical variates analyses (CVA) were used to partition morphometric diversity into within- and between- clone and tree components. CVA provides a measure, Wilks' lambda (λ), that gives a proportion of the total diversity that is due to within-group variation. Within-group replicates were 424 leaves and the groups were successively defined as 12 clones and 54 ramets obtaining λclone and λramet, respectively. 1 — λclones expresses between clone components and λclones – λramet between ramet components. Analyses were done separately for PCs calculated from nEFDs based on 20, 40, and 80 harmonics.

To test the discriminatory power of the EFDs, a classification test reassigning ramets defined by leaf averages of canonical variates (CVs) into clones identified by molecular markers was also executed. As 8 leaves were available for every tree, four leaves were used as reference samples and four as test samples. Clones represented by only one ramet were excluded from this analysis as grouping of single trees is not feasible. Clustering, tree reconstruction, CVA, and reclassification tests were done

with commands CLUSTER, TREE, CANDISC, and DISCRIM in SAS v9.1 (SAS Institute, Cary, NC).

1.4.5 Phenological and Bark Characteristics

The plots were visited once every five days during the period of leaf flushing and dates were taken when the first fully expanded leaves appeared. Every trunk was assessed visually for the color of the bark. Bark texture was evaluated as either smooth or rough.

1.5 RESULTS

1.5.1 Microsatellite Characteristics

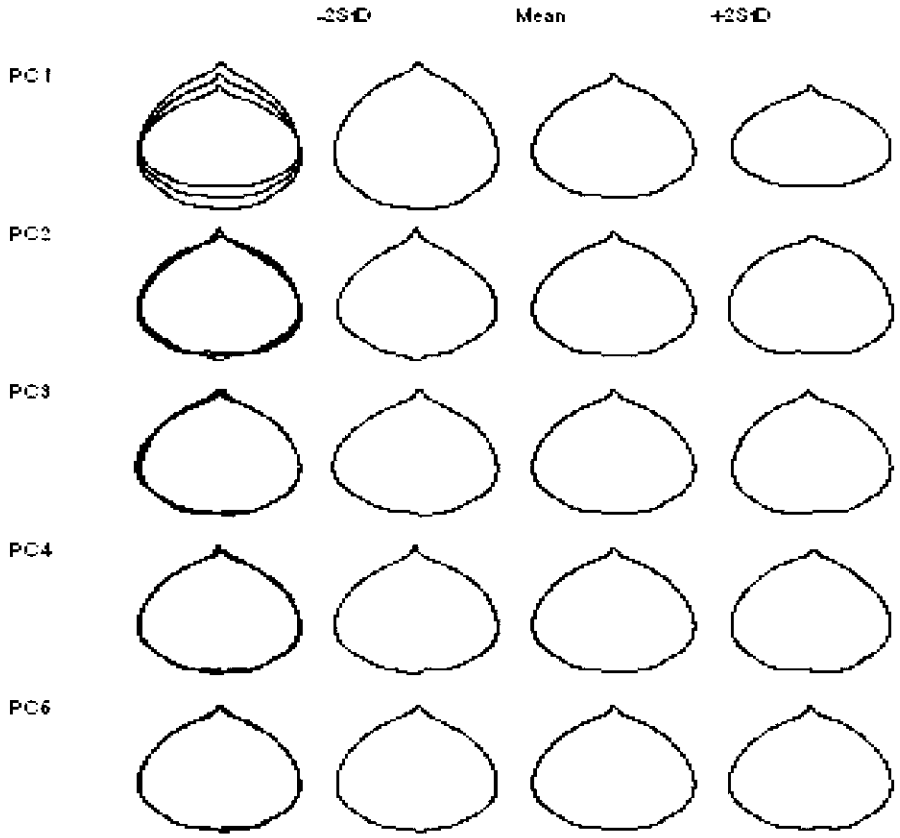
The seven microsatellite markers distinguished 18 clones among 60 aspen trees in the three sites. Six clones were represented by only one ramet. Average clonal size was 3.8 ramets and the largest clone comprised 13 ramets. The resolution power of the used set of microsatellites was high. Probability of misclassification of two different clones as one was lower than 0,01 in all clones in all sites.

1.5.2 Leaf Shape Characteristics

PCA of nEFDs identified several independent features of leaf shape variation (Fig.1.1). An increased number of harmonics resulted in a higher number of PCs (Table 1.1). Nevertheless, the cumulative contribution of the first two components accounted for over 93% of the total variance. Gaining additional PCs when employing 80 harmonics led to an increase of the total variance contained within the PCs by only 4.1%. Corresponding PCs (calculated from 20, 40, and 80 harmonics) had similar contributions to the total explained variance and seemed to reflect similar characteristics of leaf shape variation when the reconstructed contours were evaluated visually (Fig. 1.1 shows contour reconstruction of the first 5 PC scores for 80

harmonics only). PCA six first axes left out 2.7% of the diversity conveyed by nEFDs.

Figure 1.1 Contour reconstruction by inverse Fourier transformation showing the effect of the first five principal component scores calculated from 80 harmonics. Second, third, and fourth column depict the cases when the scores take -2 standard deviations, mean, and +2 standard deviations. The three contours are overlaid in the first column.



Leaf shapes reconstructed by inverse Fourier transformations indicated that the first PC was a good measure of the length to width ratio (Fig.1.1). It accounted for over 80% of the total variation of the original coefficients (Table 1.1). The second component was associated with the shape of apex that varied from pointed to flat and accounted for over 6% of the total variation. It also expressed the shape of blade base

that diverged between flat and domed. The remaining components comprising less than 3% of variation each were ascribed to other types of variation which were more difficult to explain.

Means and standard deviations of PCs based on nEFDs for 80 harmonics were calculated for the 12 multi-ramet aspen clones identified by microsatellite markers in three sites. The mean leaf shape of every clone was then drawn using the inverse Fourier transformations of these values (Fig. 1.2). Scatter plots of the within-ramet means of the first two PCs indicate variation both within and between clones and suggest possible grouping of individuals into groups (Fig. 1.3). Information contained within all PC scores is summarized by cluster analysis dendrograms in Figure 1.4.

The morphological variation described by nEFDs for 20, 40, and 80 harmonics was further subjected to the canonical variates analyses (CVAs). All Wilks' lambdas (λ) were significant at $P=0.05$ (Table 1.2). By using a higher number of harmonics and, hence, improving the precision of extracted leaf contours, a growing part of the total morphological variation was attributed to the between clone component while the foliar diversity between ramets within clones and among leaves of individual ramets had a tendency to diminish. For 80 harmonics, less than 1% of variation was found within individual trees (Table 1.2).

CVAs created linear discrimination functions that combined all input features used and presented them as new canonical variables. Microsatellite-defined clones were used as groups and mean ramet shapes (reference samples calculated from four leaves of every tree) as replicates. Clone discriminant functions were validated by reclassifying all ramets (test samples represented by means calculated from four other leaves of every ramet) to their most probable clones (Table 1.3). Eighty-seven, 93, and 96% of all aspen ramets from multi-ramet clones were correctly reassigned when using clone discriminant variables calculated from nEFDs for 20, 40, and 80

harmonics, respectively. Percentages of correctly reassigned ramets were high for most genets with the exception of KF and KI that could not be discriminated according to leaf morphology.

Table 1.1 Eigenvalues and contributions of principal components calculated from normalized elliptic Fourier descriptors based on 20, 40, and 80 harmonics.

	Eigenvalue [10^4]			Proportion [%]			Cumulative [%]		
	Nb. of harmonics			Nb. of harmonics			Nb. of harmonics		
PC	20	40	80	20	40	80	20	40	80
1	72.0	67.0	68.0	86.7	84.8	84.9	86.7	84.8	84.9
2	5.4	5.6	5.8	6.6	7.1	7.3	93.2	92.0	92.3
3		2.1	1.8		2.7	2.2		94.7	94.5
4		1.1	1.1		1.4	1.4		96.02	95.9
5			0.58			0.7			96.6
6			0.53			0.7			97.3

Figure 1.2 Mean leaf shapes of the 12 multi-ramet aspen clones identified by the microsatellite markers, single ramets clones are not included in this figure. Clones are marked by the letters; A), B), and C) show mean leaf shapes of clones from sites M, K, and W, respectively.

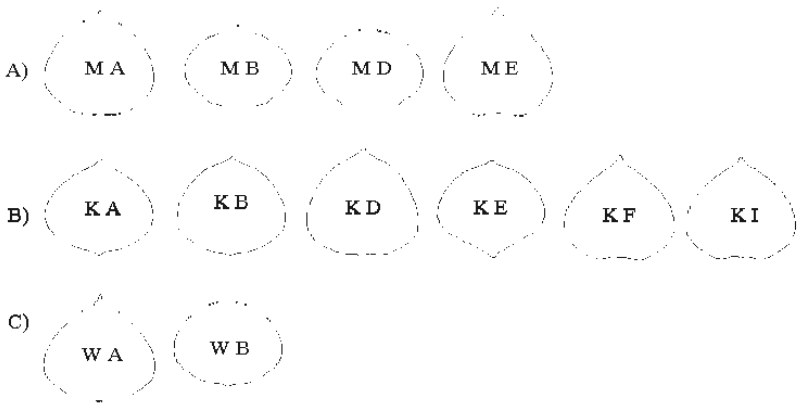
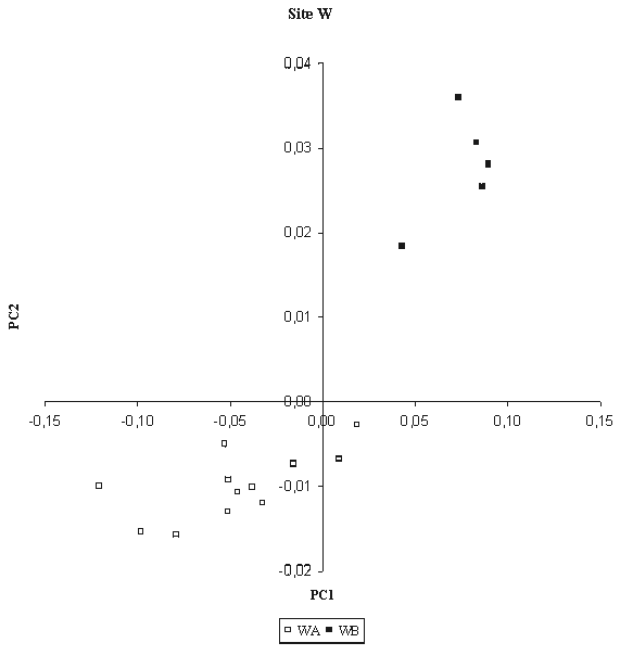
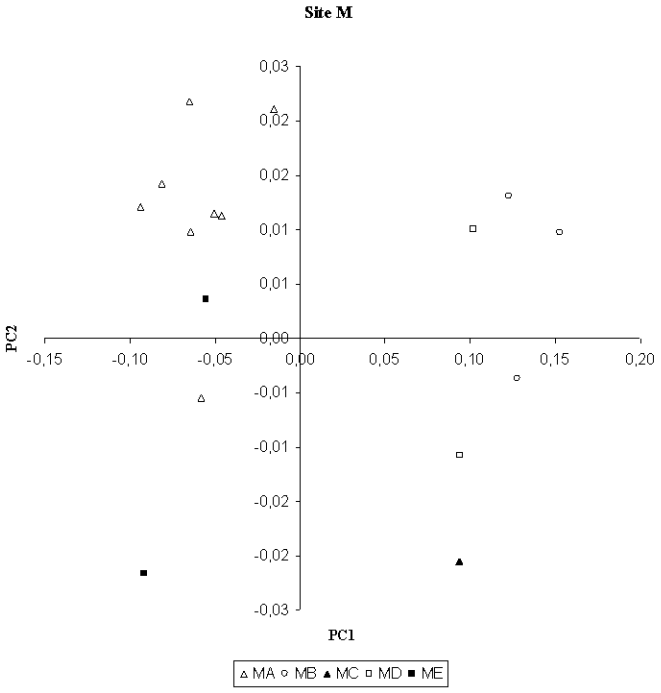


Figure 1.3 Scatter-plots of within-ramet means of the first and second principal component based on nEFDs for 80 harmonics.




Site M
0,03
0,02
0,02
0,01
0,01
0,00
-0,01
-0,01
-0,02
-0,02
-0,03
-0,15
-0,10
-0,05
0,00
0,05
0,10
0,15
0,20
PC2
PC1
MA MB MC MD ME

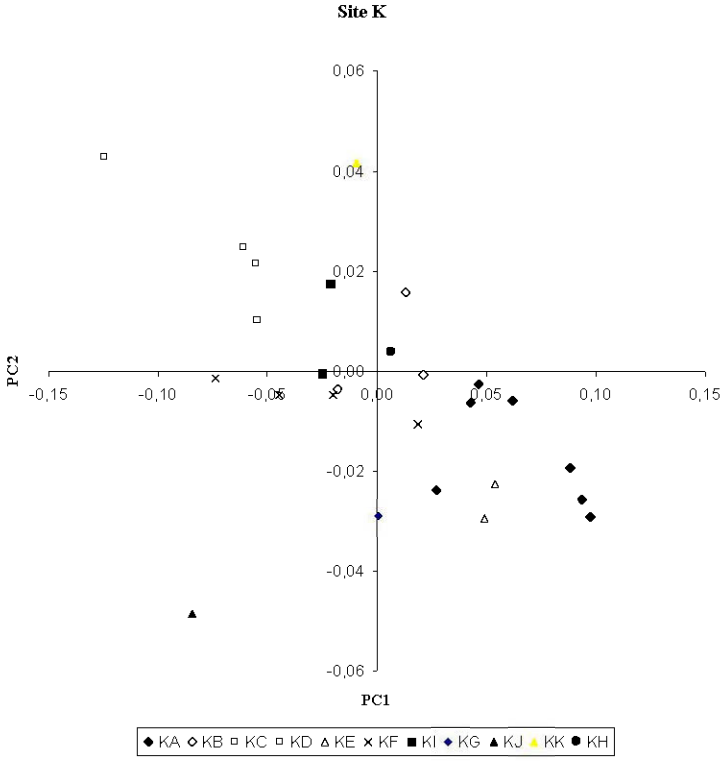
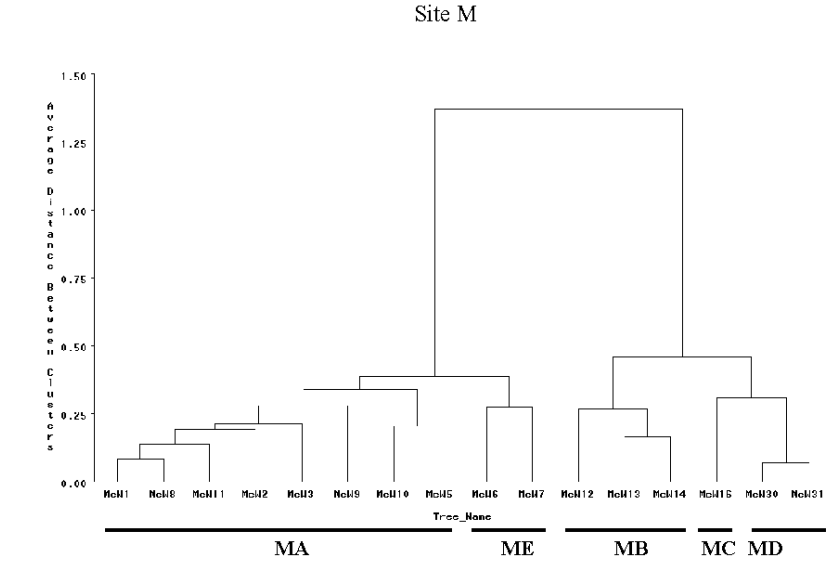
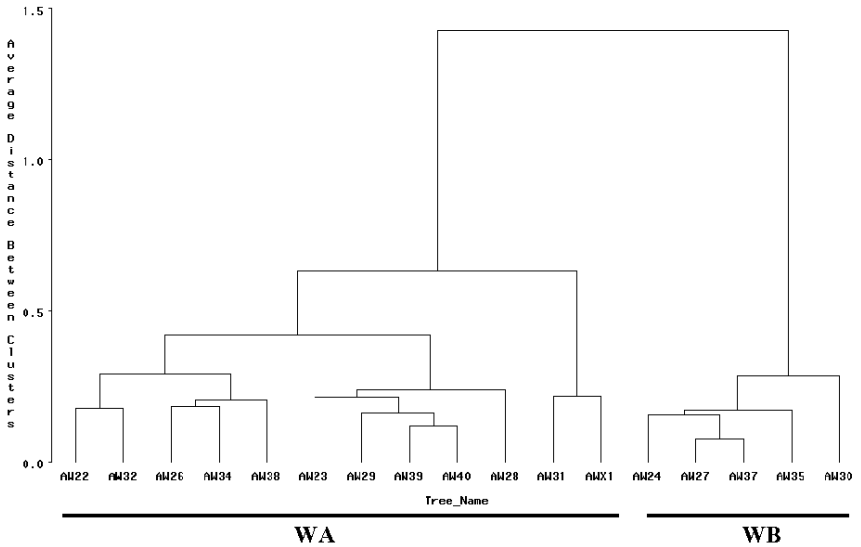

Site K
0,06
0,04
0,02
0,00
-0,02
-0,04
-0,06
-0,15
-0,10
-0,05
0,00
0,05
0,10
0,15
PC2
PC1
KA KB KC KD KE KF KI KG KJ KK KH

Figure 1.4 Cluster analysis dendrograms derived from a matrix of mean Euclidian distances based on PCs calculated from nEFDs for 80 harmonics. Outliers are marked by arrows. Clones defined by microsatellite markers are underlined. A) site M, B) site W, C) site K.

A)



B)



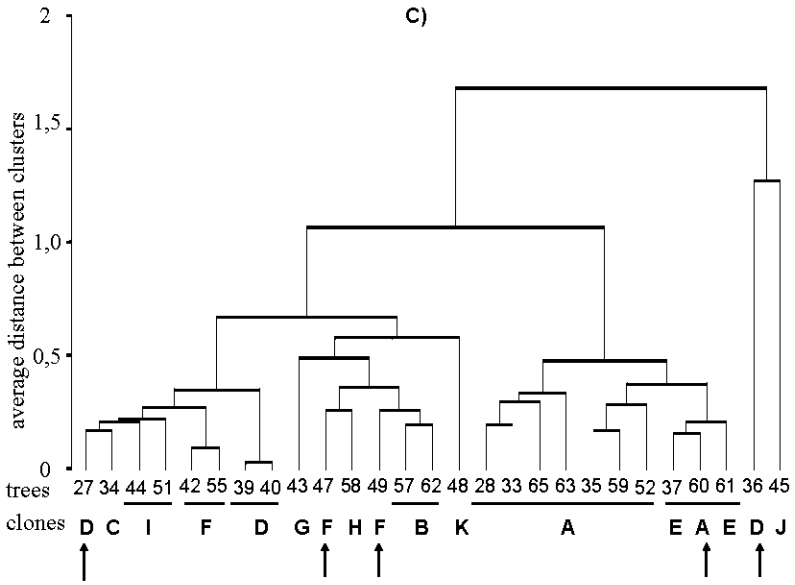


Table 1.2 Summary of canonical variates analyses of aspen leaf shape based on principal components of elliptic Fourier coefficients. Groups were successively defined as clones and ramets and individual leaves were used as replicates. All Wilks' lambdas were significant ($P<0.05$). Partitioning of the total variation based on Wilks' λ [%]

Site	No. of hrm.	between clones within sites	within clones (λ_{Clone})	between ramets within clones	within ramets (λ_{Ramet})
K	20	88.10	11.90	7.65	4.25
	40	95.91	4.09	2.93	1.16
	80	98.54	1.46	1.30	0.16
M	20	93.21	6.79	5.02	1.77
	40	94.67	5.33	4.09	1.24
	80	97.17	2.83	2.53	0.30
W	20	81.68	18.32	13.11	5.21
	40	81.08	18.92	15.51	3.41
	80	86.54	13.46	12.54	0.92

Table 1.3 Test of the discriminatory power of the EFDs. Ramets defined by leaf averages of CVs were reassigned into clones identified by molecular markers. Clone discriminant variables were calculated from nEFDs for 20, 40, and 80 harmonics. Single-ramet clones were excluded from the analysis. This method of discrimination required prior knowledge of the clones and was not used as a main method for clone identification.

	Hrm.	MA	MB	MD	ME	WA	WB	KA	KB	KD	KE	KF	KI	Total (%)
Error rate (%)	20	0	0	0	100	8	0	0	33	33	0	0	100	13
	40	0	0	0	0	0	0	0	33	0	0	0	100	6
	80	0	0	0	0	0	0	0	0	0	0	0	100	4
Misclassified as	20				WA, KI	ME			WB	MA			KF	
	40								WB				KF	
	80												KF	

1.5.3 Phenological and Bark Characteristics

Based on bark color and texture, three, two, and one phenotypes were distinguished at sites M, W, and K, respectively (Table 1.4). Two morphologically similar clones at site M appeared genetically different when using a set of microsatellites. At site W, bark morphotypes matched the microsatellite-delineated clones. Ramets of all clones in site K were uniform in bark color and type.

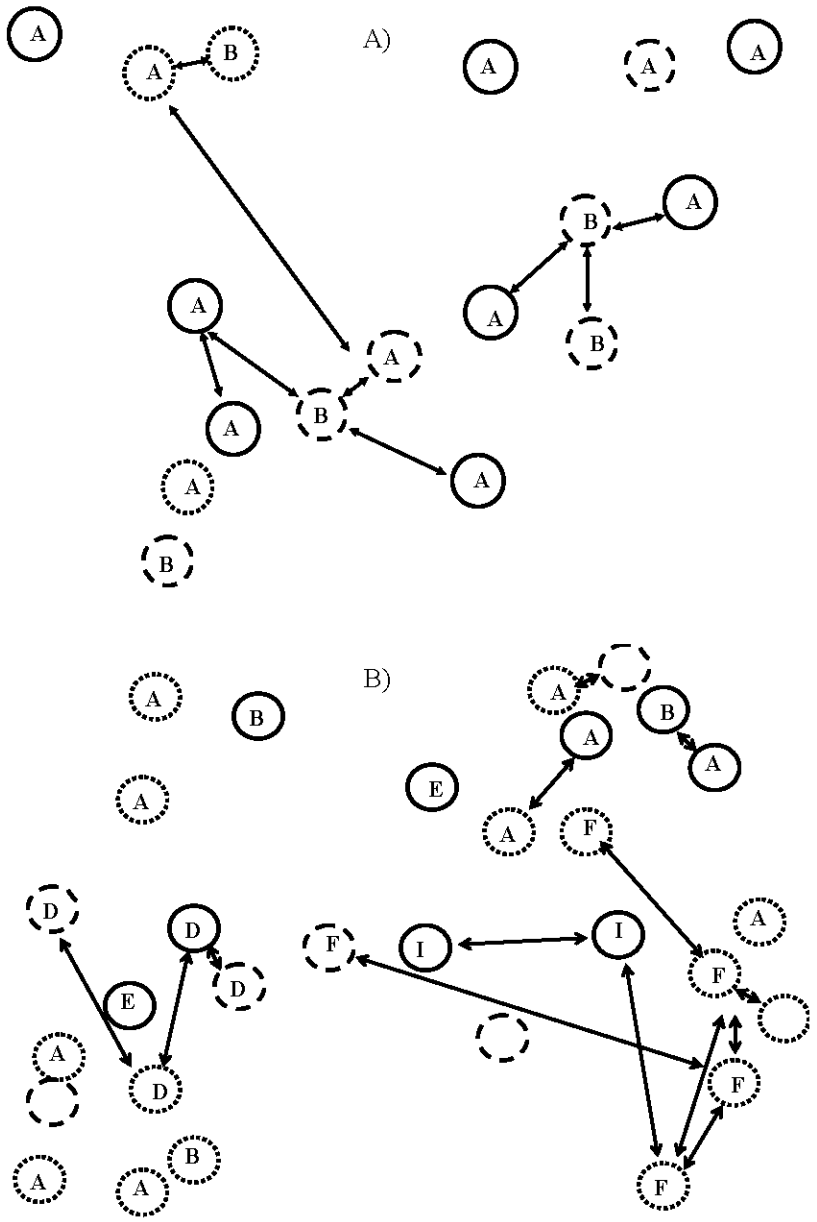
According to the timing of spring leaf flush, trees in sites M, W, and K were grouped into four, three, and three phenotypes, respectively (Table 1.4, Fig. 1.5). If bark and phenological characteristics were used together for clone identification, six, five, and three putative clones would have been distinguished. Groups defined by phenological features were not concordant with clones identified by microsatellite

markers. When comparing genetic identity and spring phenology, not all the ramets from microsatellite-defined clones produced leaves at the same time (Fig. 1.5). Seven out of 12 multi-ramet clones identified by molecular markers were heterogeneous in the timing of leaf flushing. Almost one fourth of ramets from these clones flushed at different times. Figure 1.5 show maps of sites W and K depicting leaf flush times and clones as defined by microsatellite markers. The picture also includes root maps (Jelinkova *et al.*, 2009) showing that some differentially flushing ramets had root connections to other ramets of the same or different clones.

Table 1.4 Number of clones identified by microsatellite markers and number of morphotypes defined by spring phenology and bark characteristics.

Sites	Nb. of clones	Nb. of bark phenotypes	Nb. of phenotypes according to spring phenology	Nb. of morphotypes (ramets differing in bark and/or spring phenology)
M	5	3	4	6
W	2	2	3	5
K	11	1	3	3
total	18	6	10	14

Figure 1.5 Scheme of sites K and W showing the leaf out times and clones defined by microsatellite markers . The microsatellite-defined clones are marked by letters, single ramet clones are marked by empty circles. Full line, dotted, and dashed circles mark the leaf out time: part A) full line – 15 May, dotted – 24 May, dashed – after 24 May, B) full line – before 19 May, dotted – 19 May, dashed - 23 May; Black arrows show root connections among ramets.



1.6 DISCUSSION

Trembling aspen is noted for marked variations in leaf shape. Differences in leaf morphology were reported between populations across North America (Barnes, 1966; Barnes, 1969; Gom and Rood, 1999). Here, we demonstrated that even at a very fine scale, among trees growing in close proximity, quite a remarkable variation in leaf shape could be observed (Figs. 1.2 and 1.3). Most variation was contained by the first component expressing the blade width to length ratio and distance from the insertion point to the blade maximum width (Table 1.1, Fig. 1.1). These measures were traditionally included in morphometrics (Barnes, 1966; Barnes, 1969; Barnes and Han, 1993; Suvanto and Latva-Karjanmaa, 2005) and were, thus, likely to capture a large proportion of the total variation. Other characteristics such as the shape of apex and base contained within the second component were traditionally assessed only categorically.

The automated quantitative leaf shape evaluation allowed us to make a quick assessment of 480 leaves from 60 trees in 3 sites. The cluster analysis grouped similar foliar types which in sites M and W corresponded to the clones defined by microsatellites (Fig. 1.4). Foliar morphology worked poorly in site K. We suppose that the lack of discriminatory power in this site was most likely caused by the juvenile character of the trees. This aspen stand was 23 years old and even though some trees produced catkins in the spring of the assessment, the trees were probably immature in some phenotypic traits. This was also supported by the fact the no difference in the bark type and color could be distinguished at this site (Table 1.4).

Even though the ramets from all the clones in the two mature sites were clustered correctly, in accordance with the microsatelite-defined clones, it was not possible to set a maximum value for distances within clones as a threshold for clone separation. For instance, according to the largest clone WA (Fig. 1.4B), this

maximum distance could be set to 0.6. However, under this precondition, clones MA and ME and MB, MC, MD would not be differenciated (Fig. 1.4A). On the other hand, setting this distance lower would divide the large clones WA and MA into a few other clones. This was a consequence of a varying range of leaf shape variability among clones and small distances separating the clones.

While less than 1% of leaf shape variation was located within ramets, over 12% of variation was observed among ramets at site W (Table 1.2). This indicated that there was either little within tree variation or it could be efficiently reduced by selection of leaves of the same age and from the same position in the crown. Small within plant component of leaf shape variation was also reported in other clonal species by Rumpunen and Bartish (2002) in the genus *Chaenomeles* (Rosaceae) and Persson and Gustavsson (2001) in lingonberry (*Vaccinium vitis-idaea L)*. Their observations indicated that leaf shape variation was little effected by environmental factors. Thus, little variation would be expected to be found among identical individuals. This should be particularly true when separating symmetrical and asymmetrical features of leaf shape variation as in this study since the asymmetrical component is more likely to be a consequence of environmental effects (Iwata *et al.*, 2002b). Nonetheless, despite the exclusion of asymmetrical features from our analysis, the result showed that the among ramet proportion of leaf shape variation was not negligible. To finally clarify the genetic x environmental relationship, direct field trials would be needed.

When using EFDs, one has to decide how many harmonics should be used. Employing too few could result in low precision of contour extraction while using too many may lead to introduction of random errors. Generally, from 10 to 40 harmonics were sufficient for most purposes of leaf shape analysis, however, up to 100 harmonics have also been used (McLellan and Endler, 1998; Mancuso, 1999; Persson and Gustavsson, 2001; Rumpunen and Bartish, 2002; Menesatti *et al.*, 2008). We

found 20 harmonics sufficient to suggest grouping of similar foliar types (Fig. 1.4). Nonetheless, a greater proportion of ramets was assigned correctly to microsatellite-defined clones when employing a higher number of harmonics (Table 1.3). By doing so, the clonal discriminatory power of nEFDs could be increased from 87 to 96 %. Similar misclassification rates were reached when using EFDs for plant species identification (Neto *et al.*, 2006). Discriminant analysis allowed for distinguishing 11 out of 12 multi-ramet clones (Table 1.3). Nonetheless, it is worth noticing that the discriminant function requires prior knowledge of well defined classes that are created with the help of reference samples. In this study, we applied the discriminant analysis to find out if there were features of leaf shape that can delineate the clones and not as a main method for clone identification.

Spring phenology turned out to be the least reliable trait to discriminate between clones. Many ramets in our sites that actually belonged to the same clone flushed at different times (Fig. 1.5). For instance, trees from the large clone A in site W leafed out on three different days. Some of differentially flushing ramets were obviously suppressed stems and their delayed leaf flush could be a sign of their approaching decline. Nonetheless, among the differentially flushing trees, there were even dominant trees with large diameters at breast height that showed no reduction of growth (growth ring measurements not shown). Rather, it was interesting to notice that some of differentially flushing trees were root grafted to trees from different clones (Jelinkova *et al.*, 2009). Even though little is known about the transport of substances through root grafts (Baret and DesRochers, 2011), it is tempting to speculate that even the leaf out time could be modulated by the transfer of hormones through these root connections. As natural root grafting was found to be frequent in aspen stands (DesRochers and Lieffers, 2001; Jelinkova *et al.*, 2009), we think that this phenomenon should be included among other environmental factors such as light exposure that make spring phenology an unsuitable trait for clone identification.

1.7 CONCLUSION

Foliar diversity analyzed by automated image processing has rarely been used as a tool for differentiation among genotypes of the same species (Persson and Gustavsson, 2001; Menesatti *et al.*, 2008). This is the first study in aspen that shows its quantitative measurement by normalized EFDs. In the two mature sites, the cluster analysis of leaf shape characteristics grouped together similar foliar types and reflected well clones defined by microsatellite markers. The reclassification test indicated that leaf shape contains features according to which very similar clones can be differentiated with low error rates. However, because it was not possible to set a threshold for maximum distances within clones, application of this approach of clone identification in natural aspen stands with a high number of multi-ramet clones and many singletons isdifficult.

1.8 ACKNOWLEDGEMENTS

We thank to everybody who has participated in this project namely M. Baret, E. Whitfield, and C. Pack. This work was supported by the Centre for Forest Research, National Science and Engineering Research Council, Industrial Chair NSERC-UQAT-UQAM in Sustainable Forest Management, Fonds de recherche sur la nature et les technologies, and the government of Quebec.

1.9 LITERATURE CITED

Baret M, DesRochers A (2011) Root connections can trigger physiological responses to defoliation in nondefoliated aspen suckers. Botany, 89: 753-761.

Barnes BV (1966) The clonal growth habit of American aspens. Ecology, 47: 439-447.

Barnes BV, Han FQ (1993) Phenotypic variation of chinese aspens and their relationships to similar taxa in Europe and North-America. Canadian Journal of Botany, 71: 799-815.

Barnes BV (1969) Natural variation and delineation of clonesof *Populus tremuloides* and *P. grandidentata* in northern lower Michigan. Silvae Genetica, 18: 130–142.

Bylesjo M, Segura V, Soolanayakanahally RY, Rae AM, Trygg J, Gustafsson P, Jansson S, Street NR (2008) LAMINA: a tool for rapid quantification of leaf size and shape parameters. BMC Plant Biology, 8: 82-91.

Cannon CH, Manos PS (2001) Combining and comparing morphometric shape descriptors with a molecular phylogeny: The case of fruit type evolution in *Bornean Lithocarpus* (*Fagaceae*). Systematic Biology, 50: 860-880.

Cope JS, Corney D, Clark JY, Remagnino P, Wilkin P (2012) Plant species identification using digital morphometrics: A review. Expert Systems With Applications, 39: 7562-7573.

Dayanandan S, Rajora OP, Bawa KS (1998) Isolation and characterization of microsatellites in trembling aspen (*Populus tremuloides*). Theoretical and Applied Genetics, 96: 950-956.

De Woody J, Rickman TH, Jones BE, Hipkins VD (2009) Allozyme and microsatellite data reveal small clone size and high genetic diversity in aspen in the southern Cascade Mountains. Forest Ecology and Management, 258: 687-696.

DesRochers A, Lieffers VJ (2001) The coarse-root system of mature *Populus tremuloides* in declining stands in Alberta, Canada. Journal of Vegetation Science, 12: 355-360.

Freeman H (1974) Computer processing of line drawing images. Computer Surveys, 6: 57-97.

Furuta N, Ninomiya S, Takahashi N, Ohmori H, Ukai Y (1995) Quantitative-evaluation of soybean (*Glycine-max* L MERR) leaflet shape by principal component scores based on elliptic Fourier descriptor. Breeding Science, 45: 315-320.

Gom LA, Rood SB (1999) The discrimination of cottonwood clones in a mature grove along the Oldman River in southern Alberta. Canadian Journal of Botany, 77: 1084-1094.

Iwata H, Nesumi H, Ninomiya S, Takano Y, Ukai Y (2002*a*) Diallel analysis of leaf shape variations of citrus varieties based on elliptic Fourier descriptors. Breeding Science, 52: 89-94.

Iwata H, Nesumi H, Ninomiya S, Takano Y, Ukai Y (2002*b*) The evaluation of genotype x environment interactions of citrus leaf morphology using image analysis and elliptic Fourier descriptors. Breeding Science, 52: 243-251.

Iwata H, Ukai Y (2002) SHAPE: A computer program package for quantitative evaluation of biological shapes based on elliptic Fourier descriptors. Journal of Heredity, 93: 384-385.

Jelínková H, Tremblay F, DesRochers A (2009) Molecular and dendrochronological analysis of natural root grafting in *Populus tremuloides* (*Salicaceae*). American Journal of Botany, 96: 1500-1505.

Jensen RJ, Ciofani KM, Miramontes LC (2002*a*) Lines, outlines, and landmarks: morphometric analyses of leaves of *Acer rubrum, Acer saccharinum* (*Aceraceae)* and their hybrid. Taxon, 51: 475-492.

Jensen RJ, Schwoyer M, Crawford DJ, Stuessy TF, Anderson GJ, Baeza CM, O MS, Ruiz E (2002*b*) Patterns of morphological and genetic variation among populations of *Myrceugenia fernandeziana* (*Myrtaceae*) on Masatierra island: Implications for conservation. Systematic Botany, 27: 534-547.

Kemperman J, Barnes B. (1976) Clone size in American aspens. Canadian Journal of Botany, 54: 2603 - 2607.

Kuhl FP, Giardina CR (1982) Elliptic Fourier features of a closed contour. Computer Graphics Image Processing, 18: 236-258.

Lexer C, Joseph J, van Loo M, Prenner G, Heinze B, Chase W, Kirkup D (2009) The use of digital image-based morphometrics to study the phenotypic mosaic in taxa with porous genomes. Taxon, 58: 349-364.

Liesebach H, Schneck V, Ewald E (2010) Clonal fingerprinting in the genus *Populus* L. by nuclear microsatellite loci regarding differences between sections, species and hybrids. Tree Genetics and Genomes, 6: 259-269.

Lopez-de-Heredia U, Sierra-de-Grado R, Cristobal MD Martinez-Zurimendi P, Pando V, Martin MT (2004) A comparison of isozyme and morphological markers to assess the within population variation in small populations of European aspen (*Populus tremula* L.) in Spain. Silvae Genetica, 53: 227-233.

Mancuso S (1999) Elliptic Fourier Analysis (EFA) and Artificial Neural Networks (ANNs) for the identification of grapevine (*Vitis vinifera* L.) genotypes. Vitis, 38: 73-77.

McLellan T, Endler JA (1998) The relative success of some methods for measuring and describing the shape of complex objects. Systematic Biology, 47: 264-281.

Menesatti P, Costa C, Paglia G, Pallottino F, D'Andrea S, Rimatori V, Aguzzi J (2008) Shape-based methodology for multivariate discrimination among Italian hazelnut cultivars. Biosystems Engineering, 101: 417-424.

Mock KE, Rowe CA, Hooten MB, Dewoody J, Hipkins VD (2008) Clonal dynamics in western North American aspen (*Populus tremuloides*). Molecular Ecology, 17: 4827-4844.

Namroud MC, Park A, Tremblay F, Bergeron Y (2005) Clonal and spatial genetic structures of aspen (*Populus tremuloides* Michx.). Molecular Ecology, 14: 2969-2980.

Neto JC, Meyer GE, Jones DD, Samal AK (2006) Plant species identification using Elliptic Fourier leaf shape analysis. Computers and Electronics in Agriculture, 50: 121-134.

Penaloza-Ramirez JM, Gonzalez-Rodriguez A, Mendoza-Cuenca L, Caron H, Kremer A, Oyama K (2010) Interspecific gene flow in a multispecies oak hybrid zone in the Sierra Tarahumara of Mexico. Annals of Botany, 105: 389-399.

Persson HA, Gustavsson BA (2001) The extent of clonality and genetic diversity in lingonberry (*Vaccinium vitis-idaea* L.) revealed by RAPDs and leaf-shape analysis. Molecular Ecology, 10: 1385-1397.

Rahman MH, Dayanandan S, Rajora OP (2000) Microsatellite DNA markers in *Populus tremuloides*. Genome, 43: 293-297.

Rumpunen K, Bartish IV (2002) Comparison of differentiation estimates based on morphometric and molecular data, exemplified by various leaf shape descriptors and RAPDs in the genus *Chaenomeles* (*Rosaceae*). Taxon, 51: 69-82.

Suvanto LI, Latva-Karjanmaa TB (2005) Clone identification and clonal structure of the European aspen (*Populus tremula*). Molecular Ecology, 14: 2851-2860.

Torres MAJ, Demayo CG, Siar SV (2008) Elliptic Fourier analysis of leaf outline differences between and among sixteen species of *Hoya*. Philippine Agricultural Scientist, 91: 18-28.

Viscosi V, Fortini P (2011) Leaf shape variation and differentiation in three sympatric white oak species revealed by elliptic Fourier analysis. Nordic Journal of Botany, 29: 632-640.

Wu RL (2000) Quantitative genetic variation of leaf size and shape in a mixed diploid and triploid population of *Populus*. Genetical Research, 75: 215-222.

Wu RL, Bradshaw HD, Stettler RF (1997) Molecular genetics of growth and development in *Populus (Salicaceae)* .5. Mapping quantitative trait loci affecting leaf variation. American Journal of Botany, 84: 143-153.

Wyman J, Bruneau A, Tremblay MF. (2003) Microsatellite analysis of genetic diversity in four populations of *Populus tremuloides* in Quebec. Canadian Journal of Botany, 81: 360-367.

Yoshioka Y, Iwata H, Ohsawa R, Ninomiya S (2004) Analysis of petal shape variation of *Primula sieboldii* by elliptic Fourier descriptors and principal component analysis. Annals of Botany, 94: 657-664.

CHAPTER II

MOLECULAR AND DENDROCHRONOLOGICAL ANALYSIS OF NATURAL ROOT GRAFTING IN TREMBLING ASPEN (SALICACEAE)

Article published in 2009 in Americal Journal of Botany, 96: 1500-1505

2.1 RÉSUMÉ

Le peuplier faux-tremble (*Populus tremuloides*) est une espèce d'arbre clonale qui se régénère surtout par drageonnement racinaire. En dépit d'une propagation végétative, les peuplements de tremble maintiennent de haut niveau de diversité clonale. Nous avons testé l'hypothèse suivant laqeulle le maintien de la diversité clonale pourrait être facilité par l'intégration de différents génotypes via des greffes du système racinaire. Pour vérifier cette hypothèse, les systèmes racinaires de trois stations de tremble ont été excavés et les clones ont été délimités à l'aide des marqueurs moléculaires. Nous avons constaté que les greffes racinaires étaient fréquentes indépendamment du génotype. De nombreuses racines d'arbres morts depuis plusieurs années étaient maintenues vivantes. Certaines d'entre elles n'avaient pas d'autre connexion racinaire que des greffes avec des ramets vivants de génotypes différents. Les systèmes racinaires ne comprenaient pas de génotypes uniques qui n'apparaissent pas chez les individus vivants. Néanmoins, l'intégration de racines des arbres morts aide à maintenir les systèmes racinaires extensifs, ce qui augmente les chances de survie des clones. L'interconnectivité inter- et intra-clonale résulte en formation d'unités physiologiques génétiquement diverses. Cette structure clonale pourrait avoir un impact considérable sur l'interprétation de divers processus écophysiologiques qui ont cours dans les peuplements de peuplier faux-tremble.

2.2 ABSTRACT

Trembling aspen (*Populus tremuloides*) is a clonal tree species which regenerates mostly through root suckering. In spite of vegetative propagation, aspen maintains high levels of clonal diversity. We hypothesized that the clonal diversity maintenance in this species can be facilitated by integration of different clones through natural root grafts into aspen's communal root system. In order to verify this hypothesis, we analyzed root systems of three pure aspen stands where clones had been delineated with the help of molecular markers. We found that grafting between roots was frequent regardless of their genotypes. Root system excavations revealed many living roots on trees that had been dead for several years. Some of them had no other root connections but grafts to living ramets of different clones. The uncovered root systems did not include unique genotypes that would not occur among stems. Nevertheless, acquiring roots of dead trees helps to maintain extensive root systems, which increases the chances of clone survival. Substantial interconnectivity both within clones as well as between clones due to inter-clonal grafts results in formation of large genetically diverse physiological units. Such a clonal structure can significantly affect interpretations of diverse ecophysiological processes taking place in forests of trembling aspen.

2.3 INTRODUCTION

A large number of tree species combines sexual and vegetative propagation depending on the variation of limiting biotic and abiotic factors in their distribution ranges (Eckert, 2002). In trembling aspen (*Populus tremuloides* Michx.), the balance between the two modes of reproduction is clearly shifted towards the vegetative propagation through root suckering following a disturbance (Barnes, 1966; Perala, 1990). Theory predicted that limited recruitment of seedlings in clonal species leads to a decline of their clonal diversity over time (Shapcott, 1995; Pornon *et al.,* 2000; Moriguchi *et al.*, 2001; Balloux *et al.,* 2003). Nevertheless, recent studies addressing the effect of vegetative propagation on population structure failed to find such a decline (Mayes *et al.*, 1998; Erickson and Hamrick, 2003; Pluess and Stocklin, 2004). Our findings from the southern boreal forest of eastern Canada indicated that aspen maintains a high clonal diversity along the successional gradient despite its vegetative propagation mode (Namroud *et al.*, 2005; Namroud *et al.,* 2006).

These results bring about a question of how the clonal diversity is maintained in species with extensive clonality over long periods of time. Montalvo *et al.* (1997) proposed that high diversity values could be explained by somatic mutation accumulation. Other authors asserted that high clonal diversity can be maintained by episodes of sexual reproduction no matter how rare they are (Jelinski and Cheliak, 1992; Persson and Gustavsson, 2001; Kjølner *et al.,* 2004). An additional mechanism that may contribute to the clonal diversity maintenance could possibly be sought in the structure and development of the root system.

Due to clonal growth, aspen's root system integrates roots of two types: old parental roots that produced suckers and new roots grown from suckers (DeByle, 1964; Strong and LaRoi, 1983; Desrochers and Lieffers, 2001). Part of the original parental root system continues to live even in mature aspen stands and connects

ramets that have regenerated from the same parental roots (Desrochers and Lieffers, 2001). However, the level of ramet integration into a communal root system may increase even further by natural root grafting (Barnes, 1966; Shepperd, 1993; Desrochers and Lieffers, 2001). Natural root grafting is a rarely-studied but frequent phenomenon that results in a morphological union of cambium, phloem, and xylem of two or more previously distinct roots (Graham and Bormann, 1966). Such a union may occur between roots of the same or different genotypes forming intra- or inter-clonal grafts. Nevertheless, clonal identity of roots involved in graft formation in trembling aspen has not yet been verified.

Moreover, grafts were observed not only between roots of two living trees but also between living trees and dead stumps or snags (Graham and Bormann, 1966; Eis, 1972; Stone, 1974; Desrochers and Lieffers, 2001; Fraser *et al.,* 2007). Survival of such living roots on dead stumps (LRDS) can exceed several decades and presumably depends on an influx of assimilates and other substances from the living stems to which they are grafted (Greenidge, 1955; Eis, 1972; Desrochers and Lieffers, 2001; Fraser *et al.,* 2006).

In the present study, we hypothesized that physiological integration of different clones through root grafts may allow these clones (genotypes) to remain alive after the death of the corresponding stems. Potentially, a disturbance removing the above-ground part of the stand could re-stimulate suckering in these root segments, thereby allowing these genotypes to persist after they would have normally been eliminated. From this perspective, aspen's communal root system could constitute a "genotype bank". In order to validate this hypothesis, three aspen stands were excavated and the uncovered root systems were examined for the presence of (i) root grafts, in particular the inter-clonal grafts, (ii) living roots on dead stumps and (iii) unique genotypes having no corresponding ramets among the living stems. Dendrochronological and molecular analyses, two different approaches rarely

combined in the past, were employed in this study to reconstruct the root system development with respect to grafting and to delineate the clones.

Findings of the present study may be interesting not only for the researchers investigating the processes of diversity maintenance in clonal plant species but also for a large community of scientists exploring diverse ecophysiological processes taking place in natural aspen stands. Many factors influencing these dynamic processes were identified under an assumption that stands were composed of discrete individuals or genetically uniform clones. Our results indicated that some of these conclusions may be confound by the physiological integration of different clones into a communal root system.

2.4 MATERIALS AND METHODS

2.4.1 Study Area

The three study sites (M, W, and K) were located in north-western Quebec, Canada, in the boreal forest at latitudes between 48°11' N and 48°30' N and longitudes between 78°45' W and 79°23' W. This area is part of the Northern Clay Belt characterized by post-Wisconsinian lacustrine deposits forming heavy clay soils. Climate of the region is cold and continental with an average annual temperature of 0.8°C, mean yearly precipitations of 857 mm, and a mean annual frost-free period of 64 days (Environment Canada, 1993).

Site parameters had to allow for root excavation, thus, the sites had to have a gentle slope and a nearby source of water (DesRochers and Lieffers, 2001). In order to excavate the root systems of the maximum number of ramets within minimum-size areas without intermingling of roots of other tree species, early-successional, even-aged, pure aspen stands were selected. Moreover, because incidence of root grafts is

expected to increase with stand age (Bormann and Graham, 1959; Stout, 1961; Basnet *et al.*, 1993) while aspen stem density decreases with age (Brassard *et al.*, 2008), 20 to 40-year-old stands were chosen. The three stands meeting these requirements regenerated from root suckers after clear cutting, had a minimum density of 0.5 trees per m^2, and lacked coniferous undergrowth. A plot of approximately 30m^2 was established in each of the three sites. Plots were placed in such a way that they encompassed the maximum number of clones that were identified with preliminary microsatellite analysis.

2.4.2 Excavation and Sampling

In August 2006, all stems within the plots were cut down and the root system was uncovered with a jet of water generated with a WAJAX™ forest fire pump. Depth of the mineral horizon into which plots were excavated varied between 30 and 60 cm with site M being the deepest one. This technique enabled fast removal of large volumes of soil so most of the root system could be exposed and accurately mapped.

Bark samples from all the stems, LRDS, and from both sides of grafts were taken and stored at -80°C until DNA extraction. To be able to age the grafts and distinguish original parental roots (all roots older than the corresponding ramet) and roots grown from suckers (roots of the same age as the corresponding ramet or younger), cross-sectional disks were taken both at breast height and ground level from all the stems and from every root that was at least 2 cm in diameter at its point of insertion into the stump.

Grafts were collected, dried and cut in order to verify whether the grafted roots were in a real physical union by sharing common growth rings. Moreover, by dating the growth of the first common ring, age of roots at the time when grafting was completed could be determined.

2.4.3 Dendrochronological Analysis

Grafted roots were cut into cross sections throughout the length of the grafts. Stem, root, and graft cross sections were then dried and sanded with a progressively finer grit paper starting with grade 150 and finishing with 500. To further improve visibility of growth rings, the disk surface was cut with a razor blade and the wood vessels were filled up with white chalk. Ring widths were measured with a Velmex micrometer (Velmex, Bloomfield, USA) with a precision of 1 µm. As root radial growth is often eccentric, ring widths were measured from bark to core alongside the longest radius.

To correct for the presence of missing or false rings, the disks were crossdated both graphically and statistically in the program TSAP-Win v0.55 (Frank Rinn, Heidelberg, Germany) employing a technique described by Swetnam *et al.* (1985) and Schweingruber (1989). Grafts were dated with the corresponding corrected root and stem chronologies.

2.4.4 Clone Identification

Cambial tissue was extracted from the bark samples and was used for DNA extraction using the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich Canada Ltd, Oakville, Canada) according to the manufacturer's protocol. 7 microsatellite loci PTR1, PTR2, PTR3, PTR4, PTR8, PTR5 and PTR6 (Dayanandan *et al.*, 1998; Rahman *et al.*, 2000) were amplified using dye-labelled oligonucleotide primers and Taq polymerase (Gibco, Invitrogen™ Life Technologies, Burlington, Canada). Polymerase Chain Reaction (PCR) was carried out in a 96-Well GeneAmp® PCR System 9700 (Applied Biosystems, California, USA) in a total volume of 10 µl containing: 4 µl of DNA extract, 0.625 pmol/µl of primers, 0.2 mM dNTP, 3.125 mM $MgCl_2$, 1.4 µl BSA, and 12.5 mM Tris-HCl (pH=8.0). Fragment amplification started with 10 min at 95 °C for enzyme activation and DNA strand denaturation, continued with 33 cycles of 1 min denaturation at 95 °C, 1 min

annealing at touch down temperatures decreasing by 1 °C every cycle from 60 °C to 54 °C, and 1 min primer extension at 72 °C, and terminated with 7 min final extension at 72 °C. 0.4 µl of PCR product was mixed with 0.25 µl of internal size standard (TAMRA 500 Liz) and 12 µl of deionized formamide and the mixture was heat-denaturated for 5 min. Fragments were separated by capillary electrophoresis in 3130 Genetic Analyzer (Applied Biosystems, California, USA). Allele calling was done in GeneMapper v3.7 (Applied Biosystems, California, USA).

In order to determine the resolution power of the set of microsatellites used, the round robin method was employed to estimate the probability (p_{gen}) that consecutively sampled trees that actually belong to different clones would, by chance, have a similar genotype (Parks and Werth, 1993):

$$p_{gen} = \prod_{i=1}^{l} (f_i g_i) 2^h$$

where l is the number of loci, h is the number of heterozygote loci, and f and g are the allelic frequencies of alleles f and g. Computation was done in program GenClone v1.1 (Arnaud-Haond and Belkhir, 2007). Moreover, to screen for possible somatic mutations, PCR artefacts, and scoring errors, the frequency distribution of genetic distances among multilocus genotypes (based on the number of different alleles among sampled trees) was computed in the same program.

2.5 RESULTS

2.5.1 Clone Identification

In total, 68 ramets were excavated in an area of 88m². Among these, the seven microsatellite markers identified 18 clones, with an average clonal size of 3.8 ramets

and a maximum of 13 ramets (Table 2.1). Thirty-three percent of clones had only one ramet. The resolution power of molecular markers was high. The probability of sampling two trees sharing the same genotype and being derived from a distinct sexual reproductive event, p_{gen}, was lower than 0.01, 0.001, and 0.003 in all the clones in sites W, K, and M, respectively. The frequency distribution of genetic distances did not indicate any somatic mutations or scoring errors. All clones identified in the three study sites differed by 6 or more alleles (data not shown).

Table 2.1 Site and clone characteristics.

Sites	M	W	K	All
Average age of ramets	42	39	23	35
No. of ramets	17	18	33	68
No. of ramets per m^2	0.52	0.55	1.5	0.77
No. of clones	5	2	11	18
No. of single-ramet clones	1	0	5	6
Mean no. of ramets per clone	3.4	9	3	3.78
Max. no. of ramets	9	13	11	13

2.5.2 Natural Root Grafting

Grafting was frequent averaging 0.54 grafts per tree and 0.42 grafts per excavated m^2 (Table 2.2). Number of grafts per tree as well as number of grafts per m^2 was significantly lower in site M than in site W ($\chi^2 = 9.05$, df = 1, $P < 0.01$; $\chi^2 = 9.8$, df = 1, $P < 0.01$). We found both intra- and inter-clonal grafts (Table 2.2). There was no significant difference in the number of these two graft types either when

considering both the grafted stems and LRDS together ($\chi^2 = 0.03$, df = 1, $P > 0.85$) or when counting grafts between stems and between stems and LRDS separately ($\chi^2 = 0.43$, df = 1, $P > 0.5$; $\chi^2 = 0.6$, df = 1, $P > 0.4$). More than one third of all grafts connected living stems to LRDS (Table 2.2). The number of grafts was negatively correlated with the distance between trees ($r^2=0.26$, $P=0.02$). Most grafts were formed in close proximity to stems (Table 2.2). Average age of roots at the time when the first common ring was created was 20 years but varied greatly from 2 to 41 years (Table 2.2).

2.5.3 Living Roots on Dead Stumps (LRDS)

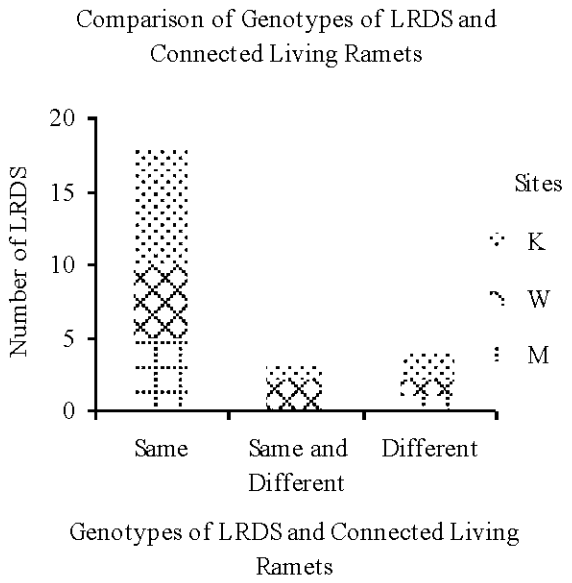
Excavation uncovered 25 LRDS belonging to 10 different clones. All the stumps, apart from one, had some root connections to at least one living bole. The only unconnected dead ramet whose roots were still alive died during the summer of excavations. Stumps of the majority of LRDS were located directly on living roots and were connected through the surviving parental roots to the living ramets of the same genotype (Figure 2.1). Almost half of them was also grafted to other roots or stems. Sixteen percent of LRDS was only integrated into the root systems of different clones. All genotypes that were present among the roots were also present among the stems.

Table 2.2 Summary of graft incidence, location, and dating of root age when grafting completed (the first common ring formation).

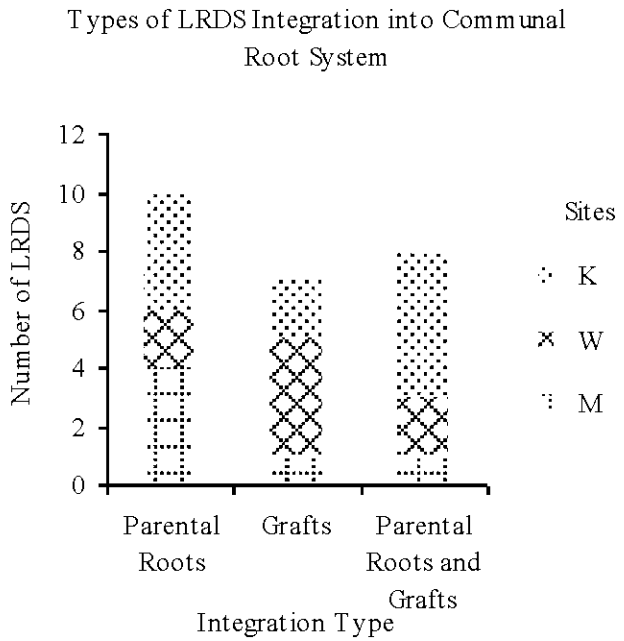
Sites	M	W	K	all
Number of grafts	3	17	17	37
Number of grafts per tree	0.176	0.944	0.515	0.544
Number of grafts per m²	0.091	0.515	0.773	0.420
Number of grafts between different clones	1	10	8	19
Number of grafts within clones	2	7	9	18
Number of grafts between living stems	2	9	10	21
Number of grafts between living stems and LRDS	1	7	7	15
Average age of roots when grafting completed	24.5	20.79	19.26	20.03
Max	25	32	41	41
Min	24	6	2	2
Average distance between grafted stems [cm]	18	60	33	43
Max [cm]	55	120	182	182
Average distance of grafts to stems [cm]	10	38	15	25
Max [cm]	27	120	182	182

Figure 2.1 Genotype A) and type of connections B) of living roots on dead stumps (LRDS) to corresponding living ramets.

A)



B)



2.5.4 Clonal Integration

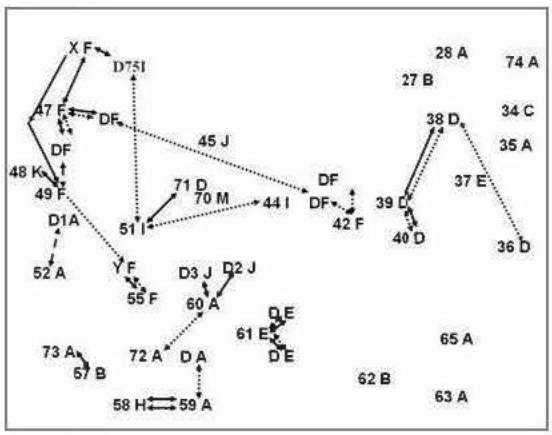
All trees had parental roots (roots older than the stem) and were, thus, of vegetative origin. Thirty-eight percent of ramets were not connected to at least another living ramet within excavated areas. Parental root connections between at least two living trees were preserved in 32% of stems. The remaining 30% of stems were attached to at least another living tree only through root grafts. Nevertheless, percentages of trees interconnected through parental roots and grafts varied greatly among sites (Table 2.3). Clonal integration was observed in all sites. Root system of the stands in sites W and K, where grafting was more frequent, is simplified in Figure 2.2. Two large networks (1 in site K and 1 in site W) connecting different clones, ramets, and LRDS through parental roots and grafts were found. The largest network integrated 10 living trees from 4 different clones (6, 2, 1, and 1 ramets) and 5 LRDS from 2 clones (1 and 4 stumps from each). The second largest root system integrated 7 living trees from 2 clones (2 and 5 ramets) and 4 LRDS from 2 clones (2 stumps from each).

Table 2.3 Integration type of aspen ramets into the communal root system.

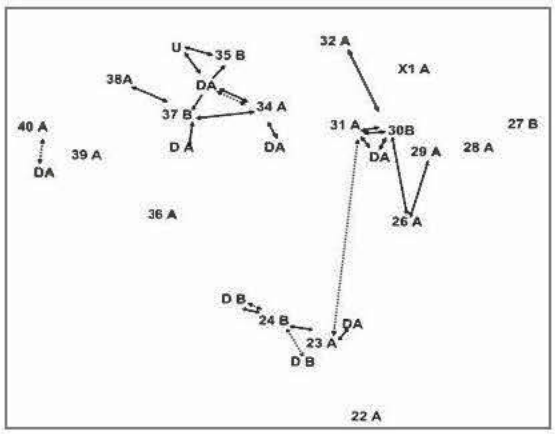
Sites	M	W	K	Aver age
Connected by parental roots only [%]	67	0	32	33
Connect. by parental roots and grafts [%]	0	9	36	19
Connected only through grafts [%]	33	91	32	48

Figure 2.2 Schemas and photographs of root systems and clonal integration in sites W and K. Living ramets are marked by numbers or letter X or Y followed by clone identification (letters A to J). Identification of living roots on dead stumps (LRDS) starts by letter D and/or a number followed by a letter indicating the clone to which they belong. Parental roots and grafts are depicted by dotted and full lines, respectively. Dashed lines show roots connecting two individuals that could not be dated because of broken or rotten cores.

Site K



Site W



2.6 DISCUSSION

This is the first study to show that ramets of different aspen clones can be integrated through root grafts into a communal root system. Even though finding grafts established between clones had been anticipated and formed the basis of our 'clone conservation' hypothesis, it was surprising to discover that, in fact, inter-clonal grafting in our sites was just as frequent as intra-clonal grafting. A greater degree of genetic similarity was predicted to lead to greater grafting as grafts within the same genotype (within a single tree) are far more common than between genotypes (Loehle and Jones, 1990). Moreover, grafting was reported to be especially frequent in species known for genetic uniformity (Stone, 1974). So how do we explain a high frequency of inter-clonal grafting in such a diverse species as trembling aspen?

Two factors may contribute to this observation: a high density and proximity of aspen ramets in young stands and aspen's clonal morphology. This study (Table 2.2) as well as reports of other authors investigating natural root grafting in trembling aspen and other tree species (Eis, 1972; Gordon and Roth, 1976; DesRochers and Lieffers, 2001; Tarroux and DesRochers, 2010) showed that tree proximity is an important factor influencing the probability of graft formation. Since grafting requires a physical contact of roots, a close tree proximity and high root density may lead to an increased grafting frequency. Moreover, aspen clones form rather loose clusters in which ramets from different clones are often spatially mixed (Namroud *et al.*, 2005). Therefore, a high root density and clones' intermingling may cause a more frequent contact between roots of different genotypes, and may, thus, result in a more frequent inter-clonal grafting in contrast to other tree species. For instance, roots of northern prickly ash cease growth as soon as they start approaching roots of different genotypes making inter-clonal grafting virtually impossible (Reinartz and Popp, 1987). Nonetheless, in trembling aspen, genetic similarity and between-individual

chemical differences seems to bear much less importance for the probability of grafting than previously suggested.

Conservation of root segments of different genotypes due to root grafts to living ramets presumes not only grafting between different clones but also its frequent occurrence. Indeed, inter-clonal grafting was frequent in two out of three sites examined in this study. The overall graft incidence ranged around 0.5 grafts per tree and was similar to observations made in aspen stands in Alberta (DesRochers and Lieffers, 2001). These findings contrast with older reports according to which natural root grafting rarely occurred in aspen (Barnes, 1966; Shepperd, 1993). Some grafts in these studies could have been overlooked as none of them employed either dendrochronological analysis or clone identification which helps differentiate grafts established close or directly on stumps from original parental root connections. Moreover, grafting frequencies in these studies might have been underestimated due to partial excavations. More than one third of all the grafts we found were located between living trees and LRDS (Table 2.2) and some of these would have gone undiscovered if they had been searched for only by partial excavation around living stems.

However, even among our sites there was some variation in graft incidence. In particular, in site M grafts were more scarce than in site W despite otherwise similar age and stem densities. This might be caused by other site characteristics known to influence graft formation such as rooting depth (Bormann and Graham, 1959; Eis, 1972). The deeper the soil, the smaller is the resulting root density which in turn gives roots fewer chances to cross and form grafts. Moreover, there might be other factors involved such as exposure of sites to wind that is believed to facilitate graft initiation due to root abrasions. Nevertheless, the causes of grafting are still poorly understood and their investigation was far beyond the scope of this study.

The dendrochronological reconstruction showed that graft formation started early after stand initiation and even trees in the youngest 23-year-old stand were commonly interconnected through root grafts (Table 2.2). The minimum root age of 2 and 6 years at the time of the first common growth ring formation indicated that the process of grafting may not take very long to be completed. The large variation in the timing of graft completion pointed to the fact that grafting was not restricted to a particular period but rather continued throughout the life of stands. Hence, there should be more grafts in old than young stands, yet a comparison of our young and middle-aged sites with old declining aspen stands failed to demonstrate such a difference (DesRochers and Lieffers, 2001). An increased graft incidence in old sands may be buffered by decreasing stem density and loss of some grafts during the stand aging. If this is true, graft loss in old stands may pose a constraint to the potential contribution of grafting to clonal diversity maintenance in this species.

The root system excavation did not uncover any 'hidden' genotypes that would not be present in the above-ground part of the stands. Most LRDS were connected by parental roots to living ramets of the same genotype (Figure 2.1) and were, therefore, likely to be a result of self-thinning, a process of density-dependent mortality in young stands (Johnstone *et al.*, 2004). Nevertheless, some LRDS had no parental connections and continued to live only due to inter-clonal grafts (Figure 2.1). Provided these stumps stay alive till the following disturbance, root suckers of these clones could appear even in places where there were no living stems of the corresponding genotypes before the disturbance.

Our concept of clone conservation in a form of LRDS largely depends on the span of time over which LRDS can stay alive. The longer their lifespan is, the better the chances are for the clone survival. Roots on dead stumps of Douglas-fir (*Pseudotsuga menziesii*) were still alive 32 years after selective cuttings (Eis, 1972). Graham and Bormann (1966) reported in their review continued growth of LRDS in

more than 50 tree species. The oldest one persisted for as long as 84 years. The timing of the death of LRDS in this study could not be determined because of the completely or partially rotten cores typically leaving only a hole in the place of a stem. Nevertheless, it is tempting to speculate that they can stay alive for many years provided the supply of carbohydrates from the living tree remains uninterrupted.

Clone survival would also be favoured by a large size of the communal root system in which roots of different clones could be 'captured'. Integration of trees into a communal root system in our young and middle-aged stands as well as in declining aspen stands in Alberta was high leaving only approximately one third of individuals unconnected (DesRochers and Lieffers, 2001). The percentage of independent trees is likely to be overestimated, as the excavated plots in this and other studies were relatively small and some trees could have been connected to trees outside the plots. This was also likely to be the reason for the substantial variation among the sites in the proportion of trees linked by parental roots (Table 2.3). In particular, site W had an obvious lack of parental root connections although the parental roots were preserved in all the trees of this site and their proportion to new roots was comparable with the other stands (data not shown). Therefore, we assume that this was an artefact caused by a coincidence of the shape of the excavated area and the shape of clones, which tend to be rather irregular due to a 'guerrilla' type of aspen's growth (Namroud *et al.*, 2005).

In the present study, we have not attempted to exhaustively explain the complex phenomenon of diversity maintenance in clonal species; we have rather tried to suggest an additional mechanism that could be involved in diversity maintenance in trembling aspen. We have not found an unassailable evidence to support our 'genotype bank' hypothesis. Nevertheless, this study showed that grafting in aspen can be extensive regardless of the root genotype and that there is a substantial interconnectivity among aspen trees both through surviving parental roots and root

grafts. From this perspective, aspen stands should be seen as large genetically diverse physiological units rather than discrete individuals or genetically uniform clones. Even though the root systems that we uncovered did not include roots of unique genotypes, acquiring roots of dead trees either through parental roots or root grafts helps to maintain extensive root systems, which is crucial for clone survival (Pelton, 1953; Tappeiner *et al.*, 1991). Finally, excavation of larger plots in late-successional stands with only a few aspen trees per hectare left would probably have better chances of discovering unique genotypes in order to finally validate or reject our hypothesis.

2.7 ACKNOWLEDGEMENTS

The authors thank to M. Baret, E. Whitfield, L. Blackburn, Ch. Pack, K. Naydenov, and C. Krause for their help and advice and to anonymous reviewers for critical reading of the manuscript. This study was supported by the Centre for Forest Research, National Science and Engineering Research Council, Industrial Chair in Sustainable Forest Management, Fonds de recherche sur la nature et les technologies, and the government of Quebec.

2.8 LITERATURE CITED

Arnaud-Haond S, Belkhir K (2007) GENCLONE: a computer program to analyse genotypic data, test for clonality and describe spatial clonal organization. Molecular Ecology Notes, 7: 15-17.

Balloux F, Lehmann L, De Meeus T (2003) The population genetics of clonal and partially clonal diploids. Genetics, 164: 1635-1644.

Barnes BV (1966) The clonal growth habit of American aspens. Ecology, 47: 439-447.

Basnet K, Scatena FN, Likens GE, Lugo AE (1993) Ecological consequences of root grafting in Tabonuco (*Dacryodes excelsa*) trees in the Luquillo experimental forest, Puerto Rico. Biotropica, 25: 28-35.

Bormann FH, Grahman FJ (1959) The occurrence of natural root grafting in eastern white pine, *Pinus strobes* L., and its ecological implications. Ecology, 40: 677-691.

Brassard BW, CHen HYH, Wang JR, Duinker PN (2008) Effects of time since stand-replacing fire and overstory composition on live-tree structural diversity in the boreal forest of central Canada. Canadian Journal of Forest Research, 38: 52-62.

Dayanandan S, Rajorar OP, Bawa KS (1998) Isolation and characterization of microsatellites in trembling aspen (*Populus tremuloides*). Theoretical and Applied Genetics, 96: 950-956.

Debyle NV (1964) Detection of functional intraclonal root connections by traces and excavation. Forest Science, 10: 386-396.

Desrochers A, LieffersVJ (2001) The coarse-root system of mature *Populus tremuloides* in declining stands in Alberta. Journal of Vegetation Science, 12: 355-360.

Eckert CG (2002) The loss of sex in clonal plants. Evolutionary Ecology, 15: 501–520.

Eis S (1972) Root grafts and their silvicultural implications. Canadian Journal of Forest Research, 2: 111-120.

Environment Canada (1993) Climate Normals in Canada. Environment Canada, Service of Atmospheric Environment, Ottawa, Ontario.

Erickson DL, Hamrick JL (2003) Genetic and clonal diversity for *Myrica cerifera* along a spatiotemporal island chronosequence. Heredity, 90: 25-32.

Fraser EC, Lieffers VJ, Landhausser SM (2006) Carbohydrate transfer through root grafts to support shaded trees. Tree Physiology, 26: 1019-1023.

Fraser EC, Lieffers VJ, Landhausser SM (2007) The persistence and function of living roots on lodgepole pine snags and stumps grafted to living trees. Annual Forest Science, 64: 31–36.

Gordon DE, Roth LF (1976) Root grafting of Port-Orford-cedar- An infection route for root rot. Forest Science, 22: 276-278.
Graham BF, Bormann FH (1966) Natural root grafts. Botanical Reviews, 32: 255-292.

Greenidge KNH (1955) Studies in the physiology of forest trees. III. The effect of drastic interruption of conducting tissues on moisture movement. American Journal of Botany, 42: 582-587.

Jelinski DE, Cheliak WM (1992) Genetic diversity and spatial subdividion of *Populus tremuloides* (*Salicaceae*) in a heterogeneous landscape. American Journal of Botany, 79: 728-736.

Johnstone JF, Chapin FS, Foote J, Kemmett S, Price K, Viereck L (2004) Decadal observations of tree regeneration following fire in boreal forests. Candian Journal of Forest Research, 34: 267-273.

Kjølner S, Săstad SM, Tabertetand P, Brochmann C (2004) Amplified fragment length polymorphism versus random amplified polymorphic DNA markers: Clonal diversity in *Saxifraga cernua*. Molecular Ecology, 13: 81-86.

Loehle C, Jones RH (1990) Adaptative significance of root grafting in trees. Functional Ecology, 4: 268-271.

Mayes SG, Mcginley MA, Werth CR (1998) Clonal population structure and genetic variation in sand-shinnery oak, *Quercus havardii* (*Fagaceae*). American Journal of Botany, 85: 1609-1617.

Montalvo A, Conard S, Conkle M, Hodgskiss P (1997) Population structure, genetic diversity, and clone formation in *Quercus chrysolepis* (*Fagaceae*). American Journal of Botany, 84: 1553–1564.

Moriguchi Y, Matsumoto A, Saitom, Tsumura Y, Taira H (2001) DNA analysis of clonal structure of an old growth, isolated forest of *Cryptomeria japonica* in a snowy region. Canadian Journal of Forest Research, 31: 377-383.

Namroud MC, Park A, Tremblay F, Bergeron Y (2005) Clonal and spatial genetic structures of aspen (*Populus tremuloides* Michx.). Molecular Ecology, 14: 2969-2980.

Namroud MC, Leduc A, Tremblay F, Bergeron Y (2006) Simulations of clonal species genotypic diversity – trembling aspen (*Populus tremuloides*) as a case study. Conservation Genetics, 7: 415-426.

Parks J, Werth C (1993) A study of spatial features of clones in a population of bracken fern, *Pteridium aquilinum* (Dennstaedtiaceae). American Journal of Botany, 80: 537-544.

Pelton J (1953) Studies on the life-history of *Symphoricarpos occidentalis* Hook. in Minnesota. Ecological Monographs, 23: 17-39.

Perala DA (1990) *Populus tremuloides* Michx. Quaking aspen. Silvics of North America (eds R.M. Burns and B. H. Honkala), pp. 555-569, US Department of Agriculture Forest Service, Washington, DC.

Persson HA, Gustavsson BA (2001) The extent of clonality and genetic diversity in lingonberry (*Vaccinium vitisidaea* L.) revealed by RAPDs and leaf-shape analysis. Molecular Ecology, 10: 1385-1397.

Pluess AR, Stocklin J (2004) Population genetic diversity of the clonal plant *Geum reptans* (*Rosaceae)* in the Swiss Alps. American Journal of Botany, 91: 2013-2021.

Pornon A, Escaravage N, Thomas O, Taberlet P (2000) Dynamics of genotypic structure in clonal *Rhododendron ferrugineum* (*Ericaceae*) populations. Molecular Ecology, 9: 1099-1111.

Rahman MH, Dayanandan S, Rajora PO (2000) Microsatellite DNA marker in *Populus tremuloides*. Genome, 43: 293-297.

Reinartz JA, Popp JW (1987) Structure of clones of northern prickly ash (*Xanthoxylum americanum*). American Journal of Botany, 74: 415-428.

Schweingruber FH (1989) Tree rings: Basics and applications of dendrochronology. Kluwer Academic Publishers, Dordrecht.

Shapcott A (1995) The spatial genetic structure in natural populations of the Australian temperate rainforest tree *Atherosperma moshatum* (Labill.) (*Monimiaceae)*. Heredity, 74: 28-38.

Shepperd WD (1993) Initial growth, development, and clonal dynamics of regenerated aspen in the Rocky Mountains. USDA Forest Service, Research Paper RM-312. Rocky Mountain Forest and Range Experiment Station, Fort Collins, CO.

Stone EL (1974) The communal root system of red pine: growth of girdled trees. Forest Science, 20: 294-305.

Stout BB (1961) Season influences the amount of backflash in a red pine plantation. Journal of Forestry, 59: 897-898.

Strong WL, Laroi GH (1983) Root-system morphology of common boreal forest trees in Alberta, Canada. Canadian Journal of Forest Research, 13: 1164-1173.

Swetnam TW, Thompson MA, Sutherland EK (1985) Using dendrochronology to measure radial growth of defoliated trees. USDA Forest Service, Agriculture Handbook, 639: 1-39.

Tappeiner J, Zasada J, Ryan P, Newton M (1991) Salmonberry clonal and population structure: the basis for a persistent cover. Ecology, 72: 609-618.

Tarroux E, DesRochers A (2010) Frequency of root grafting in naturally and artificially regenerated stands of *Pinus banksiana*: influence of site characteristics. Canadian Journal of Forest Research 40:861–871.

CHAPTER III

HERBIVORE-SIMULATED INDUCTION OF DEFENSES IN CLONAL NETWORKS OF TREMBLING ASPEN (*POPULUS TREMULOIDES*)

Article published in 2012 in Tree Physiology 32: 1348-1356.

3.1. RÉSUMÉ

Le peuplier faux-tremble (*Populus tremuloides*) est une espèce d'arbre clonale qui possède un système racinaire complexe par lequel les arbres d'un même clone ou de clones différents sont reliés. Les connections racinaires ont été étudiées en relation avec le partage de ressources, mais la nature, les quantités et l'ampleur de ce qui est partagé entre les arbres sont relativement peu connus. Dans cette étude, nous avons posé l'hypothèse suivant laquelle l'induction de défense systémique pourrait se propager à travers les liens racinaires et déclencher les défenses dans les ramets voisins avant l'arrivée des parasites. Le modèle d'expression temporelle des inhibiteurs de la trypsine de type Kunitz (*KTI*) et les gènes de dihydroflavonol réductase (*DFR*), deux indicateurs de défense chez les peupliers, a été suivi èa l'aide d'une PCR quantitative en temps réel. L'expression a été quantifiée dans les feuilles systémiques de plantes blessées et non blessées qui partageaient la même racine parentale et chez les échantillons plants témoins, cultivés dans des pots séparés. Les plantes communicantes non traitées n'ont pas présenté de résistance induite contre une attaque simulée d'herbivore. Bien que les gènes défensifs aient été induits dans les plants blessés, les plantes communicantes non traitées ont produit un modèle d'expression similaire aux échantillons témoins non-communicants. Les connexions entre racines ne mènent pas automatiquement à l'induction des mécanismes de défense qui sont exprimés dans les plantes directement exposées aux dommages simulant une attaque d,insectes phytophages. Il semble plutôt que d'autres moyens de communication, tel que l'émission de substances volatiles, puissent servir de voies de transmission des signaux entre plantes voisines.

3.2 ABSTRACT

Trembling aspen (*Populus tremuloides*) as a clonal tree species possesses a complex root system through which trees of the same or different clones are connected. Root connections have been studied with respect to resource sharing, but the nature, quantities or extent of what is shared between trees is relatively unknown. In this study we posed the hypothesis that systemic defense induction signals could also spread through these root networks and trigger defenses in neighboring ramets before arrival of pests. Temporal expression pattern of Kunitz trypsin inhibitors (*KTI*s) and dihydroflavonol reductase (*DFR*) genes, two markers of poplar defense, were followed by quantitative real-time PCR. The expression was quantified in systemic leaves of wounded and healthy plants that shared the same parental root and in untreated controls grown in separate pots. Untreated interconnected plants did not show induced resistance upon herbivore simulated attack. Although wound-treated ramets induced defense genes, untreated interconnected plants produced expression pattern similar to non-connected controls. Root connections do not automatically lead to induction of defensive traits that are expressed in plants directly under damage thought to simulate herbivory. Rather, it seems that other communication means such as air borne volatiles can serve as signal transmission pathways among neighbouring plants.

3.3 INTRODUCTION

Physiological integration through physical root connections in plants has been thoroughly studied in various plant species for the last three decades. Most studies dealt with the mechanisms of carbohydrate, water, and mineral nutrients sharing (Stone, 1974; Kozlowski, 1992; Stuefer *et al.*, 1994; Alpert, 1996; Stuefer *et al.*, 1998; Magori *et al.*, 2003; Nilsson and D'Hertefeldt, 2008; Liu *et al.*, 2009; Du *et al.*, 2010; Mony *et al.*, 2011). Other authors investigated the physiological integration in terms of exploitation of patchy resources (Evans and Cain, 1995; Shumway, 1995; Hutchings and Wijesinghe, 1997; Ikegami *et al.*, 2008), resistance to environmental stresses (Pennings and Callaway, 2000; Yu *et al.*, 2008), or improvement of competitive ability (Oborny *et al.*, 2000; Peltzer, 2002; Yu *et al.*, 2009). Much less attention has been paid to circulation of non-resource agents such as pathogens (Bruhn *et al.*, 1991; He *et al.*, 2000; Koubek and Herben, 2008) and hormones (Alpert *et al.*, 2002). In 2004, Stuefer and Gomez proposed that clonal plant networks could represent highways for long-distance transport of systemic defense induction signals. Sharing of these signals among network members could allow an early response to impending herbivore attacks by upregulation of defense traits. To date, however, such a network-specific early-warning system has only been studied in a single species of stoloniferous herb *Trifolium repens* L. (Gomez and Stuefer, 2006; Gomez *et al.*, 2007; Gomez *et al.*, 2008; Gomez *et al.*, 2010).

Clonal trees are extremely long-lived and successful life-forms that possess efficient defense mechanisms (Maleck and Dietrich, 1999; Bruxelles and Roberts, 2001; Philippe and Bohlmann, 2007; Chen *et al.*, 2009; Duplessis *et al.*, 2009; Ralph, 2009). Physical barriers and constitutively produced phytochemicals make up the first line of defense. However, when these barriers are breached, inducible defenses take over reducing herbivory (Philippe and Bohlmann, 2007). Some inducible biochemicals directly inhibit insect growth and development, while others serve as

airborne signals that reduce herbivory indirectly by deterring herbivores or attracting their predators or parasites (Havill and Raffa, 2000). In the genus *Populus*, defense mechanisms against herbivory are well-understood and many genes encoding enzymes involved in induced direct or indirect defenses and their corresponding spatial and temporal expression profiles are known (Haruta *et al.*, 2001a; Haruta *et al.*, 2001b; Peters and Constabel, 2002; Arimura *et al.*, 2004; Christopher *et al.*, 2004; Wang and Constabel, 2004; Lawrence *et al.*, 2006; Ralph *et al.*, 2006; Tsai *et al.*, 2006; Major and Constabel, 2008; Philippe *et al.*, 2009; Philippe *et al.*, 2010).

Amongst the 1100 genes upregulated upon herbivory feeding, the Kunitz protease inhibitors (KPIs) feature as a prominent marker of poplar defense response. *KPIs* belong to a large and rapidly evolving gene family whose members are differentially expressed throughout the plant (Haruta *et al.*, 2001a; Christopher *et al.*, 2004; Lawrence *et al.*, 2006; Major and Constabel, 2006; Ralph *et al.*, 2006; Miranda *et al.*, 2007; Philippe *et al.*, 2009). They show diverse biochemical properties and functional specialization against various types of proteases in insect gut where they intervene with normal digestion leading to loss of essential amino acids (Major and Constabel, 2008). Among them, mRNA encoding for Kunitz trypsin inhibitors (KTIs) were found to be one of 10 most abundant expressed sequence tags (ESTs) in leaf transcriptome following wounding simulated herbivory suggesting their important role in poplar defense (Christopher *et al.*, 2004) .

Besides protein-based anti-herbivore defense, poplars produce substantial amounts of phytochemicals functioning as anti-nutrients or feeding deterrents (Philippe and Bohlmann, 2007). Salicin-based phenolic glycosides, hydroxycinnamate derivates and flavonoid-derived condensed tannins represent an array of complex compounds that can comprise up to one-third of *Populus* leaf dry mass (Tsai *et al.*, 2006). The last-mentioned condensed tannins are produced by the flavonoid biosynthetic pathway. Dihydroflavonol reductase (DFR), a key enzyme of

this pathway, is encoded by a single-copy gene in the genome of trembling aspen (Peters and Constabel, 2002). Both its expression and enzyme activity was shown to be dramatically induced 24 hours after herbivory in local and as well as systemic leaves (Peters and Constabel, 2002). Levels of condensed tannins correlated with negative impacts on the performance of gypsy moth larvae and forest tent caterpillars (Hemming and Lindroth, 1995; Hwang and Lindroth, 1997; Osier *et al.*, 2000; Osier and Lindroth, 2001).

Herbivore feeding initiates production of defense induction signals at the site of the damage. These systemic signals travel together with assimilate movement through the vascular architecture of phloem (Davis *et al.*, 1991; Jones *et al.*, 1993; Metraux *et al.*, 2002). Even though the nature of these signals is not yet well-known, their movement seems to be affected by the source-sink gradient and constrained by vascular connectivity (Arnold and Schultz, 2002; Arnold *et al.*, 2004; Babst *et al.*, 2005). Hence, maximum systemic upregulation was observed in leaves directly connected to the damaged leaves. However, Major and Constabel (2007) clearly demonstrated that some insect herbivory defense genes are upregulated even in roots of plants with methyl jasmonate treated or wounded leaves. Their findings imply that systemic signals are transmitted not only upward from damaged to the top systemic leaves but also downward, basipetally, from shoots to roots.

In this work, we wanted to verify if systemic defense induction signals can spread through the clonal root system of trembling aspen (*Populus tremuloides*) and trigger expression of defense traits in undamaged neighboring ramets. The aspen clonal root system is made up not only by new roots grown from suckers but also original parental roots that gave rise to the new sucker generation. These original parental roots can be found even in mature aspen stands where they keep connecting stems (ramets) that have regenerated from the same parental roots (DeByle, 1964; Strong and LaRoi, 1983; DesRochers and Lieffers, 2001; Jelinkova *et al.*, 2009).

Moreover, besides connecting genetically identical ramets, aspen root systems also integrate stems of different clones (Jelinkova *et al.*, 2009). These inter-clonal connections are enabled by the existence of natural root grafts, which result in morphological union of cambium, phloem, and xylem of previously distinct roots (Graham and Bormann, 1966). Trembling aspen creates large physiological networks rather than stands of discrete individuals or clones.

We followed the temporal expression pattern of Kunitz trypsin inhibitor (*KTI*) and dihydroflavonol reductase (*DFR*) genes using quantitative real-time PCR. The expression was quantified in systemic leaves of wounded and healthy plants sharing the same roots and in untreated controls. To our knowledge, this is the first study attempting to investigate clonal networks in respect to information sharing among network members in long-living tree species.

3.4 MATERIALS AND METHODS

3.4.1 Plant material

Different genotypes of *Populus tremuloides* Michx. originating from the mixed boreal forest of northwestern Quebec, Canada, were collected as root cuttings in autumn 2007 and stored at 4°C in moist peat moss. In spring 2008, 60-cm-long root segments were transferred into large pots filled with PRO-MIX® BX potting substrate (75 -85% sphagnum peat moss, perlite, vermiculite, limestone, and micronutrients). Planted root cuttings were kept in a greenhouse under the conditions promoting root suckering (16/8-h photoperiod, 28 °C, and 60% humidity). Suckers used for the experiment were from 12 to 15 weeks old and approximately 1.5 m tall and had only one main stem bearing around 30 leaves.

3.4.2 Stress treatment simulating insect herbivory

Aspen suckers growing on the same root (stem bases not farther than 30 cm from each other) were selected for the experiment. The sucker growing at the root's proximal end underwent a stress treatment and the one at the distal part was left untreated. A third plant in a separate pot was designated as a control. The stressed, untreated, and control plants were placed so they would be at the same distance from each other. The experiment was done in biological replicates I – III (three separate experiments comprised of a stressed, untreated, and control plant) using plant material of the same genotype. This genotype had been selected in a preliminary experiment as the one exhibiting the strongest systemic response to the stress treatment.

Leaves of all plants were numbered basipetally according to the leaf plastochron index (LPI) with the first unfolded leaf being designated as leaf 1 (Constabel *et al.*, 2000). Leaves at LPI 8 – 25 of the stressed plants were wounded by crunching the blade margins with pliers 3 times at one hour intervals (Major and Constabel, 2006). Leaves at LPI 7 – 3 of stressed, untreated, and control plants were collected across 5 time points. Collection started with the leaves at LPI 7 before the wound treatment (time 0) and continued after 24, 36, 72, and 94 hours from the beginning of the treatment. In this way, at every time point, LPI of the controls corresponded to LPI of stressed and untreated plants. Sampled leaf tissue was immediately frozen in liquid nitrogen and then stored at -70 °C until RNA extraction.

3.4.3 Selection of target genes and normalization standards

Gene expression was followed in 2 target genes: dihydroflavonol reductase gene (*DFR*; GenBank AY147903) and Kunitz trypsin inhibitor III gene (*KTI*; GenBank AF349441-AF349443). These genes were selected based on the following criteria: 1) a maximum increase in the systemic (in untreated leaves of stressed plants) expression after wounding, 2) known direct roles in anti-herbivore defense, 3)

a selection representing different types of antiherbivore defense (phytochemical and proteolytic), and 4) sequences of *P. tremuloides* available in public databases. Two house-keeping genes, actin (*ACT*) and translation initiation factor 5A (*TIF*), were chosen as endogenous standards for transcript abundance normalization (Brunner *et al.*, 2004). These genes were shown not to be significantly up or down regulated by herbivory feeding (Ralph *et al.*, 2006) and hence represented suitable candidates for robust normalization.

3.4.4 Primer and TaqMan probe design

Gene-specific primers (Table 3.1) were designed in FastPCR v.6.0 software (Kalendar, 2008) using a stringent set of criteria. Primer specificity was verified *in silico* by megablast against the nucleotide database at NCBI, by gel electrophoresis in Agilent Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany), and by sequencing of PCR amplicons.

Dual-labeled TaqMan probes with Black Hole Quencher dyes™ (Biosearch Technologies, Novato, USA; Table 3.1) were designed to meet the following parameters: melting temperature range 68 – 70, guanine-cytosine content range 30-80%, melting temperature range at 3'end 34-48, and a maximum length of 30 bp. Runs of more than 3 Gs and Cs and no more than 2 Gs or Cs in a row within the last 5 nucleotides at 3'end were avoided whenever possible. All probes were designed in forward orientation and so that annealing would take place close to the forward primer. Specificity verification was done by nucleotide BLAST against the NCBI database.

Table 3.1 Gene-specific primers and probes used for the real-time amplification of Kunitz trypsin inhibitors (*KTI*s) and dihydroflavonol reductase (*DFR*) genes, two markers of poplar defense, and actin (*ACT*) and translation initiation factor 5A (*TIF*) used as endogenous standards for transcript abundance normalization.

Gene	Primers 5'-3'	Probe 5'-3'	Amplicon size (bp)	Amplicon Tm (°C)	PCR efficiency (%)
ACT	tcagcaccttccagcagatg tcaccgcactgttcggaacttag	cgacgagtctggcccatccatcgtccacag	102	77.0	101.1
TIF	tgcaaggttgttgaggtttccac ggaacatcacagttgtgagatgagg	acaggcaagcacggacatgctaagtgccac	134	73.5	99.7
KTI	accatgtggaagattgaattgaggc aagctgatactgattggtaccacc	agcgcgaggattcgttgtgaccacaggag	123	74.5	97.8
DFR	cggatcatggctaattatgcgactac tgatgcctttggcagttccag	aggttatgctgtccgagccaccgtccga	115	75.0	108.1

3.4.5 Quantitative real-time PCR assay

Frozen leaf tissue was ground in liquid nitrogen and total RNA was isolated using Qiagen RNeasy Plant MiniKit according to the manufacturer's instructions. Real-time PCR was done in two steps. The total RNA was first reverse transcribed to cDNA with AffinityScript™ QPCR cDNA Synthesis Kit (Stratagene, La Jolla, USA) and 1µg of the total RNA. Quantitative PCR was done in a total reaction volume of 25µl containing 10x PCR buffer, 3.5mM of magnesium chloride, 300nM of each primer, 300nM of probe, 0.8mM dNTP, 30nM of reference ROX dye, 1.25 U of SureStart *Taq* DNA polymerase and 1µl of cDNA template. A two-step amplification protocol started with 10 minutes of denaturation at 95°C and was followed by 40 cycles of 15-second denaturation at 95°C and 1-minute annealing and synthesis at 60 °C. Amplification and fluorescence data collection was done in optical 96-well plates

on Mx3005P® System (Stratagene, La Jolla, USA). All PCR reactions were run in three technical replicates.

3.4.6 Data analysis and statistics

Raw fluorescence data (R) was normalized to ROX reference dye (R_n) and the amplification plots were corrected employing the adaptive base line algorithm (ΔR_n) in MxPro™ QPCR software (Stratagene, La Jolla, USA). Amplification based threshold was used to obtain threshold cycle values (C_t) in the same program. Standard curves were created using five dilutions of PCR products. The real-time PCR efficiency values were calculated from the slope according to the equation $E = 10^{[-1/slope]}$ in REST-384© v.2 software (Pfaffl *et al.*, 2002). The same software was adopted to calculate relative concentrations of target mRNA (expression ratios) using the $\Delta\Delta C_t$ method. The calculated expression ratios were tested for significance by a Pair Wise Fixed Reallocation Randomization Test©. Each of the five time points was analyzed separately, gene expression was related within the same plant to time 0. Expression ratios were presented both in log-scale and as fold-changes in comparison to time 0.

3.5 RESULTS

3.5.1 Expression of KTI

All stressed plants responded to the simulated herbivory by a statistically significant upregulation of *KTI* gene expression (Table 3.2, Fig. 3.1*a*). In replicates II and III, the levels of *KTI* transcript abundance peaked 24 hours after treatment with an approximately 34-fold increase and then gradually decreased. In replicate I, *KTI* gene was upregulated five times at the 36-hour time point and significantly downregulated at 96-hour time point.

No control plant showed upregulation of *KTI* gene 24 hours after treatment (Table 3.2, Fig. 3.1*a*). There was on average a 2.4 fold increase of transcript abundance 36 hours after treatment. This transient change was statistically significant in replicate II, followed by a significant decrease in all replicates.

In untreated plants, expression pattern of *KTI* gene greatly resembled the one of healthy controls (Table 3.2, Fig.3.1*a*). Untreated suckers did not respond to the simulated herbivory immediately after treatment, nevertheless they showed a similar transient increase of *KTI* transcript abundance 36 hours after treatment as well as a similar drop at the 72-hour time point.

3.5.2 Expression of DFR

Expression of *DFR* gene was significantly up regulated in all stressed plants (Table 3.3, Fig.3.2). Number of *DFR* transcripts started increasing at first sampling 24 hours after treatment and reached the maximum levels at 36-hour time point. No significant increase of *DFR* mRNA abundance was observed in either control or untreated plants 24 hours after treatment. Similar to the *KTI* expression pattern, there was a transient but statistically significant increase of *DFR* transcript abundance in both untreated and control plants 36 hours after treatment.

Table 3.2 Temporal pattern of *KTI* relative expression in control (C), stressed (S) and untreated (UT) plants of *Populus tremuloides* after simulated herbivory.
*expression of *KTI* gene significantly different ($P < 0.05$) from time 0
**expression of *KTI* gene significantly different ($P < 0.001$) from time 0

Relative expression of *KTI* gene in stressed and untreated aspen suckers sharing the same root												
			factor					log-value				
sampling time [hours after treatment]			replicate I	replicate II	replicate III	average	SE	replicate I	replicate II	replicate III	average	SE
	24	C	-2.7	-1.5		-2.1	0.8	-1.5	-0.6		-1.0	0.6
		S	3.3*	34.8*	33.8**	24.0	18.0	1.7	5.1	5.1	4.0	2.0
		UT	-1.2*			-1.0		-0.3			-0.3	
	36	C	2.1	3.7*	1.3	2.4	1.2	1.1	1.9	0.4	1.1	0.8
		S	5.1	12.6**	20.9**	12.9	7.9	2.4	3.7	4.4	3.5	1.0
		UT	5.3		3.5*	4.4	1.3	2.4		1.8	2.1	0.4
	72	C	-12.8**	-4.2**	-7.2**	-8.1	4.4	-3.7	-2.1	-2.8	-2.9	0.8
		S		3.6	8.3**	6.0	3.4		1.8	3.1	2.4	0.9
		UT	-3.0		-3.3	-3.2	0.2	-1.6		-1.7	-1.7	0.1
	96	C	-4.8*	-1.2	-1.3	-2.4	2.1	-2.3	-0.3	-0.4	-1.0	1.1
		S	-2.7**		1.6*	-0.6	3.0	-1.4		0.6	-0.4	1.5
		UT	-8.2*		-2.5	-5.4	4.0	-3.0		-1.3	-2.2	1.2

Figure 3.1 Temporal pattern of Kunitz trypsin inhibitors (*KTI*s) relative expression in *Populus tremuloides* after simulated herbivory. Time-course of transcript abundance was followed over 5 time points in systemic leaves of stressed and untreated plants sharing the same root and in controls. Gene expression was related within the same plant to time 0.

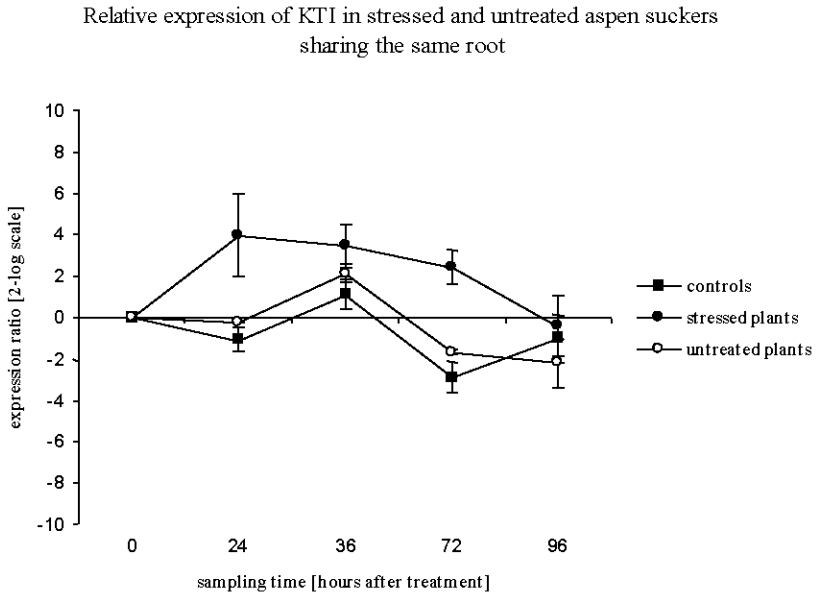
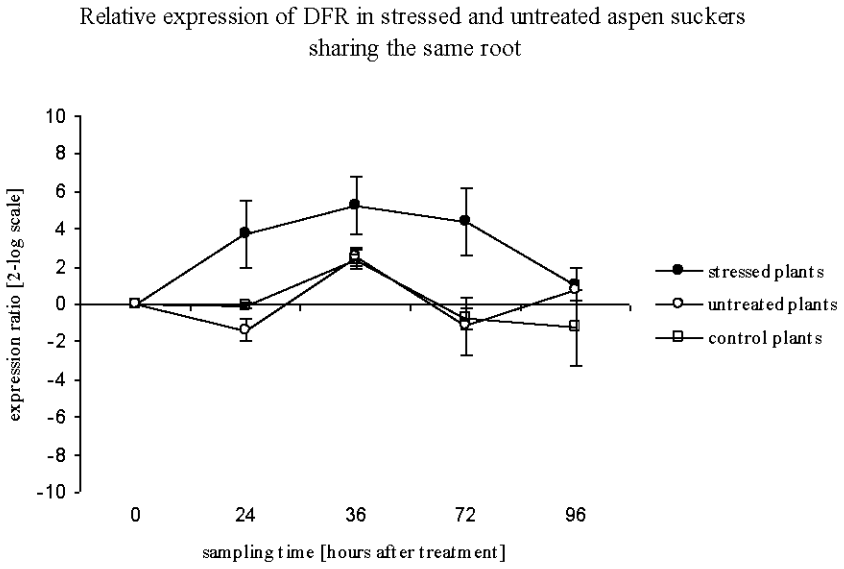


Table 3.3 Temporal pattern of *DFR* relative expression in control (C), stressed (S) and untreated (UT) plants of *Populus tremuloides* after simulated herbivory.
*expression of *DFR*gene significantly different ($P < 0.05$) from time 0
**expression of *DFR* gene significantly different ($P < 0.001$) from time 0

Relative expression of *DFR* gene in stressed and untreated aspen suckers sharing the same root												
			factor					log-value				
sampling time [hours after treatment]			replicate I	replicate II	replicate III	average	SE	replicate I	replicate II	replicate III	average	SE
	24	C	1.0		-1.1	-0.1	1.5	0.1		-0.2	-0.1	0.2
		S	11.7**	50.4*	4.3**	22.1	1.8	3.6	5.7	2.1	3.8	1.8
		UT	-3.5**	-1.9		-2.7	1.1	-1.8	-0.9		-1.4	0.6
	36	C	6.6**	4.0*		5.3	1.8	2.7	2.0		2.4	0.5
		S	80.5**		18.1**	49.3	1.5	6.3		4.2	5.3	1.5
		UT	4.7*	8.5**	4.7**	6.0	2.2	2.3	3.1	2.2	2.5	0.5
	72	C	-1.5*	-1.3	-2.6	-1.8	0.7	-0.6	-0.3	-1.4	-0.8	0.6
		S	38.8**	45.4*	5.1**	29.8	1.8	5.3	5.5	2.4	4.4	1.8
		UT	-4.7		-1.0	-2.8	2.6	-2.2		-0.1	-1.1	1.5
	96	C	-5.2	-5.4*	2.1**	-2.8	4.3	-2.4	-2.4	1.1	-1.2	2.0
		S	3.2		1.4*	2.3	0.9	-1.7		0.4	-1.1	0.9
		UT			1.6	1.6				0.7	0.7	

Figure 3.2 Temporal pattern of dihydroflavonol reductase (*DFR*) relative expression in *Populus tremuloides* after simulated herbivory. Time-course of transcript abundance was followed over 5 time points in systemic leaves of stressed and untreated plants sharing the same root and in controls. Gene expression was related within the same plant to time 0.



3.6 DISCUSSION

In this work, we found no evidence that interconnected ramets of trembling aspen are able to systemically induce resistance upon herbivore simulated attack. While wound-treated ramets showed systemic induction of defense genes, untreated interconnected suckers produced expression pattern similar to controls. This was the first study attempting to investigate clonal networks in respect to information sharing among root systems in long-living tree species. Even though trembling aspen comprises an important component of the boreal forest (Bradshaw *et al.*, 2000) and as that many aspects of its biology have been thoroughly studied, root connections in this clonal species and their ecological significance has received considerably less attention.

It is unlikely that the lack of systemic defense induction in sibling ramets was caused by factors such as marker gene selection. Kunitz-type trypsin inhibitor is among the genes most strongly induced in both mature and young leaves and in almost all plant tissues including roots (Major and Constabel, 2007). Dihydroflavonol reductase is a marker for the biosynthesis of condensed tannins (Robbins *et al.*, 1998). They function as feeding deterrents and their formation was repeatedly shown to be induced by wounding and insect attacks (Osier *et al.*, 2000; Osier and Lindroth, 2001; Kao *et al.*, 2002; Peters and Constabel, 2002; Tsai *et al.*, 2006). Thus, both genes represent good markers of the systemic defense response.

We used a highly sensitive and robust detection method. Quantitative real-time PCR assays using gene specific primers showed a significant upregulation of the selected genes in systemic leaves of wound-treated aspen ramets. These ramets showed upregulation by more than 30 and 80 times in comparison to the same plant before treatment. Even though the systemic defense induction tends to be much weaker than the local response (Haruta *et al.*, 2001a; Christopher *et al.*, 2004), the

plier-wounding we applied was sufficient to provoke a profound systemic effect. We observed a rather substantial variation of upregulation intensity among biological replicates. As the plants used were genetically identical, we assume that this variation can be contributed mainly to differences in damage applied to leaves. It was shown that damaging different proportion of leaves produced varying intensity of defense response (Haruta *et al.*, 2001a). Hence, among replicate variation could be diminished by better standardization of wound treatment.

In addition, we assume that the lack of defense response in interconnected sibling ramets was not caused by time point selection in this experiment. Our sampling times stretched from 24 to 96 hours after mechanical wounding. With respect to *DFR* gene expression, Peters and Constabel (2002) did not observe any delay between local and systemic upregulation. *DFR* transcripts started accumulating in both local and systemic leaves 12 hours after treatment and reached the maximum levels after 24 hours. Similarly, Haruta *et al.* (2001*a*, 2001*b*) observed only small time lapse of *KTI* gene expression in systemic and local leaves. While a local increase in mRNA levels was detectable already 6 hours after treatment, systemic leaves started upregulating *KTI* gene expression only at the 24 hour-time point. Moreover, 24 hours after treatment, *KTI* mRNA was also detectable in roots of damaged plants (Major and Constabel, 2007). Hence, the time needed for producing a systemic defense signal at the site of damage, transmitting the signal within a plant body, and activating defense mechanisms in undamaged parts seems to be very short. However, very few studies have investigated the systemic activation time in relation to physiological integration in clonal plant species. Gomez *et al.* (Gomez *et al.*, 2010) reported that undamaged ramets of clover (*Trifolium repens*) had become defense-induced 38-51 hours after the initial attack. Nonetheless, they measured defense induction as a reduction in leaf palatability which required protein synthesis. Changes at the transcript level would, thus, have proceeded much faster. Hence, we presume a

potential induction of defense mechanism in interconnected sibling ramets would take no longer than 96 hours after insect feeding.

An absence of systemic defense response in interconnected sibling ramets could be a consequence of functional coupling between a dominant sap flow and spatial expression of induced defense in clonal plant networks. Our experimental design provided homogeneous environmental as well as developmental conditions (all saplings of equal size and age). Under such conditions, the prevailing sap flow presumably together with systemic defense induction signals follow usual trajectories in fast growing aspen plants. In general, the direction of these trajectories is governed by a physiological source-sink relationship and is limited by vascular plant architecture (Davis *et al.*, 1991; Jones *et al.*, 1993; Metraux *et al.*, 2002; Arnold *et al.*, 2004). While in healthy plants, fast growing young leaves represent a strong source, plants undergoing foliar herbivory partially divert photoassimilate flow to stems and roots (Babst *et al.*, 2005; Babst *et al.*, 2008). The increased carbon allocation to roots probably allows basipetally induced systemic signaling in roots (Jones *et al.*, 1993; Major and Constabel, 2007). Nevertheless, the subsequent pathways of these signals through clonal root networks has only been explored in a single species of a stoloniferous herb *Trifolium repens* (Gomez and Stuefer, 2006). Gomez and Stuefer (2006) showed that defense induction was dependent on the presence of a photoassimilate sink. Older ramets did not receive a defense induction signal from younger ramets unless the predominant phloem flow was reversed by means of basal shading.

It is also possible that the potential spread of defense induction signals is limited by the union between the sucker and mother root to only one direction. Brown (1935) explored the morphology of aspen shoots regenerated by root suckering. He found that root and stem cells were aligned towards the distal side of the mother root enlarging this side more than the proximal one. In accordance with this finding, Baret

and DesRochers (2011) showed that suckers located distally could influence physiology of their neighbors located proximally on the same mother root. It remains unclear if proximal trees could physiologically interact with trees located distally.

We detected a transient, however, significant upregulation of *KTI* and *DFR* genes in both non-treated sibling ramets interconnected to wounded plants and controls. This peak appeared 36 hours after treatment and was significant in almost all replicates. We assume that it was a response to herbivore-induced plant volatiles (HIPV) released from wounded leaves. In experiments using glass chambers, HIPV can upregulate expression of some defense traits in intact leaves (Frost *et al.*, 2008). In their experiments, *DFR* gene expression was induced provided the intact leaves were exposed to HIPV at high concentrations. By contrast, the *KTI1* gene did not respond to volatile exposure. As far as we know, there is no available information on volatile-induced *KTI*3 gene expression. As even the control plants responded with defense induction, other factors cannot be excluded.

This study has focused specifically at the expression of two representative genes of poplar defense after herbivory simulated by mechanical wounding. It has been shown that responses to mechanical wounding and insect feeding are qualitatively similar, nontheless, they differ quantitatively (Major and Constabel, 2006). To clarify the role of aspen root networks in antiherbivore defense, further studies employing real insect feeding are needed.

3.7 CONCLUSION

Even though root connections can trigger physiological responses to defoliation in nondefoliated aspen suckers (Baret and DesRochers, 2011), our results

suggest that these root pathways do not automatically lead to induction of defensive traits that are expressed in plants directly under herbivore attack. Rather, it seems that other communication means such as air borne volatiles can serve as signal transmission pathways among neighbouring plants as indicated by a transient mRNA accummulation of defense marker genes.

3.8 ACKNOWLEDGEMENTS

The authors thank P. Wildová for help and advice and anonymous reviewers for critical reading of the manuscript. This study was supported by the Centre for Forest Research, National Science and Engineering Research Council, Industrial Chair in Sustainable Forest Management, Fonds de recherche sur la nature et les technologies, and the government of Quebec.

3.9 LITERATURE CITED

Alpert P (1996) Nutrient sharing in natural clonal fragments of *Fragaria chiloensis*. Journal of Ecology, 84:395-406.

Alpert P, Holzapfel C, Benson JM (2002) Hormonal modification of resource sharing in the clonal plant *Fragaria chiloe*nsis. Functional Ecology, 16:191-197.

Arimura G, Huber DPW, Bohlmann J (2004) Forest tent caterpillars (Malacosoma disstria) induce local and systemic diurnal emissions of terpenoid volatiles in hybrid poplar (*Populus trichocarpa x deltoides*): cDNA cloning, functional characterization, and patterns of gene expression of (-)-germacrene D synthase, PtdTPS1. Plant Journal, 37:603-616.

Arnold T, Appel H, Patel V, Stocum E, Kavalier A, Schultz J (2004) Carbohydrate translocation determines the phenolic content of *Populus* foliage: a test of the sink-source model of plant defense. New Phytologist, 164:157-164.

Arnold TM, Schultz J (2002) Induced sink strength as a prerequisite for induced tannin biosynthesis in developing leaves of *Populus*. Oecologia, 130:585-593.

Babst BA, Ferrieri RA, Gray DW, Lerdau M, Schlyer DJ., Schueller M, Thorpe MR, Orians CM (2005) Jasmonic acid induces rapid changes in carbon transport and partitioning in *Populus*. New Phytologist, 167:63-72.

Babst BA, Ferrieri RA, Thorpe MR, Orians CM (2008) *Lymantria dispar* herbivory induces rapid changes in carbon transport and partitioning in *Populus nigra*. Entomologia Experimentalis Et Applicata, 128:117-125.

Baret M, DesRochers A (2011) Root connections can trigger physiological responses to defoliation in nondefoliated aspen suckers. Botany, 89:753-761.

Bradshaw HD, Ceulemans R, Davis J, Stettler R (2000) Emerging model systems in plant biology: Poplar (*Populus*) as a model forest tree. Journal of Plant Growth Regulation, 19:306-313.

Brown AB (1935) Cambial activity root habit and sucker shoot development in two species of poplar. New Phytologist, 34: 163-179.

Bruhn JN, Pickens JB, Stanfield DB (1991) Probit analysis of oak wilt transmission through root grafts in red oak stands. Forest Science, 37:28-44.

Brunner AM, Yakovlev IA, Strauss SH (2004) Validating internal controls for quantitative plant expression studies., BMC Plant Biology, 4:14-20.

Bruxelles GL, Roberts MR (2001) Signals Regulating Multiple Responses to Wounding and Herbivory, Critical Reviews in Plant Sciences 20:487-521.

Chen, F, Liu C, Tschaplinski TJ, Zhao N (2009) Genomics of Secondary Metabolism in *Populus:* Interactions with Biotic and Abiotic Environments. Critical Reviews in Plant Sciences, 28:375-392.

Christopher ME, Miranda M, Major IT, Constabel CP (2004) Gene expression profiling of systemically wound-induced defenses in hybrid poplar. Planta, 219:936-947.

Constabel CP, Yip L, Patton JJ, Christopher ME (2000) Polyphenol oxidase from hybrid poplar. Cloning and expression in response to wounding and herbivory. Plant Physiology, 124: 285-295.

Davis JM., Gordon MP, Smit BA (1991) Assimilate movement dictates remote sites of wound-induced gene-expression in poplar leaves. Proceedings of the National Academy of Sciences of the United States of America 88:2393-2396.

DeByle N (1964) Detection of functional intraclonal root connections by traces excavation., Foresty Science, 10:386-396.

DesRochers A, Lieffers VJ (2001) The coarse-root system of mature *Populus tremuloides* in declining stands in Alberta, Canada. Journal of Vegetation Science, 12:355-360.

Du J, Wang N, Alpert P, Yu M, Yu F, Dong M (2010) Clonal integration increases performance of ramets of the fern *Diplopterygium glaucum* in an evergreen forest in southeastern China. Flora, 205:399-403.

Duplessis S, Major I, Martin F, Seguin A (2009) Poplar and Pathogen Interactions: Insights from Populus Genome-Wide Analyses of Resistance and Defense Gene Families and Gene Expression Profiling. Critical Reviews in Plant Sciences, 28:309-334.

Evans JP, Cain ML (1995) A spatially explicit test of foraging behavior in a clonal plant. Ecology, 76:1147-1155.

Frost CJ, Mescher MC, Dervinis C, Davis JM, Carlson JE, De Moraes CM (2008) Priming defense genes and metabolites in hybrid poplar by the green leaf volatile cis-3-hexenyl acetate. New Phytologist, 180:722-733.

Gomez S, Latzel V, Verhulst Y, Stuefer JF (2007) Costs and benefits of induced resistance in a clonal plant network. Oecologia, 153:921-930.

Gomez S, Onoda Y, Ossipov V, Stuefer JF (2008) Systemic induced resistance: a risk-spreading strategy in clonal plant networks? New Phytologist, 179:1142-1153.

Gomez S, Stuefer JF (2006) Members only: induced systemic resistance to herbivory in a clonal plant network. Oecologia, 147:461-468.

Gomez S, van Dijk W, Stuefer JF (2010) Timing of induced resistance in a clonal plant network. Plant Biology, 12:512-517.

Graham BF, Bormann FH (1966) Natural root grafts., Botanical Reviews, 32:255-292.

Haruta M, Major IT, Christopher ME, Patton JJ, Constabel CP (2001*a*). A Kunitz trypsin inhibitor gene family from trembling aspen (*Populus tremuloides* Michx.): cloning, functional expression, and induction by wounding and herbivory. Plant Molecular Biology, 46:347-359.

Haruta M, Pedersen JA, Constabel CP (2001*b*). Polyphenol oxidase and herbivore defense in trembling aspen (*Populus tremuloides*): cDNA cloning, expression, and potential substrates. Physiologia Plantarum, 112:552-558.

Havill NP, Raffa KF (2000) Compound effects of induced plant responses on insect herbivores and parasitoids: implications for tritrophic interactions. Ecological Entomology, 25:171-179.

He CX, Li WB, Ayres AJ, Hartung JS, Miranda VS, Teixeira DC (2000) Distribution of *Xylella fastidiosa* in citrus rootstocks and transmission of citrus variegated chlorosis between sweet orange plants through natural root grafts. Plant Disease, 84:622-626.

Hemming JDC, Lindroth RL (1995) Intraspecific variation in aspen phytochemistry - effects on performance of gypsy moths and forest tent caterpillars. Oecologia, 103:79-88.

Hutchings MJ, Wijesinghe DK (1997) Patchy habitats, division of labour and growth dividends in clonal plants. Trends in Ecology and Evolution, 12:390-394.

Hwang SY, Lindroth RL (1997) Clonal variation in foliar chemistry of aspen: Effects on gypsy moths and forest tent caterpillars. Oecologia, 111:99-108.

Ikegami M, Whigham DF, Werger MJA (2008) Optimal biomass allocation in heterogeneous environments in a clonal plant - Spatial division of labor. Ecological Modelling, 231:156-164.

Jelinkova H, Tremblay F, DesRochers A (2009) Molecular and dendrochronological analysis of natural root grafting in *Populus tremuloides* (*Salicaceae*). American Journal of Botany, 96:1500-1505.

Jones CG, Hopper RF, Coleman JS, Krischik VA (1993) Control of systemically induced herbivore resistance by plant vascular architecture. Oecologia, 93:452-456.

Kalendar R (2008) FastPCR: a PCR primer and probe design and repeat sequence searching software with additional tools for the manipulation and analysis of DNA and protein., www.biocenter.helsinki.fi/bi/programs/fastpcr.htm.

Kao Y, Harding SA, Tsai C (2002) Differential expression of two distinct phenylalanine ammonia-lyase genes in condensed tannin-accumulating and lignifying cells of quaking aspen. Plant Physiology, 130:796-807.

Koubek T, Herben T (2008) Effect of systemic diseases on clonal integration: modelling approach. Evolutionary Ecology, 22:449-460.

Kozlowski TT (1992) Carbohydrate sources and sinks in woody plants. Botanical Review, 58:107-222.

Lawrence SD, Dervinis C, Novak N, Davis JM (2006) Wound and insect herbivory responsive genes in poplar. Biotechnology Letters, 28:1493-1501.

Liu H, Yu F, He W, Chu Y, Dong M (2009) Clonal integration improves compensatory growth in heavily grazed ramet populations of two inland-dune grasses. Flora, 204:298-305.

Magori K, Oborny B, Dieckmann U, Meszena G (2003) Cooperation and competition in heterogeneous environments: the evolution of resource sharing in clonal plants. Evolutionary Ecology Research, 12:787-817.

Major IT, Constabel CP (2006) Molecular analysis of poplar defense against herbivory: comparison of wound- and insect elicitor-induced gene expression. New Phytologist, 172:617-635.

Major IT, Constabel CP (2007) Shoot-root defense signaling and activation of root defense by leaf damage in poplar. Canadian Journal of Botany, 85:1171-1181.

Major IT, Constabel CP (2008) Functional analysis of the Kunitz trypsin inhibitor family in poplar reveals biochemical diversity and multiplicity in defense against herbivores. Plant Physiology, 146:888-903.

Maleck K, Dietrich RA (1999) Defense on multiple fronts: How do plants cope with diverse enemies? Trends in Plant Science, 4:215-219.

Metraux J, Nawrath C, Genoud T (2002) Systemic acquired resistance. Euphytica, 124:237-243.

Miranda M, Ralph SG, Mellway R, White R, Heath MC, Bohlmann J, Constabel CP (2007) The transcriptional response of hybrid poplar (*Populus trichocarpa x P-deltoides*) to infection by *Melampsora medusae* leaf rust involves induction of flavonoid pathway genes leading to the accumulation of proanthocyanidins. Molecular Plant-Microbe Interactions, 20:816-831.

Mony C, Garbey M, Smaoui M, Benot ML (2011) Large scale parameter study of an individual-based model of clonal plant with volunteer computing. Ecological Modelling, 222:935-946.

Nilsson J, D'Hertefeldt T (2008) Origin matters for level of resource sharing in the clonal herb *Aegopodium podagraria.* Evolutionary Ecology, 22:437-448.

Oborny B, Kun A, Czaran T, Bokros S (2000) The effect of clonal integration on plant competition for mosaic habitat space. Ecology, 81:3291-3304.

Osier TL, Hwang S, Lindroth RL (2000) Effects of phytochemical variation in quaking aspen *Populus tremuloides* clones on gypsy moth Lymantria dispar performance in the field and laboratory. Ecological Entomology, 25:197-207.

Osier TL, Lindroth RL (2001) Effects of genotype, nutrient availability, and defoliation on aspen phytochemistry and insect performance. Journal of Chemical Ecology, 27:1289-1313.

Peltzer DA (2002) Does clonal integration improve competitive ability? A test using aspen (*Populus tremulo*ides [*Salicaceae*]) invasion into prairie. American Journal of Botany, 89:494-499.

Pennings SC, Callaway RM (2000) The advantages of clonal integration under different ecological conditions: A community-wide test. Ecology, 81:709-716.

Peters DJ, Constabel CP (2002) Molecular analysis of herbivore-induced condensed tannin synthesis: cloning and expression of dihydroflavonol reductase from trembling aspen (*Populus tremuloides*). Plant Journal, 32:701-712.

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST (c)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Research, 30: 36-46.

Philippe RN, Bohlmann J (2007) Poplar defense against insect herbivores. Canadian Journal of Botany, 85:1111-1126.

Philippe RN, Ralph SG, Kulheim C, Jancsik SI, Bohlmann J (2009) Poplar defense against insects: genome analysis, full-length cDNA cloning, and transcriptome and protein analysis of the poplar Kunitz-type protease inhibitor family. New Phytologist, 184:865-884.

Philippe RN, Ralph SG, Mansfield SD, Bohlmann J (2010) Transcriptome profiles of hybrid poplar (*Populus trichocarpa x deltoides*) reveal rapid changes in undamaged, systemic sink leaves after simulated feeding by forest tent caterpillar (*Malacosoma disstria*). New Phytologist, 188:787-802.

Ralph SG (2009) Studying Populus Defenses Against Insect Herbivores in the Post-Genomic. Critical Reviews in Plant Sciences, 28:335-345.

Ralph S, Oddy C, Cooper D, Yueh H, Jancsik S, Kolosova N, Philippe RN, Aeschliman D, White R, Huber D, Ritland C, Benoit F, Rigby T, Nantel A, Butterfield YSN, Kirkpatrick R, Chun E, Liu J, Palmquist D, Wynhoven B, Stott J, Yang G, Barber S, Holt RA, Siddiqui A, Jones SJM, Marra MA, Ellis BE, Douglas CJ, Ritland D, Bohlmann J (2006) Genomics of hybrid poplar (*Populus trichocarpa x deltoides*) interacting with forest tent caterpillars (*Malacosoma disstria*): normalized and full-length cDNA libraries, expressed sequence tags, and a cDNA microarray for the study of insect-induced defences in poplar. Molecular Ecology, 15:1275-1297.

Robbins MP, Bavage AD, Strudwicke C, Morris P. 1998. Genetic manipulation of condensed tannins in higher plants II. Analysis of birdsfoot trefoil plants harboring antisense dihydroflavonol reductase constructs. Plant Physiology, 116:1133-1144.

Shumway SW (1995) Physiological integration among clonal ramets during invasion of disturbance patches in a new-england salt-marsh. Annals of Botany, 76:225-233.

Stone EL (1974) The Commumal Root System of Red Pine: Growth of Girdled Trees., Forest Science, 20:294-305.

Strong WL, LaRoi GH (1983) Root-system morphology of common boreal forest trees in Alberta, Canada., Canadian Journal of Forest Research, 13:1164-1173.

Stuefer JF, During HJ, Dekroon H (1994) High benefits of clonal integration in 2 stoloniferous species, in response to heterogeneous light environments. Journal of Ecology, 82:511-518.

Stuefer JF, During HJ, Schieving F (1998) A model on optimal root-shoot allocation and water transport in clonal plants. Ecological Modelling, 111:171-186.

Tsai CJ, Harding SA, Tschaplinski TJ, Lindroth RL, Yuan YN (2006) Genome-wide analysis of the structural genes regulating defense phenylpropanoid metabolism in *Populus*. New Phytologist, 172:47-62.

Wang JH, Constabel CP (2004) Three polyphenol oxidases from hybrid poplar are differentially expressed during development and after wounding and elicitor treatment. Physiologia Plantarum, 122:344-353.

Yu F, Wang N, Alpert P, He W, Dong M. 2009. Physiological integration in an introduced, invasive plant increases its spread into experimental communities and modifies their structure. American Journal of Botany, 96:1983-1989.

Yu F, Wang N, He W, Chu Y, Dong M (2008) Adaptation of rhizome connections in drylands: Increasing tolerance of clones to wind erosion. Annals of Botany, 102:571-577.

GENERAL CONCLUSION

Clone Identification

Employing digital analysis of leaf shape for clone identification helped to overpass the problem of subjective assessment of some leaf shape features when using traditional morphometrics. Nonetheless, as the evaluation is quantitative and identification of clones requires a creation of separate categories, a threshold needs to be set to define the clone borders. This proved to be uneasy in natural aspen stands with a high number of genets, varying levels of leaf shape variation, and numerous clones represented by a single ramet.

The first chapter also pointed to the problematic use of spring phenology for clone identification. We have shown that uniformly flushing patches of aspen trees can be composed of more than one clone and vice versa trees differing in their time of leaf out can in fact belong to the same clone. This is especially true in aspen stands that have not reached full maturity. Hence, we strongly discourage the use of this trait for clone delineation in forest management practices.

We showed that aspen stands in the north western boreal forests of Quebec are made up of a high number of clones represented by a limited number of trees and that the clones are often spatially intermixed. For current ecosystemic management that takes inspiration from natural forest dynamics and attempts at imitating the natural setup, this means that even for instance poplar plantation should be composed of differing genotypes creating a mosaic of genetically different individuals rather than large areas of genetically identical trees.

Natural Root Grafting and Clone Survival

In our second chapter, we made use of a detailed descriptive retrospective approach. We consider this approach appropriate with respect to the study objectives

and when taking into account the amount of information available in the current 'grafting' literature. Yet, this strategy does not allow us to address the studied phenomenon more 'mechanistically'. Even though we found no specific instances of genotype conservation in the communal root system of aspen, we showed that roots of different genotypes can be preserved due to inter-clonal grafting. Hence, our hypothesis remains theoretically possible and could have important implications for other researchers studying population structure and dynamics in clonal tree species.

Even though we found no unique genotypes among aspen roots that would be missing among stems, we assume that the survival of roots of dead ramets increases the chances of clone survival over time. Namroud *et al.* (2006) built a model to simulate clonal species genotypic diversity over long periods of time. Their model showed the relationship between the number of ramets per genet and the length of the genet survival. The larger the initial number of ramets per genet was, the longer the genet survived (Namroud *et al.*, 2006). Living roots of dead ramets could, thus, contribute to the clone longevity. Nonetheless, it is not known whether these roots preserve their ability to produce suckers and how the resprouting from these roots is affected by apical dominance of the connected neighboring living ramets. Further studies would be needed to verify whether these roots can truly participate in suckering of a new cohort of aspen ramets.

Our results fill up an important knowledge gap in fundamental aspects of clonal structure and development. The presented findings deal with neither a rare phenomenon nor a rare species. Natural root grafting is a process that was discovered in more than 50 tree species. Yet, the number of papers addressing this phenomenon using up-to-date techniques is sparse. Similarly, a number of species exhibiting some form of a clonal growth is far from being negligible.

Ecological significance of natural root grafting

Moreover, the second chapter may be interesting not only for the researchers investigating the processes of diversity maintenance in clonal plant species but also for a large community of scientists exploring natural stand dynamics. Many factors influencing dynamic processes in natural stands were identified under an assumption that these stands were composed of discrete individuals or genetically uniform clones. Our study indicated that some of these conclusions may be confound by the physiological integration of different clones. These findings significantly affects interpretations of diverse ecophysiological processes taking place in forests of trembling aspen or other clonal tree species.

Most ecophysiological studies still consider trees as discrete individuals competing among each other for space and resources largely overlooking the fact that many tree species share a communal root system either through mother root connections or root grafts (Graham and Bormann, 1966). We have shown that in young and middle-aged aspen stands almost two thirds of aspen trees were integrated into the communal root system. Similar was true for declining aspen stands in Alberta (Desrochers and Lieffers, 2001). Hence, aspen remains part of a large entity throughout the succession. Moreover, we have shown that due to unions of previously distinct root systems through root grafts, even different clones are often interlinked creating a genetically diverse unites.

Integration of aspen ramets into the communal root system is likely to directly influence the process of mortality. For long, a synchronous die out of aspen stands was mainly related to the genetic uniformity of trees or to the fact that the whole stand was initiated at the same time and, thus, reached the age of senescence at once. Nonetheless, it is also possible that pathogens can be transmitted through the communal root system speeding up the spread of infections from infected to healthy trees (Bruhn *et al.*, 1991). Interconnectivity among aspen trees could also influence

mortality indirectly by diverting the flow of water from suppressed to more vigorous trees within the communal root system. It was suggested that this could accelerated the process of self-thinning in dense young aspen stands (Krasny and Johnson, 1992).

Root connections among aspen trees are also likely to have an impact on stand structure. Members of a communal root system can share resources redistributing them among network members (Graham and Bormann, 1966). In this way, less vigorous trees can be supported by their more dominant neighbors (Basnet *et al.*, 1993; Fraser *et al.*, 2006; Tarroux and DesRochers, 2011). Prolonged survival of such weaker trees can help to maintain the stand integrity preventing other species from establishing at the site. Similarly, capturing of root systems of dead trees through root grafts keep the space occupied making the resources available for the remaining living trees and maximizing resource acquisition from the site.

Grafting of root systems also increases stand resistance to wind stress by improving the mechanical stability of interconnected members (Basnet *et al.*, 1993). Moreover, it can improve wind resistance indirectly by enhancing the survival of less vigorous trees through carbohydrate transfer (Fraser *et al.*, 2006). Death of suppressed trees would open up the canopy exposing the remaining trees to increased wind pressure.

The excavations undertaken in our study also indicated that while many stems were eliminated from the stand during self-thinning of dense young aspen regeneration, roots of many continued to live as a part of the communal root system. This communal root system is, thus, extensive which is important both for the survival of aspen clones and for maintaining of aspen component in many ecosystems. These findings help to explain why aspen can colonize perturbated areas with high speed even when the above grown parts of aspen before the disturbance were neither dense nor extensive and why even a small number of aspen trees per

hectare can regenerate a pure aspen stand (Mitton and Grant, 1980; Mitton and Grant, 1996; Bergeron, 2000).

Finally, it was suggested that natural root grafting could have a real adaptive value. Tarroux and DesRochers (2010) showed in jack pine (*Pinus banksiana*) that grafting was especially frequent in sandy soils. Formation of root connection that allows redistribution of resources among interconnected members could represent an adaptation to nutrient poor environments improving the chances of species survival in such nutrient limited conditions.

Anti-herbivore defenses

While the results of our second chapter completed quite well the picture of the structure and development of aspen root systems, it remains largely unknown what and to which extent is shared between interconnected trees and what are the consequences of such physiological integration for diverse ecophysiological processes and forest dynamics. Explicit studies demonstrating such possible impacts in perennial species are rare and their results are contradictory (Hellstrom *et al.*, 2006; Klimesova *et al.*, 2009; Baret and DesRochers, 2011). Baret and DesRochers (2011) showed that trees sharing a root system can physiologically interact with one another. Their study indicated that a defoliation stress could impact an interconnected sucker and cause compensatory photosynthesis in intact ramets. Another study of *Linaria vulgaris*, a clonal root-sprouting perennial herb, reported no carbon transfer between intact and damaged plants despite their physiological integration (Hellstrom *et al.*, 2006). Our results suggested that common root pathways do not automatically result in induction of defensive traits expressed in plants directly under herbivore attack. Suckers sharing the same mother root responded to the simulated herbivory in the same way as unconnected controls.

Nontheless, it is worth noticing that this pioneer study was done under specific conditions. Firstly, we used only one direction of the stress treatment that was applied to the proximal suckers while the DFR and KTI expression was measured in the distal suckers. The reasoning behind this choice was that the defense induction signals are believed to flow through phloem together with photootoassimilates (Metraux *et al.*, 2002). In general, the photoassimilates travel from leaves to roots from their proximal to distal parts where they meet the respiration needs and growth of fine roots. However, treatments in the opposite direction could have a different outcome. Moreover, the study of Baret and Desrocher (2011) showed that the interaction between the defoliated and nondefoliated suckers was more pronounced if the defoliated sucker was dominant and the untreated one was suppressed. In our study, both interconnected plants were of equal size. The same study also showed that the compensatory photosynthesis was observable only under water limited conditions. In the year with abundant rain fall, no interaction between the defoliated and nondefoliated sucker was detected. As the spread of defense induction signals presumably depends on the photoassimilate flow, the defense trait induction in clonal networks is also likely to be modulated by environmental conditions.

While there is no doubt that if trees are interconnected through their roots, they have the possibility to interact; direct evidence from experimental studies is missing. More work needs to be done to clarify whether interconnected trees can communicate signals about occurring herbivory through their root systems or if they rather use other communication pathways as suggested by our results.

LITERATURE CITED

Arimura G, Huber D, Bohlmann J (2004) Forest tent caterpillars (*Malacosoma disstria*) induce local and systemic diurnal emissions of terpenoid volatiles in hybrid poplar (*Populus trichocarpa x deltoides*): cDNA cloning, functional characterization, and patterns of gene expression of (-)-germacrene D synthase, PtdTPS1. Plant Journal, 37: 603-616.

Arnold T, Appel H, Patel V, Stocum E, Kavalier A, Schultz J (2004) Carbohydrate translocation determines the phenolic content of *Populus* foliage: a test of the sink-source model of plant defense. New Phytologist, 164:157-164.

Arnold TM, Schultz J (2002) Induced sink strength as a prerequisite for induced tannin biosynthesis in developing leaves of *Populus*. Oecologia, 130:585-593.

Babst BA, Ferrieri RA, Gray DW, Lerdau M, Schlyer DJ., Schueller M, Thorpe MR, Orians CM (2005) Jasmonic acid induces rapid changes in carbon transport and partitioning in *Populus*. New Phytologist, 167:63-72.

Babst BA, Ferrieri RA, Thorpe MR, Orians CM (2008) *Lymantria dispar* herbivory induces rapid changes in carbon transport and partitioning in *Populus nigra*. Entomologia Experimentalis Et Applicata, 128:117-125.

Bailey J, Schweitzer J, Rehill B, Irschick D, Whitham T, Lindroth R (2007) Rapid shifts in the chemical composition of aspen forests: an introduced herbivore as an agent of natural selection. Biological Invasions, 9: 715-722.

Balloux F, Lehmann L, De Meeus T (2003) The population genetics of clonal and partially clonal diploids. Genetics, 164: 1635-1644.

Baret M, DesRochers A (2011) Root connections can trigger physiological responses to defoliation in nondefoliated aspen suckers. Botany, 89: 753-761.

Barnes BV (1966) The clonal growth habit of American aspens. Ecology, 47: 439-447.

Barnes BV, Han FQ (1993) Phenotypic variation of chinese aspens and their relationships to similar taxa in Europe and North-America. Canadian Journal of Botany, 71: 799-815.

Barnes BV (1969) Natural variation and delineation of clonesof *Populus tremuloides* and *P. grandidentata* in northern lower Michigan. Silvae Genetica, 18: 130–142.

Basnet K, Scatena FN, Likens GE, Lugo AE (1993) Ecological consequences of root grafting in tabonuco (*Dacryodes excelsa*) trees in the Luquillo experimental forest, Pueto Rico. Biotropica 25: 28-35.

Barton K, Koricheva J (2010) The ontogeny of plant defense and herbivory: characterizing general patterns using meta-analysis. American Naturalist 175,: 481-493.

Bergeron Y (2000) Species and stand dynamics in the mixed woods of Quebec's southern boreal forest. Ecology, 81: 1500-1516.

Bradshaw H, Ceulemans R, Davis J, Stettler R (2000) Emerging model systems in plant biology: Poplar (*Populus*) as a model forest tree. Journal of Plant Growth Regulation, 19: 306-313.

Brilli F, Ciccioli P, Frattoni M, Prestininzi M, Spanedda A, Loreto F (2009) Constitutive and herbivore-induced monoterpenes emitted by *Populus* x *euroamericana* leaves are key volatiles that orient *Chrysomela populi* beetles. Plant Cell and Environment, 32: 542-552.

Bruhn JN, Pickens JB, Stanfield DB (1991) Probit analysis of oak wilt transmission through root grafts in red oak stands. Forest Science 34: 28-44.

Christopher M, Miranda M, Major I, Constabel C (2004) Gene expression profiling of systemically wound-induced defenses in hybrid poplar. Planta, 219: 936-947.

Constabel CP, Yip L, Patton JJ, Christopher ME (2000) Polyphenol oxidase from hybrid poplar. Cloning and expression in response to wounding and herbivory. Plant Physiology, 124: 285-295.

Constabel P (2003) Molecular analysis of herbivore-induced condensed tannin synthesis: cloning and expression of dihydroflavonol reductase from trembling aspen (*Populus tremuloides*). Plant Journal , 32l: 605-605.

Cope JS, Corney D, Clark JY, Remagnino P, Wilkin P (2012) Plant species identification using digital morphometrics: A review. Expert Systems With Applications, 39: 7562-7573.

Dayanandan S, Rajorar OP, Bawa KS (1998) Isolation and characterization of microsatellites in trembling aspen (*Populus tremuloides*). Theoretical and Applied Genetics, 96: 950-956.

De Woody J, Rickman TH, Jones BE, Hipkins VD (2009) Allozyme and microsatellite data reveal small clone size and high genetic diversity in aspen in the southern Cascade Mountains. Forest Ecology and Management, 258: 687-696.

Dervinis C, Frost CJ, Lawrence SD, Novak NG, Davis JM (2010) Cytokinin primes plant responses to wounding and reduces insect performance. Journal of Plant Growth Regulation, 289-296

Desrochers A, LieffersVJ (2001) The coarse-root system of mature *Populus tremuloides* in declining stands in Alberta. Journal of Vegetation Science, 12: 355-360.

Dicke M, Hilker M (2003) Induced plant defences: from molecular biology to evolutionary ecology. Basic and Applied Ecology, 4: 3-14.

Donaldson J, Lindroth R (2008) Effects of variable phytochemistry and budbreak phenology on defoliation of aspen during a forest tent caterpillar outbreak. Agricultural and Forest Entomology, 10: 399-410.

Duplessis S, Major I, Martin F, Seguin A (2009) Poplar and pathogen interactions: Insights from *Populus* genome-wide analyses of resistance and defense gene families and gene expression profiling. Critical Reviews in Plant Sciences, 28: 309-334.

Erickson DL, Hamrick JL (2003) Genetic and clonal diversity for *Myrica cerifera* along a spatiotemporal island chronosequence. Heredity, 90: 25-32.

Fraser EC, Lieffers VJ, Landhausser SM (2006) Carbohydrate transfer through root grafts to support shaded trees. Tree Physiology, 26: 1019-1023.

Frey B, Lieffers V, Landhausser S, Comeau P, Greenway K (2003) An analysis of sucker regeneration of trembling aspen. Canadian Journal of Forest Research, 33: 1169-1179.

Germain H, Seguin A (2011) Innate immunity: has poplar made its BED? New Phytologist, 189: 678-687.

Graham B, Bormann F (1966) Natural root grafts. Botanical Reviews, 32: 255-292.

Griffis-Kyle K, Beier P (2003) Small isolated aspen stands enrich bird communities in southwestern ponderosa pine forests. Biological Conservation, 110: 375-385.

Haruta M, Pedersen J, Constabel C (2001b) Polyphenol oxidase and herbivore defense in trembling aspen (*Populus tremuloides*): cDNA cloning, expression, and potential substrates. Physiologia Plantarum, 112: 552-558.

Haruta M, Major I, Christopher M, Patton J, Constabel C (2001a) A Kunitz trypsin inhibitor gene family from trembling aspen (*Populus tremuloides* Michx.): cloning, functional expression, and induction by wounding and herbivory. Plant Molecular Biology, 46: 347-359.

Hellstrom K, Kytoviita M, Tuomi J, Rautio P (2006) Plasticity of clonal integration in the perennial herb *Linaria vulgaris* after damage. Functional Ecology, 20: 413-420.

Jensen RJ, Ciofani KM, Miramontes LC (2002) Lines, outlines, and landmarks: morphometric analyses of leaves of *Acer rubrum, Acer saccharinum* (Aceraceae) and their hybrid. Taxon, 51: 475-492.

Jones CG, Hopper RF, Coleman JS, Krischik VA (1993) Control of systemically induced herbivore resistance by plant vascular architecture. Oecologia, 93:452-456.

Kemperman JA, Barnes BV (1976) Clone size in American aspens. Canadian Journal of Botany, 54: 2603 - 2607.

Krasny ME, Johnson EA (1992) Stand development in aspen clones. Canadian Journal of Forest Research 22: 1424-1429

Liesebach H, Schneck V, Ewald E (2010) Clonal fingerprinting in the genus *Populus* L. by nuclear microsatellite loci regarding differences between sections, species and hybrids. Tree Genetics and Genomes, 6: 259-269.

Lindroth R (2001) Adaptations of quaking aspen for defense against damage by herbivores and related environmental agents. USDA Forest Service Proceedings, 18: 273-284.

Lopez-de-Heredia U, Sierra-de-Grado R, Cristobal MD Martinez-Zurimendi P, Pando V, Martin MT (2004) A comparison of isozyme and morphological markers to assess the within population variation in small populations of European aspen (*Populus tremula* L.) in Spain. Silvae Genetica, 53: 227-233.

Major IT, Constabel CP (2006) Molecular analysis of poplar defense against herbivory: comparison of wound- and insect elicitor-induced gene expression. New Phytologist, 172:617-635.

McLellan T, Endler JA (1998) The relative success of some methods for measuring and describing the shape of complex objects. Systematic Biology, 47: 264-281.

Metraux J, Nawrath C, Genoud T (2002) Systemic acquired resistance. Euphytica, 124: 237-243.

Mock KE, Rowe CA, Hooten MB, Dewoody J, Hipkins VD (2008) Clonal dynamics in western North American aspen (*Populus tremuloides*). Molecular Ecology, 17: 4827-4844.

Montalvo A, Conard S, Conkle M, Hodgskiss P (1997) Population structure, genetic diversity, and clone formation in *Quercus chrysolepis* (*Fagaceae*). American Journal of Botany, 84: 1553–1564.

Namroud M, Park A, Tremblay F, Bergeron Y (2005a) Clonal and spatial genetic structures of aspen (*Populus tremuloides* Michx.). Molecular Ecology, 14: 2969-2980.

Namroud M, Tremblay F, Bergeron Y (2005b) Temporal variation in quaking aspen (*Populus tremuloides*) genetic and clonal structures in the mixedwood boreal forest of eastern Canada. Ecoscience, 12: 82-91.

Namroud M, Leduc A, Tremblay F, Bergeron Y (2006) Simulations of clonal species genotypic diversity - trembling aspen (*Populus tremuloides*) as a case study. Conservation Genetics, 7: 415-426.

Neto JC, Meyer GE, Jones DD, Samal AK (2006) Plant species identification using Elliptic Fourier leaf shape analysis. Computers and Electronics in Agriculture, 50: 121-134.

Osier T, Hwang SY, Lindroth RL (2000) Effects of phytochemical variation in quaking aspen *Populus tremuloides* clones on gypsy moth *Lymantria dispar* performance in the field and laboratory. Ecological Entomology, 24: 197-207

Osier T, Lindroth R (2001) Effects of genotype, nutrient availability, and defoliation on aspen phytochemistry and insect performance. Journal of Chemical Ecology, 27: 1289-1313.

Parry D, Herms D, Mattson W (2003) Responses of an insect folivore and its parasitoids to multiyear experimental defoliation of aspen. Ecology, 84: 1768-1783.

Persson HA, Gustavsson BA (2001) The extent of clonality and genetic diversity in lingonberry (*Vaccinium vitis-idaea* L.) revealed by RAPDs and leaf-shape analysis. Molecular Ecology, 10: 1385-1397.

Peters D, Constabel C (2002) Molecular analysis of herbivore-induced condensed tannin synthesis: cloning and expression of dihydroflavonol reductase from trembling aspen (*Populus tremuloides*). Plant Journal, 32: 701-712

Philippe R, Ralph S, Kulheim C, Jancsik S, Bohlmann J (2009) Poplar defense against insects: genome analysis, full-length cDNA cloning, and transcriptome and protein analysis of the poplar Kunitz-type protease inhibitor family. New Phytologist, 184: 865-884.

Philippe R, Ralph S, Mansfield S, Bohlmann J (2010) Transcriptome profiles of hybrid poplar (*Populus trichocarpa x deltoides*) reveal rapid changes in undamaged, systemic sink leaves after simulated feeding by forest tent caterpillar (*Malacosoma disstria*). New Phytologist, 188: 787-802.

Philippe R, Bohlmann J (2007) Poplar defense against insect herbivores. Canadian Journal of Botany, 85: 1111-1126.

Pluess AR, Stocklin J (2004) Population genetic diversity of the clonal plant *Geum reptans* (*Rosaceae*) in the Swiss Alps. American Journal of Botany, 91: 2013-2021.

Rahman MH, Dayanandan S, Rajora PO (2000) Microsatellite DNA marker in *Populus tremuloides*. Genome, 43: 293-297.

Ralph S, Oddy C, Cooper D, Yueh H, Jancsik S, Kolosova N, Philippe R, Aeschliman D, White R, Huber D, Ritland C, Benoit F, Rigby T, Nantel A, Butterfield Y, Kirkpatrick R, Chun E, Liu J, Palmquist D, Wynhoven B, Stott J, Yang G, Barber S, Holt R, Siddiqui A, Jones S, Marra M, Ellis B, Douglas C, Ritland K, Bohlmann J (2006) Genomics of hybrid poplar (*Populus trichocarpa x deltoides*) interacting with forest tent caterpillars (*Malacosoma disstria*): normalized and full-length cDNA libraries, expressed sequence tags, and a cDNA microarray for the study of insect-induced defences in poplar. Molecular Ecology, 15: 1275-1297.

Ralph S (2009) Studying populus defenses against insect herbivores in the post-genomic. Critical Reviews in Plant Sciences, 28: 335-345.

Robison D, Raffa K (1997) Effects of constitutive and inducible traits of hybrid poplars on forest tent caterpillar feeding and population ecology. Forest Science, 43: 252-267.

Romme WH, Turner MG, Tuskan GA, Reed RA (2005) Establishment, persistence, and growth of aspen (*Populus tremuloides*) seedlings in Yellowstone National Park. Ecology, 86: 404-418.

Rumpunen K, Bartish IV (2002) Comparison of differentiation estimates based on morphometric and molecular data, exemplified by various leaf shape descriptors and RAPDs in the genus *Chaenomeles* (Rosaceae). Taxon, 51: 69-82.

Scrosati R (2002) An updated definition of genet applicable to clonal seaweeds, bryophytes, and vascular plants. Basic and Applied Ecology, 3: 97-99.

Shepperd WD (1993) Initial growth, development, and clonal dynamics of regenerated aspen in the Rocky Mountains. USDA Forest Service, Research Paper RM-312. Rocky Mountain Forest and Range Experiment Station, Fort Collins, CO.

Suvanto LI, Latva-Karjanmaa TB (2005) Clone identification and clonal structure of the European aspen (*Populus tremula*). Molecular Ecology, 14: 2851-2860.

Stuefer J, Gomez S, Van Molken T (2004) Clonal integration beyond resource sharing: implications for defence signalling and disease transmission in clonal plant networks. Evolutionary Ecology, 18: 647-667.

Tarroux E, DesRochers A (2010) Frequency of root grafting in naturally and artificially regenerated stands of *Pinus banksiana*: influence of site characteristics. Canadian Journal of Forest Research 40:861–871.

Tarroux E, DesRochers A (2011) Effect of natural root grafting on growth response of jack pine (*Pinus banksiana*; Pinaceae) American Journal of Botany, 98: 967-974.

Tsai C, Harding S, Tschaplinski T, Lindroth R, Yuan Y (2006) Genome-wide analysis of the structural genes regulating defense phenylpropanoid metabolism in *Populus*. New Phytologist, 172: 47-62.

Viscosi V, Fortini P (2011) Leaf shape variation and differentiation in three sympatric white oak species revealed by elliptic Fourier analysis. Nordic Journal of Botany, 29: 632-640.

Wang J, Constabel P (2004) Three polyphenol oxidases from hybrid poplar are differentially expressed during development and after wounding and elicitor treatment. Physiologia Plantarum, 122: 344-353.

Wyman J, Bruneau A, Tremblay MF (2003) Microsatellite analysis of genetic diversity in four populations of *Populus tremuloides* in Quebec. Canadian Journal of Botany, 81: 360-367.